Vitamin D Hydroxylating Enzymes and Analogue in Parathyroid Tumors and Breast Cancer

ULRIKA SEGERSTEN
Dissertation presented at Uppsala University to be publicly examined in Rosénalsen, Kvinnokliniken, Akademiska sjukhuset Ing 95, Uppsala, Tuesday, November 8, 2005 at 09:15 for the degree of Doctor of Philosophy (Faculty of Medicine). The examination will be conducted in Swedish.

Abstract

In hyperparathyroidism (HPT) raised serum concentrations of ionized calcium is caused by increased secretion of parathyroid hormone (PTH) by parathyroid tumors. Active vitamin D, 1α,25-dihydroxyvitamin D₃, is known to suppress PTH secretion and to reduce proliferation of parathyroid tumor cells.

The aim of this thesis was to examine expression of vitamin D hydroxylating enzymes, regulating the activation and inactivation of vitamin D and to study effects of vitamin D analogues, in parathyroid tumors and breast cancer.

The vitamin D activating enzyme, CYP27B1/25-hydroxylvitamin D₃, 1α-hydroxylase (1α-hydroxylase) and the vitamin D inactivating enzyme CYP24A1/25-hydroxylvitamin D₃, 24-hydroxylase (24-hydroxylase) were expressed in parathyroid tumors and breast cancer.

The parathyroid tumors had raised expression levels of 1α-hydroxylase and reduced levels of 24-hydroxylase in comparison to normal parathyroid glands, indicating ability for endogenous activation of vitamin D. The expression of 1α-hydroxylase may be of therapeutic advantage for local activation of non-1α-hydroxylated vitamin D analogues in tumor cells, thereby reducing unwanted hypercalcemic effects.

Three of five selected low calcemic vitamin D analogues had as efficient PTH suppressing effect, in bovine parathyroid cells, as three vitamin D analogues used clinically for treatment of secondary HPT.

The non-1α-hydroxylated vitamin D analogue EB1285 showed antiproliferative and PTH suppressive effects as well as transcriptional activity in parathyroid and breast tumor cells, respectively.

Ketoconazole, an inhibitor of vitamin D hydroxylating enzymes, suppressed PTH secretion and potentiated the effect of vitamin D analogues. Combined treatment with vitamin D analogues and specific 24-hydroxylase inhibitors may be important for future therapy.

Keywords: hyperparathyroidism, breast cancer, vitamin D, CYP27B1, CYP24A1, vitamin D analogues, ketoconazole, vitamin D receptor

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ISSN 1651-6206
ISBN 91-554-6361-4
urn:nb:n:se:uu:diva-6008 (http://urn.kb.se/resolve?urn=urn:nbn:se:uu:diva-6008)
To my family
List of papers

This thesis is based on following papers, which will be referred to in the text by their Roman numerals.


V **Ulrika Segersten**, Per Hellman, Göran Åkerström & Gunnar Westin (2005). Potentiating inhibitory effects of the non-1α-hydroxylated precursor vitamin D analogue of EB1089 (EB1285) and ketoconazole in parathyroid cells. Submitted.

*These authors contributed equally.

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### Abbreviations

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<tr>
<td>1α-hydroxylase</td>
<td>CYP27B1/25-hydroxyvitamin D₃, 1α-hydroxylase</td>
</tr>
<tr>
<td>1α(OH)D₂</td>
<td>doxercalciferol/1α-hydroxyvitamin D₂</td>
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<td>1,25(OH)₂D₃</td>
<td>calcitriol/1α,25-dihydroxyvitamin D₃</td>
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<td>25(OH)D₃</td>
<td>25-hydroxyvitamin D₃</td>
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<td>25-hydroxylase</td>
<td>CYP27A1/vitamin D₁ 25-hydroxylase</td>
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<td>24-hydroxylase</td>
<td>CYP24A1/25-hydroxyvitamin D₁ 24-hydroxylase</td>
</tr>
<tr>
<td>19-norD₂</td>
<td>19-nor-1α,25-dihydroxyvitamin D₂</td>
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<tr>
<td>BRCA</td>
<td>breast cancer gene</td>
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<td>CASR</td>
<td>calcium-sensing receptor</td>
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<tr>
<td>cDNA</td>
<td>complementary deoxyribonucleic acid</td>
</tr>
<tr>
<td>CMF</td>
<td>cyclophosphamide, methotrexate, 5-fluorouracil</td>
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<tr>
<td>CYP</td>
<td>cytochrome P450</td>
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<td>ER</td>
<td>estrogen receptor</td>
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<tr>
<td>ESRD</td>
<td>end-stage renal disease</td>
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<tr>
<td>GAPDH</td>
<td>glyceraldehyde-3-phosphate dehydrogenase</td>
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<td>HPT</td>
<td>hyperparathyroidism</td>
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<tr>
<td>LRP2</td>
<td>low density lipoprotein receptor-related protein 2</td>
</tr>
<tr>
<td>MEN</td>
<td>multiple endocrine neoplasia</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<td>OCT</td>
<td>22-oxacalcitriol</td>
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<tr>
<td>pHPT</td>
<td>primary hyperparathyroidism</td>
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<tr>
<td>PR</td>
<td>progesterone receptor</td>
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<td>PTH</td>
<td>parathyroid hormone</td>
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<td>RB</td>
<td>retinoblastoma</td>
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<tr>
<td>RT-PCR</td>
<td>reverse transcriptase-polymerase chain reaction</td>
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<td>RXR</td>
<td>retinoid X receptor</td>
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<tr>
<td>SERM</td>
<td>selective estrogen receptor modulator</td>
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<tr>
<td>sHPT</td>
<td>secondary hyperparathyroidism</td>
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<tr>
<td>VDDR</td>
<td>vitamin D-dependent rickets</td>
</tr>
<tr>
<td>VDR</td>
<td>vitamin D receptor</td>
</tr>
<tr>
<td>VDRE</td>
<td>vitamin D response element</td>
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Introduction

The parathyroid gland
The overall regulatory role of the parathyroid glands in the systemic calcium homeostasis is to maintain the tight regulation of the serum calcium concentration. In the majority of individuals, two superior and two inferior parathyroid glands are symmetrically located on the posterior part of the thyroid gland (1). The superior glands are mostly situated above the intersection of the recurrent laryngeal nerve and the inferior thyroid artery, whereas the inferior glands are commonly located on the lower thyroid pole (1). The parenchyma consists predominantly of chief cells and generally more sparse oxyphil cells, interspersed with fat cells (2). The chief cells secrete the 84 amino acid parathyroid hormone (PTH) and store intracellular fat, while oxyphil cells contain a notable level of mitochondria.

Physiology
Extra- and intracellular calcium exert a number of important functions, including blood clotting, cell division and as a second messenger. PTH and active vitamin D, calcitriol/1α,25-dihydroxyvitamin D₃ (1,25(OH)₂D₃) are the major hormones precisely regulating the extracellular Ca²⁺-level in the body. Hypocalcemia is instantly sensed by the parathyroid chief cells, inducing secretion of PTH, which in turn increases the serum calcium level by stimulating osteoclastic bone resorption and reabsorption of calcium in the kidney (3, 4) (Fig. 1).

Figure 1. Calcium regulation. See text for details.
In addition, PTH enhances the renal CYP27B1/25-hydroxyvitamin D$_3$ 1α-hydroxylase (1α-hydroxylase) activity, increasing the renal production of 1,25(OH)$_2$D$_3$ (5). The active form of vitamin D stimulates the intestinal calcium reabsorption and potentiates the actions of PTH on bone and kidney (3, 4). Furthermore, 1,25(OH)$_2$D$_3$ exerts negative feedback-regulation on PTH mRNA levels and reduces parathyroid cell proliferation (6-10) (Fig. 1).

The calcium-sensing ability of the parathyroid gland

There is an inverse relationship between extracellular Ca$^{2+}$-concentrations and the PTH secretion described by a sigmoidal dose response-curve (11, 12). The set-point is defined as the extracellular Ca$^{2+}$-concentration required to lower the PTH secretion to half maximum. The set-point falls into the range of the physiological free Ca$^{2+}$-concentration (1,10-1,25 mM) (13), where the curve is particularly steep, reflecting a marked capacity to change PTH secretion in response to minimal alterations in surrounding Ca$^{2+}$.

The calcium-sensing mechanism of the parathyroid gland involves the 120 kD calcium-sensing receptor (CASR). The CASR receptor gene is located on chromosome 3 and was originally cloned from bovine parathyroid cells (14). Extracellular Ca$^{2+}$-binding to CASR affects intracellular inositol-phosphate levels and intracellular Ca$^{2+}$-levels, regulating the PTH mRNA levels and PTH secretion (15-17).

Inactivating mutations in one allele of CASR is found in familial hypocalcuric hypercalcemia (FHH), whereas both alleles are mutated in neonatal severe hyperparathyroidism (NSHPT) (18). FHH is characterized by mild hypercalcemia and hypocalcuria with normal serum levels of PTH and size of parathyroid glands (19). On the contrary, NSHPT is associated with marked parathyroid enlargement, severe hypercalcemia in infancy and requires urgent treatment, generally total parathyroidectomy. A variant of FHH has been described recently, characterized by hypercalcemia and hypocalcuria and has been linked to an atypical mutation in CASR (20).

Another parathyroid cell surface receptor is the low density lipoprotein receptor-related protein 2 (LRP2) (21, 22). The LRP2 550 kD glycoprotein is also expressed in renal proximal tubule cells, epididymal epithelial cells and placental cytotrophoblast cells (22-24). In human pathological parathyroid cells monoclonal antibodies to LRP2 inhibited the rise of intracellular Ca$^{2+}$-concentration with reduced PTH secretion, in a high extracellular Ca$^{2+}$-environment (25-27). Interestingly, the LRP2 receptor is involved in the endocytic uptake of 25-hydroxyvitamin D$_3$ (25(OH)D$_3$) in renal tubule cells (28).
Hyperparathyroidism

Hyperparathyroidism (HPT) results from tumor development or hyperplasia of the parathyroid glands, causing excessive production of PTH and raised serum calcium levels. At the cellular level all variants of the disease appear to be characterized by proliferation of parathyroid cells and an abnormal set-point of the calcium regulated PTH secretion due to an altered Ca$^{2+}$-sensing ability of the parathyroid cells.

Primary hyperparathyroidism

The prevalence of sporadic primary HPT (pHPT) is approximately 1% of the adult population and the predisposition for pHPT rises with age, female sex and irradiation to the neck (29-32). About 85% of pHPT is caused by a solitary adenoma, whereas the rest accounts for mainly chief cell hyperplasia (15%) with enlargement of two or more parathyroid glands (33). Parathyroid carcinoma represents less than 1% of all cases of pHPT (34). Historically, pHPT was associated with severe bone and renal disease, whereas today the majority of the patients present mild hypercalcemia causing vague manifestations such as neuromuscular weakness, fatigue and cognitive symptoms (2, 31). Furthermore, pHPT appears to be associated with cardiovascular risk factors such as hypertension, insulin-resistance and dyslipidemia (31, 35-37). The therapy for pHPT is removal of pathological parathyroid tissue, leading to normocalcemia in about 95-99% of the patients.

Familial forms of HPT are multiple endocrine neoplasia type 1 (MEN1), multiple endocrine neoplasia type 2 (MEN2A), HPT-jaw tumor (HPT-JT) syndrome and familial isolated HPT (FIHPT).

The MEN1 syndrome is caused by inactivating mutations of the MEN1 tumor suppressor gene at 11q13, encoding the protein menin (19, 38, 39). Parathyroid, anterior pituitary, endocrine pancreatic and duodenal tumors are the classical lesions of the MEN1 syndrome.

Both the MEN type 2A and B syndromes are due to mutations in the tyrosine kinase receptor, RET proto-oncogene and the syndromes are associated with medullary thyroid carcinoma and pheochromocytoma (40). Less than 30% of the MEN2A patients have HPT, whereas HPT is not part of the MEN2B syndrome (41).

The HPT-JT syndrome, characterized by HPT, ossifying jaw fibromas and renal cysts and tumors, is associated with a high incidence of parathyroid carcinomas (42, 43). Mutations in a suggested tumor suppressor gene, HRPT2, are common in patients of the HPT-JT syndrome and appear to occur also in sporadic parathyroid carcinoma (44-46).

Familial isolated HPT (FIHPT) is a familial form of HPT with low risk of developing other tumors than neoplasia of the parathyroid gland. As HPT-JT, FIHPT is also associated with higher incidence of parathyroid carcinoma
and the disease has been mapped to both the MEN1 and the HPT-JT locus (47-49).

Chromosomal rearrangements of the parathyroid adenomatosis gene 1 (PRAD1/cyclin D1) involving the PTH promoter and the cyclin D1 gene with resulting cyclin D1 overexpression, has been reported in less than 5%, whereas overexpression of cyclin D1, from other causes, is demonstrated in 20-40% of sporadic parathyroid adenomas (50). Moreover, 30% of pHPT patients display loss of heterozygosity (LOH) on chromosome 11q13, corresponding to the MEN1 locus and somatic mutations of the other allele is seen in 15% (33). Transgenic mice exhibiting either parathyroid-specific cyclin D1 overexpression or deletion of the MEN1 gene developed symptoms of pHPT with hypercalcemia and parathyroid neoplasia (51, 52).

In sporadic parathyroid carcinoma overexpression of cyclin D1 is rather common, in contrast to infrequent LOH at 11q13 (34). Interestingly, LOH on chromosome 13 including the retinoblastoma (RB) tumor suppressor gene and abnormal expression of the RB protein is reported in the majority of parathyroid carcinomas, but is uncommon in parathyroid adenomas (53).

Secondary hyperparathyroidism

Secondary HPT (sHPT) is a more or less constant complication of chronic renal failure (CRF) and may also rarely occur in association with malabsorption of vitamin D. In addition, sHPT is relatively common in patients subjected to long-term lithium therapy. The inability of the uremic kidney to respond to PTH results in reduced levels of renally produced 1,25(OH)2D3, hypocalemia and hyperphosphatemia. Inappropriate 1,25(OH)2D3 levels cause inefficient growth-regulation of parathyroid cells initially leading to diffuse polyclonal hyperplasia, which ultimately tend to transform into multinodular hyperplasia (54). The hypocalemia and hyperphosphatemia stimulate the parathyroid gland to produce and secrete PTH and also contribute to further stimulation of glandular growth (17). sHPT is associated with initial hypocalemia and later in gradually more advanced end-stage renal disease (ESRD) with sometimes marked tendency to hypercalcemia.

In 5/6-nephrectomized uremic-sHPT rats elevated vitamin D levels, hypophosphatemia or hypercalcemia reduced the PTH serum levels and uremia-induced parathyroid hyperplasia (9, 17, 55). At the molecular level active vitamin D, hypophosphatemia or hypercalcemia promote growth inhibition in hyperplastic parathyroid tissue by inducing the expression of the cell-cycle restricting molecule p21/WAF1/CIP1 or preventing the increase of the growth stimulating factor, transforming growth factor-α and its receptor the epidermal growth factor-receptor (55).

Standard treatment of sHPT consists of active vitamin D, calcium supplementation and phosphate-restriction in diet and by using phosphate-
binding compounds. Parathyroidectomy is the final option in patients resistant to this therapy.

Hypercalcemia needs to be avoided in sHPT since it may cause increased mortality in ESRD-patients due to vascular calcification resulting in cardiovascular complications (56). Thereby, calcium-free phosphate-binders and low calcemic vitamin D analogues need to be developed for treatment of sHPT.

Novel drugs in treatment of sHPT are the calcimimetic drugs. These compounds sensitize CASR to its ligand, Ca\(^{2+}\), resulting in suppressed PTH secretion leading to decreased serum calcium and phosphate levels (56).

The effects of vitamin D analogues and calcimimetics are dependent on the expression of the vitamin D receptor (VDR) and CASR, respectively. In early stages of sHPT, VDR and CASR are still expressed to a certain level. However, in advanced nodular sHPT, both VDR and CASR are extensively reduced (57-60) resulting in insensitivity to vitamin D analogues or calcimimetics.

Notably, not only in sHPT but also in pHPT both VDR and CASR mRNA and protein levels are reduced (57-61). In addition, both sHPT and pHPT tumors demonstrated reduced mRNA levels of the cell cycle restricting proteins p18, p21 and p27 (62). Moreover, nodular hyperplasia expressed increased levels of cyclin D1 and the proliferation marker Ki67 compared to diffuse hyperplasia (63). Furthermore, LOH at 11q13, the MEN1 locus, is a rare event in sHPT (64).
Breast cancer

Breast cancer is one of the most common forms of malignances in the western world. In Sweden, more than 6000 women each year are diagnosed with breast cancer and the age corrected incidence has increased annually the last 20 years (65-67).

Histology and classification

Anatomically, the mammary glandular structure consists of 15-20 organized lobes each composed by a variable number of lobules (67). Each lobule is composed by multiple acini with terminal ducts. Both ducts and lobules of the breast tissue consist of a single inner layer of epithelial cells and an outer layer of myoepithelial cells. Except for the glandular mammary tissue the breast consists mainly of fat and connective tissue.

The WHO breast cancer classification (68) divides breast cancer into in situ carcinomas, invasive carcinomas and Paget’s disease. Cancer in situ, non-invasive tumors, are classified as ductal cancer in situ (DCIS) and lobular cancer in situ (LCIS). Another classification system for pathological evaluation made by Fisher and co-workers divide invasive breast carcinoma into ductal (75%), medullary (6%), lobular (5%), mucinous (2%) and tubular (1%) carcinomas. Other rare histological types are papillary, apocrine, adenocystic and Paget’s disease carcinomas.

Risk factors and etiology

Risk factors for developing breast cancer are early menarche, late menopause, ionizing radiation, while early age at first childbirth decreases the risk of breast cancer (69). Today, oral contraceptives and hormonal replacement therapy are considered as established risk factors (70). Notably, the exposure of estrogen to breast tissue represents a great risk factor and endocrine therapy is mostly focused on diminishing the exposure of estrogen in order to treat and prevent recurrence of breast cancer.

Familial breast cancer

About 5-10% of breast cancers are caused by genetic predisposition and nearly 80% of these harbour germline mutations in the breast cancer susceptibility genes BRCA1 or BRCA2 (71). Both genes are involved in DNA repair and gene transcription (72). The risk of developing breast cancer is 60-80% if one of these genes is mutated (73).
Prognostic and predictive factors, and link to therapeutic approach

There are several well-established prognostic and predictive factors related to breast cancer. True prognostic factors appear to remain independent of other factors and seem to predict the natural outcome of the disease. Predictive factors are related to biological features of individual patients and may provide information for selection of therapy.

The well-established prognostic factors today are lymph node status and primary tumor size. A clinical classification of breast tumors (clinical stages I-IV) is developed from the TNM system (74) which is based on the size of the primary tumor (T) lymph node status (N) and distant metastases (M).

Tubular arrangement, mitotic cells and nuclear pleomorphism are considered in histological grading, which is another way to determine the malignancy and differentiation of breast carcinoma (75). In addition, S-phase fraction and DNA-ploidy status are other factors of importance in breast cancer evaluation (76).

The estrogen receptor (ER) and progesterone receptor (PR) status of the tumor have been used to select patients which might respond to endocrine therapy such as tamoxifen (77). Patients co-expressing ER and PR have the highest likelihood to respond to anti-estrogenic compounds (78). Tamoxifen and raloxifene, belong to the selective estrogen receptor modulators (SERMs), which bind to ER and block the action of estrogen in breast cancer tissue (79, 80). In postmenopausal women, both compounds have anti-estrogenic effects in breast cancer and estrogenic effects in bone (79, 81). Today, tamoxifen is well established as a breast cancer drug, whereas raloxifene has been used in osteoporosis, but is also being tested in breast cancer trials (80).

In addition to SERMs, aromatase inhibitors are used as endocrine therapy in breast cancer. The aromatase enzyme is involved in the production of estrogen and inhibition leads to drastically reduced levels of estrogen in favour of an efficient treatment (82).

Although ER is expressed in approximately 70-80% of breast carcinomas only about 50% respond to endocrine therapy (78, 83). Another problem with endocrine therapy is the development of resistance, which may occur with tamoxifen in initially responding patients (82).

Another predictive marker used routinely is the HER2/Neu/ERBB2 proto-oncogene, a receptor belonging to the epidermal growth factor receptor family. Approximately 15-30% of breast cancers overexpress HER2/Neu and these patients have a poor clinical outcome in comparison to HER2/Neu-negative patients (77). Gene amplification/overexpression of HER2/Neu is correlated with shorter disease-free survival unrelated to the node status (84). Furthermore, HER2/Neu overexpression is associated with poor benefit of tamoxifen and cyclophosphamide, methotrexate, 5-fluorouracil (CMF)-
therapy (77). A monoclonal antibody, trastuzumab/Herceptin, targeting the HER2/Neu protein has an antiproliferative effect and promote apoptosis in HER2/Neu positive-breast cancer cells (85, 86). In randomized clinical trials Herceptin immunotherapy has demonstrated increased clinical benefit in patients expressing HER2/Neu (87, 88).

Treatment
For early-stage breast cancer, clinical stage I and II, several randomized clinical studies have demonstrated that the survival rate is the same, whether modified radical mastectomy or breast-conservative surgery plus radiation are performed (89). To exert breast-conservative surgery the tumor cannot be diffuse and free/clear surgical margins must be determined.

Women with larger tumors (4 cm) or multiple tumors in the same breast, need modified radical mastectomy. However, clinically more advanced tumors, of stage III-IV, surgery is performed mainly to obtain local control of the tumor.

Whether chemo-, endocrine or combined therapies are used depend on various predictive factors, stage of cancer and the patient-response. Chemotherapy may include use of antracyclines (adriamycin), taxanes (paclitaxel) and cisplatin (90-92). Often the therapeutic response is improved when several compounds are combined, such as for the CMF-therapy (see above).
Vitamin D

Active vitamin D₃, 1,25(OH)₂D₃ (Fig. 2) has numerous functions in the body. Except for the impact on calcium metabolism, 1,25(OH)₂D₃ has anti-proliferative effect with potential influence on several malignances such as cancer of the breast, colon, prostate and leukaemia (93). The growth inhibitory and differentiating effects in psoriatic keratinocytes is also well-established (94). Furthermore, 1,25(OH)₂D₃ acts in the immune system by suppressing the T-helper 1 (Th-1) response and enhancing the phagocytic activity of macrophages (95, 96). In addition, 1,25(OH)₂D₃ or vitamin D analogues appear to have potential therapeutic effects in diabetes, rheumatoid arthritis and by reducing hypertension (93).

Here, focus will be on the vitamin D regulating enzymes and the effects of vitamin D analogues in sHPT and breast cancer.

![Figure 2](image)

Figure 2. Chemical structure of active vitamin D₃, 1,25(OH)₂D₃. The numbers refer to the carbon atoms in the molecule.
Cytochrome P450 enzymes

The vitamin D activating/inactivating enzymes, 1α-hydroxylase, CYP27A1/vitamin D$_3$ 25-hydroxylase (25-hydroxylase) and CYP24A1/25-hydroxyvitamin D$_3$ 24-hydroxylase (24-hydroxylase) belong to the type I (mitochondrial) cytochrome P450 (CYP) enzyme family catalyzing monooxygenase reactions (97). Bioactivation by CYP enzymes also includes steroid hormone synthesis, bile acid synthesis and degradation of drugs and foreign chemicals. CYP enzymes such as aromatase, 17α-hydroxylase or hepatic enzymes involved in drug metabolism are categorized as type II CYP enzymes located in the endoplasmatic reticulum (ER) (97).

The CYP enzymes are part of an electron transport chain situated in the inner membrane of mitochondria or the microsomal compartment of ER. For the mitochondrial enzymes, including the vitamin D regulating enzymes, the electron transport chain consists of the flavoprotein ferredoxin reductase and the iron-sulfur protein ferredoxin (98). The catalytic activity is dependent on NADPH and oxygen. Sequence alignment of the vitamin D CYP enzymes reveals conserved regions corresponding to heme- and ferredoxin binding sites in the C-terminal (99).

Vitamin D metabolism

Cholecalciferol (vitamin D$_3$) is produced in the skin by ultraviolet-light cleavage of the B-ring in 7-dehydrocholesterol (100). In addition, vitamin D$_3$ as well as ergocalciferol (vitamin D$_2$) are provided by dietary sources. Both vitamin D$_2$ and vitamin D$_3$ are processed in the same way in the liver and kidney, but from now on focus will be mainly on vitamin D$_3$.

Initially, vitamin D$_3$ is 25-hydroxylated in the liver by CYP27A1/25-hydroxylase (101, 102) (Fig. 3). This 25-hydroxylase has also been described in other tissues such as kidney, duodenum, lung, and keratinocytes (101, 103). However, the contribution by this enzyme to vitamin D hydroxylation has been questioned. For example, CYP27A1/25-hydroxylase does not 25-hydroxylate vitamin D$_2$ and there are mice and cerebrotendinous xanthomatosis patients lacking a functional enzyme without obvious 25-OH vitamin D$_3$ deficiency (99). Instead these mice and patients have impaired bile acid synthesis due to the non-functioning CYP27A1/25-hydroxylase. Recently, several other 25-hydroxylating enzymes have been reported in different species (99). A strong candidate in humans is the microsomal enzyme CYP2R1 expressed in human liver, 25-hydroxylating both vitamin D$_2$ and vitamin D$_3$ (104, 105).
Figure 3. Vitamin D activation/inactivation.

1,25(OH)₂D₃ is produced by 1α-hydroxylation of 25(OH)D₃ (Fig. 3) mainly in the proximal convoluted tubule cells of the kidney, but the 1α-hydroxylase (106-108) is also expressed in other parts of the nephron such as the distal convoluted tubules and the cortical collecting ducts (109). Even though the systemic levels of 1,25(OH)₂D₃ is due to production in the kidney, expression of 1α-hydroxylase has also been reported in keratinocytes, macrophages, prostate cells, colon adenocarcinoma, cultured bone cells, testis, brain and islet cells of the pancreas (106, 110-118). The extrarenally produced 1,25(OH)₂D₃ is believed to have an autocrine/paracrine roll. For instance, keratinocytes lacking a functional 1α-hydroxylase proliferate and are less differentiated both in vitro and in vivo in the presence of 25(OH)D₃ compared to control cells (119).

As described earlier, the renal production of 1,25(OH)₂D₃ has a major effect on bone, intestine and the parathyroid gland and is thereby tightly regulated by PTH and 1,25(OH)₂D₃. To obtain optimal 1,25(OH)₂D₃ levels, PTH not only up-regulates 1α-hydroxylase, but also decreases the mRNA stability of 24-hydroxylase, the degrading enzyme of 1,25(OH)₂D₃ (5, 120-122). The negative feed-back mechanism is strong, since high levels of 1,25(OH)₂D₃ instantly down-regulates 1α-hydroxylase and enhances the mRNA levels of 24-hydroxylase through the two vitamin D response elements (VDREs) in the 24-hydroxylase gene promoter (5, 123, 124). In conclusion, both 1α-hydroxylase and 24-hydroxylase are strictly regulated by PTH and 1,25(OH)₂D₃.

Recently, a mechanism for how PTH and 1,25(OH)₂D₃ regulate 1α-hydroxylase expression in the kidney has been reported (125). A novel transcription factor named VDIR situated at two E-box-like motifs in the 1α-hydroxylase promoter, distinct from a VDRE, binds to coactivators in re-
response to PTH resulting in up-regulation of the 1α-hydroxylase gene. However, upon 1,25(OH)2D3 exposure the VDIR binds to ligand-activated VDR leading to dissociation of coactivators and recruitment of a corepressor-complex resulting in repression of transcription.

The extrarenal regulation of 1α-hydroxylase is reported to differ from the regulation of the renal counterpart. Interestingly, neither in macrophages nor keratinocytes did 1,25(OH)2D3 suppress 1α-hydroxylase mRNA or 1α-hydroxylase activity to the same extent as demonstrated in the kidney (5, 112, 114, 126, 127). Notably, in keratinocytes the reduction of 1α-hydroxylase activity was rather due to degradation of the 1α-hydroxylase-substrate, 25(OH)D3, by 1,25(OH)2D3-induced 24-hydroxylase (126, 127).

The 24-hydroxylase ultimately degrades 1,25(OH)2D3 to the biliary excretory form calcitroic acid, referred to as the C-24 oxidation pathway. The steps of the C-24 oxidation pathway are 24-hydroxylation, 24-oxidation, 23-hydroxylation, C-23-24 cleavage, formation of 1α(OH)-23-OH-24,25,26,27 tetranor-D3 and finally calcitroic acid as the end product (128) (Fig. 4).

![Figure 4. C-24 oxidation pathway.](image-url)
Notably, the other natural 24-hydroxylase substrate, 25(OH)D₃, is also degraded according to the C-24 oxidation pathway. In addition, 24-hydroxylase is ubiquitously expressed in the body and not only in the classical calcium-regulating organs such as kidney, bone and intestine (129).

In sarcoidosis and tuberculosis activated macrophages produce high levels of 1,25(OH)₂D₃ due to 1α-hydroxylase-activity in these cells (113, 130). The dysregulation of 1,25(OH)₂D₃ might be explained by a recent report (131), presenting macrophages expressing a RNA splice variant of 24-hydroxylase inactive to degrade 1,25(OH)₂D₃.

There are several CYP inhibitors available disturbing the binding of the oxygen to the heme iron and the docking of the substrate to the active site of the CYP enzyme. Ketoconazole and liarozole are examples of unspecific CYP inhibitors, but recently more specific inhibitors for 1α- and 24-hydroxylase have been developed (94, 132).

**Gene regulation by vitamin D**

Active vitamin D exerts its function through VDR, which belongs to the nuclear hormone receptor superfamily. Nuclear receptors such as retinoic, thyroid, estradiol and progesterone receptors are example of other members of this receptor family. VDR expression in a vast number of tissues is crucial for 1,25(OH)₂D₃ genomic effects. Binding of 1,25(OH)₂D₃ to the receptor introduces a conformational change in VDR essential for the liganded VDR to dimerize with primarily the retinoid X receptor (RXR) (96). The ligand/VDR/RXR complex binds to the VDREs in promoters of vitamin D responsive genes. Some genes are up-regulated by vitamin D such as 24-hydroxylase, p21, osteocalcin and osteopontin, whereas other genes are suppressed such as PTH, 1α-hydroxylase and c-myc (133). The mechanism for how 1,25(OH)₂D₃ either up- or down-regulates genes involves the recruitment of either coactivators or corepressors by the ligand/VDR/RXR at the promoter (96, 125).

1,25(OH)₂D₃ also performs rapid non-genomic responses affecting calcium membrane transport and signal transduction, involving VDR (134, 135). Expression of VDR not only in the nucleus but also in the cytoplasm and the cytoplasmic membrane may explain the involvement of VDR in these rapid effects of 1,25(OH)₂D₃ (136, 137).
Hereditary vitamin D-dependent rickets type I and II

Classical nutritional rickets is due to lack of sunlight exposure and dietary vitamin D. Hereditary rickets is caused by mutations in 1α-hydroxylase or VDR genes, both situated on chromosome 12q13-14. There is also another form of hereditary rickets named hypophosphatemic (HYP) rickets mapped to chromosome X. Mutations in 1α-hydroxylase cause vitamin D-dependent rickets type I (VDDR-I) associated with normal 25(OH)D3 and low 1,25(OH)2D3 serum levels, since 1α-hydroxylase is inactive (97). In VDDR type II (VDDR-II), where VDR is mutated, the 1,25(OH)2D3 serum levels are high in combination with a tissue insensitivity to 1,25(OH)2D3 (138). Except for different 1,25(OH)2D3 serum levels the symptoms for VDDR-I and –II are similar, characterized by hypocalcemia, hypophosphatemia, secondary HPT and bone malformation (97, 138). Moreover, mice lacking a functioning 1α-hydroxylase or VDR exhibit similar manifestations as VDDR-I and -II patients (139-142).

Vitamin D analogues

Apart from being essential in calcium homeostasis, 1,25(OH)2D3 has a therapeutic effect in several disorders such as hyperproliferative, autoimmune and endocrine disease. Unfortunately, active vitamin D causes hypercalcemia and thereby vitamin D analogues are being developed and used, with less side effects. Usually, structural modifications are introduced in the side-chain of 1,25(OH)2D3 to alter the catabolic rate. Moreover, structural changes in the side-chain most certainly alter the affinity for vitamin D binding protein (DBP) giving the vitamin D analogue a shorter half-life in serum in combination with enhanced tissue accessibility (143). In the vitamin D analogues first produced, the A-ring was mostly unchanged to preserve the VDR affinity. However in new hybrid vitamin D analogues, A-ring modifications have been introduced with success, implying that optimal VDR transcriptional activity is not only reached through VDR affinity but by producing a stable ligand/VDR/RXR/cofactor complex (100). Except for vitamin D analogues representing VDR agonists, also VDR antagonists and non-agonists have been developed, inducing unique conformational changes in the VDR (144).
Vitamin D analogues and secondary hyperparathyroidism

Today in Sweden, the vitamin D compounds Rocaltrol® (Hoffman-La Roche) or Etalpa® (Leo Pharma.) corresponding to 1,25(OH)2D3 and the prehormone alfacalcidol (1α(OH)D3) respectively, are in clinical use for sHPT to decrease serum PTH levels and proliferation of parathyroid cells. In other countries vitamin D analogues are in clinical use for sHPT such as 22-oxacalcitriol (OCT) and 26,27-F6-calcitriol (falecalcitriol) in Japan, and 19-nor-1α,25-dihydroxyvitamin D2 (19-norD2) and doxercalciferol/1α-hydroxyvitamin D2 (1α(OH)D2) in the United States (Fig. 5) (145).

In in vitro and animal experiments, OCT has been shown to reduce PTH synthesis and secretion with less hypercalcemic effects than 1,25(OH)2D3 (146-148). Unfortunately, in long-term clinical trials or at high dosage with OCT hypercalcemic episodes occur in sHPT patients (149-151). In addition, a recent comparative study showed no significant difference between OCT and 1,25(OH)2D3 with respect to serum PTH, calcium or phosphate levels during treatment (151).

Falecalcitriol is another vitamin D analogue used for treatment of sHPT (152, 153). In a clinical trial, falecalcitriol demonstrated better PTH suppres-
sive effect than alfacalcidol, but there was no difference between the two compounds regarding the moderate increase in serum calcium (153).

19-norD$_2$ has been convincing in down-regulating PTH, causing minimal hypercalcemia (154-160). An explanation for the low hypercalcemic effect by 19-nor D$_2$ in rats, could be that 19-norD$_2$ is less efficient than both 1,25(OH)$_2$D$_3$ and 1α(OH)D$_2$, in stimulating bone resorption and intestinal absorption of calcium and phosphate (161, 162). In a clinical multicenter study, 19-norD$_2$ suppressed PTH more rapidly than calcitriol with fewer sustained hypercalcemic episodes (160). Interestingly, 19-norD$_2$ was related to a 16% survival-advantage in comparison to calcitriol during 36 months of treatment in hemodialysis patients (163). This advantage in survival is probably due to less inflammatory and cardiovascular events in 19-norD$_2$-treated hemodialys patients (164, 165).

Clinical studies claimed that 1α(OH)D$_2$ safely controlled sHPT, even if cases of hypercalcemia were reported (166, 167). Unfortunately, no clinical study comparing 1,25(OH)$_2$D$_3$ with 1α(OH)D$_2$ has yet been performed.

Recently, a new vitamin D analogue, 1α-hydroxy-3-epi-vitamin D$_3$, was shown to exhibit low calcemic and PTH suppressive effects in uremic rat (168). Future studies will reveal its efficiency in sHPT patients.
Vitamin D analogues and breast cancer

Ever since the discovery of VDR expression and antiproliferative characteristics of 1,25(OH)_2D_3 in breast cancer (169-174), the interest of developing the ultimate vitamin D analogue for treatment of breast cancer has been an important goal.

The vitamin D analogue, mostly studied in breast cancer is EB1089 (Fig. 6), which is 50% less calcemic compared to 1,25(OH)_2D_3 in rats, and several times more potent regarding antiproliferative effect in the cell types studied (133). Even though, EB1089 has an elongated and polyunsaturated side-chain to prevent EB1089 from degradation by 24-hydroxylation, the biological half-life of EB1089 is about the same as for 1,25(OH)_2D_3 in rats. However, EB1089 enhances transcriptional activity more effectively than 1,25(OH)_2D_3 probably due to increased stability of the liganded VDR/RXR/cofactor complex at the VDRE (133).

The antiproliferative properties of EB1089 and 1,25(OH)_2D_3 in breast cancer cell lines are due to inhibition of cell cycle progression and promotion of proapoptotic events (175). The majority of MCF-7 cells, a breast cancer cell line, exposed to EB1089 are retained in the G0-G1 stage. The cell cycle restricting gene p21 is up-regulated, the RB protein is prevented from phosphorylation and the protein level of the proto-oncogene c-myc is decreased (175-177). Apoptotic events such as involvement of the bcl-2 family proteins, disrupted mitochondria and production of reactive oxygen species are also reported in MCF-7 cells exposed to vitamin D analogues (178-180). Furthermore, the apoptotic pathway of EB1089 is reported to be independent of caspases, (178, 180-182), but whether the tumor suppressor gene p53 is involved in the apoptotic effects is unclear, due to contradicting reports (145, 178). p53 is mutated in 20-30% of breast carcinomas (83) and thereby it is of major importance to develop treatments independent of a functional p53.

Numerous in vitro and in vivo studies have demonstrated that EB1089 has an antiproliferative and proapoptotic effect in breast cancer (179, 183-185). In addition, EB1089 sensitizes the breast cancer cell line or tumor to irradiation, antiestrogens, vitamin A, paclitaxel, cisplatin and doxorubicin (186-193). Chemotherapy has severe side effects and if a vitamin D analogue could decrease the dosage it would be of great advantage. In addition, clinical trials with EB1089 have been performed in breast, colorectal, pancreatic and hepatocellular cancer (194-196). Unfortunately, the clinical trials have not been as successful as required and thereby the development of EB1089 has been discontinued (145).

Other vitamin D analogues tested to a lesser extent in breast cancer cells or animal models are OCT, calcipotriol (MC903), 1α-hydroxy-24-ethylcholecalciferol (1α(OH)_2D_3), 20-epi and 16-ene-vitamin D analogues.

Both in vitro and in vivo OCT inhibited proliferation of ER-positive and ER-negative breast cancer cells (197, 198). OCT alone or in combination
with an aromatase inhibitor had a growth inhibitory effect in 7,12-
dimethylbenzanthracene (DMBA) induced rat mammary tumors without
causing hypercalcemia (199).

Topical application of MC903 in breast cancer patients resulted in a par-
tial response in 3 out of 14 patients (200). Also in vivo studies in rat revealed
an antitumorigenic activity of MC903 (201).

Growth inhibitory and cell differentiating effects were demonstrated in
T47D mammary tumor cells exposed to 1α(OH)D₃ (202). Interestingly,
1α(OH)D₃ prevented DMBA induced preneoplastic lesions in mammary
gland organ cultures (203) and in vivo in rats (204).

Both the 20-epivitamin D analogue, CB1093 and the 16-ene-vitamin D
analogue 16-ene-23-yne-19nor-26,27-F6-D₃ had antiproliferative potential of
MCF-7 cell xenografts in mice alone or in combination with paclitaxel (191,
205).
Aims of the study

The specific aims of the study were:

- to investigate whether 1α-hydroxylase (CYP27B1) is expressed in the parathyroid glands and quantitatively determine expression of 1α-hydroxylase and 24-hydroxylase (CYP24A1) in parathyroid tumors of HPT and breast cancer. In parathyroid tumors, the aim was also to examine the expression level of 25-hydroxylase (CYP27A1).

- to select and evaluate new vitamin D analogues in primary bovine parathyroid cells for future treatment of sHPT.

- to examine whether a non-1α-hydroxylated precursor vitamin D analogue has an inhibitory effect on PTH secretion in bovine parathyroid cells and on cell proliferation in human pathological parathyroid cells.

- to investigate whether a non-1α-hydroxylated precursor vitamin D analogue could be biologically active in breast cancer cells expressing 1α-hydroxylase.

- to study the effect of the CYP inhibitor ketoconazole on PTH suppression as well as the transcriptional activity of vitamin D compounds in bovine parathyroid cells and breast cancer cells, respectively.
Materials and Methods

Summary of materials and methods

The following is a brief summary of the materials and methods used in this thesis. A more detailed description can be found in the individual papers.

Subjects
(Paper I, II, IV and V)
All clinical data and tissue specimens, except for two parathyroid carcinomas provided by prof. Henning Dralle, Germany, were collected from the clinical routine at Uppsala University Hospital. Approval of ethical committee and informed consent were achieved.

Immunohistochemistry
(Paper I, IV and V)
Acetone-fixed cryosections of 6 μm were immersed in 0,3% H₂O₂ to quench the endogenous peroxidase activity followed by blockage using an avidin-biotin blocking kit (Vector Laboratories, Inc., Burlingame, CA) or normal goat serum. The tissue sections were exposed to the 1α-hydroxylase sheep polyclonal peptide antiserum (109, 206) diluted 1/300 (Paper I and V) or 1/50 (Paper IV) or the VDR rabbit polyclonal peptide antiserum (sc-1008, Santa Cruz Biotechnology, Inc., Santa Cruz, California, USA) diluted 1/400 (Paper IV and V), for 90 min at room temperature. After 30 min of incubation with secondary antibodies, the biotinylated donkey anti-sheep IgG (diluted 1/500) (Paper I, IV and V) or the biotinylated goat anti-rabbit IgG (diluted 1/200) (Paper IV and V) an avidin-biotin complex (Vector Laboratories, Inc.) were applied. The sections were developed with 3-amino-9-ethylcarbazole followed by counterstaining with Mayer’s hematoxylin. Negative controls corresponded to sections exposed to the 1α-hydroxylase antiserum or VDR antiserum preincubated with an excess of the 1α-hydroxylase or the VDR (sc-1008P, Santa Cruz Biotechnology Inc.) immunizing peptides.

In addition, acetone-fixed MCF-7 cells were also subjected to the 1α-hydroxylase-staining procedure (Paper IV).
RNA isolation and cDNA synthesis
(Paper I - V)
The total RNA was extracted using TriZol reagent (Invitrogen Corp., Carlsbad, CA), treated with RQ1 Dnase I (Promega, Madison, WI) before further purification with proteinase K (Paper I, II, IV) or using Rneasy Mini kit columns (Qiagen, Hilden, Germany) (Paper III and V). The total RNA was reverse transcribed to cDNA using the First Strand cDNA Synthesis Kit (Amersham Biosciences, Uppsala, Sweden).

RT-PCR
(Paper I and II)
Expression of 1α-hydroxylase (Paper I), 25-hydroxylase and 24-hydroxylase (Paper II) mRNA were demonstrated in parathyroid, kidney and liver specimens by RT-PCR analysis. All primers were mRNA-specific and the PCR fragments were confirmed by sequence analysis using the ABI prism dye terminator cycle sequencing ready kit (Applied Biosystems, Foster City, CA).

Real-time quantitative RT-PCR
(Paper I and II)
The real-time quantitative RT-PCR was performed with two mRNA-specific primers and a hybridizing probe inbetween (207, 208). The probe has a flourescent reporter dye at the 5' end and a quencher dye attached to the 3' end. On the probe, the close vicinity of the energy emission of the quencher inhibits the reporter dye emission. During extension the 5’ to 3’ exonuclease activity of the Taq DNA polymerase degrades the probe separating the reporter from the quencher resulting in increased fluorescence corresponding to the initial levels of target fragment. The fluorescence detection was performed with the ABI PRISM 7700 Sequence Detector (Applied Biosystems). The mRNA levels of 1α-hydroxylase, 24-hydroxylase and 25-hydroxylase were determined in normal parathyroid tissues, parathyroid tumors and carcinoma. The housekeeping gene glyceraldehyde-3-phosphate dehydrogenase (GAPDH) was used as internal standard. The PCR reaction contained 5 µl cDNA template, 1x TaqMan buffer A, 5,5 mM MgCl₂, 200 µM of dATP, dCTP, dGTP and 400 µM dUTP, 200 nM probe, 100 nM of each primer, 0.01 U AmpErase® UNG and 0.05 U AmpliTaq™ Gold (Applied Biosystems). Each cDNA sample was analyzed in triplicate.
Semi-quantitative RT-PCR
(Paper III, IV and V)
Semi-quantitative RT-PCR analysis to examine the expression of relative mRNA levels was performed using GAPDH (Paper III and V) or 28SrRNA (Paper IV) as internal controls. The number of PCR cycles avoiding saturation was determined for each PCR reaction. The identity of each PCR fragment was confirmed with sequence analysis using the ABI prism dye terminator cycle sequencing ready kit (Applied Biosystems). Using mRNA-specific primers the expression levels of human 1α-hydroxylase (Paper IV), human 24-hydroxylase (Paper IV), human VDR (Paper IV), bovine PTH (Paper III and V) and bovine 24-hydroxylase (Paper V) were examined. The PCR reactions were performed in Gene Amp 9700 thermal cycler (Applied Biosystems) with thermal cycler conditions carefully tested for each PCR transcript. After separating the PCR reactions on a 2% ethidium bromide stained agarose gel the intensity of each PCR transcript was quantified by Molecular Analysis software (BioRad Lab., Richmond, CA).

Parathyroid cell dispersion
(Paper III and V)
Dispersed cells were prepared from biopsies of normal bovine parathyroid glands from adult cattle or pathological human parathyroid glands obtained during surgery from patients with primary or secondary HPT, as described previously (209). After the biopsies were minced with scissors the cell suspensions were prepared by shaking for 30-45 min (for human) and 120 min (for bovine) in 1 mg/ml collagenase (Sigma, St Louis, Missouri, USA), 0.05 mg/ml DNase I, 1.5% bovine serum albumin and 1.25 mM Ca\(^{2+}\). In addition, the cell suspension was purified by centrifugation through 25% and 75% standard isotonic Percoll (Amersham Biosciences Europe, Germany). The cell viability routinely exceeded 95% with the Trypan blue exclusion test.

PTH secretion in bovine parathyroid cells
(Paper III and V)
Bovine parathyroid cells were seeded in 6-well plates (10\(^6\) cells/well), and cultured for 4 hours in DMEM/Ham’s F-12, 1 mM total calcium, 4% fetal bovine serum, 15 mM HEPES, 100 U/ml penicillin, 100 µg/ml streptomycin, 5 µg/ml insulin, 2 mM glutamine and 1% nonessential amino acids (210). After 4 hours medium was replenished with the same ingredients as above, apart from 1mg/ml bovine serum albumin instead of serum, addition of holotransferrin to 5 µg/ml, vehicle, vitamin D analogues (Paper III and V), ketoconazole (Paper V) or ketoconazole in combination with vitamin D analogues (Paper V). Once, 28 hours before harvest the serum free medium with
the different test compounds were replenished. The cells were exposed to the test compounds for totally 60 hours, followed by a 4 hour long PTH secretion test. Measurement of the PTH protein concentration in the collected medium was performed with a radioimmunoassy kit (Peninsula Lab Inc., San Carlos, CA) and were related to total RNA extracted from each well. Experiments were done at least in triplicates.

**Calcium metabolism in rat**

(Paper III)

Daily for 7 days a fixed concentration of 1,25(OH)$_2$D$_3$ and increasing concentrations of OCT, EB1089, GS1590, CB1393, MC1598 and HEP187 were administrated orally to LEW/mol female rats. Urinary calcium from day 3 to 7 was collected and measured. The concentration for each vitamin D analogue having the same effect on urinary calcium in normal rat as 1,25(OH)$_2$D$_3$ (0.5µg/kg per day) was determined. The calcemic activity for each vitamin D analogue in comparison to 1,25(OH)$_2$D$_3$ corresponded to the ratio between the concentration determined for each vitamin D analogue and 1,25(OH)$_2$D$_3$ (0.5µg/kg per day).

**VDR binding assay**

(Paper III)

$^3$H-1,25(OH)$_2$D$_3$ (Amersham Biosciences) and increasing concentrations of 1,25(OH)$_2$D$_3$ or the vitamin D analogues, OCT, EB1089, GS1590, CB1393, MC1598 and HEP187 were incubated for 60 min at 22 ºC with intestinal VDR of rachitic chicken (Amersham Biosciences). Free and VDR-bound $^3$H-1,25(OH)$_2$D$_3$ were separated and the receptor bound $^3$H-1,25(OH)$_2$D$_3$ was measured in a beta-counter. The concentration of 1,25(OH)$_2$D$_3$ or of each vitamin D analogue causing 50% displacement of VDR bound $^3$H-1,25(OH)$_2$D$_3$ was determined.

**Transient transfection in MCF-7 cells**

(Paper III and IV)

Transient transfection studies with MCF-7 cells were performed using DOTAP liposomal transfection reagent (Roche, Mannheim, Germany) (Paper III) or FuGENE 6 reagent (Roche Diagnostics Scandinavia AB, Bromma, Sweden) (Paper IV). The cells were cotransfected with a luciferase reporter gene plasmid with four vitamin D response elements (pMWM-30, M.W. Madsen, unpublished), an expression vector for VDR (pSG5-VDR) and a reference gene (CMV-LacZ) following exposure to a variety of vitamin D compounds (Paper III and IV), ketoconazole (Paper IV) or vitamin D com-
pounds in combination with ketoconazole (Paper IV). The luciferase activity was measured luminometrically and related to β-galactosidase activity.

Proliferation assay
(Paper III and V)
Proliferation assay was performed in MCF-7 cells (Paper III) or as earlier described (211) in primary cells of sHPT and pHPT patients (Paper V). After exposure of the different vitamin D analogues for 5 days (Paper III) and for totally 72 hours (Paper V) the cells were incubated with ³H-thymidine for the last 4 hours (Paper III) or 24 hours (Paper V) of culture. The cells were harvested and the intracellular radioactivity measured.

Statistical analysis
(Paper I-V)
Statistical analysis was performed with Stat View 5.0 (SAS Institute, Inc., Cary NC). In all papers Student's unpaired t test was used and the values are presented as the arithmetical mean ± SEM or as geometrical mean (in parentheses) multiplicative SD for gland weight (Paper I). P < 0.05 was considered significant.
Results and Discussion

Summary of observations and discussions
Paper I and II

25-hydroxyvitamin D$_3$ 1α-hydroxylase (CYP27B1), 25-hydroxyvitamin D$_3$ 24-hydroxylase (CYP24A1) and vitamin D$_3$ 25-hydroxylase (CYP27A1) in parathyroid tumors

Our group has earlier identified and cloned the human LRP2 receptor and it was suggested a role in calcium-sensing of the parathyroid gland (21, 22, 26). However, the function of LRP2 in this tissue is still unclear. Interestingly, LRP2 has been shown to exert reabsorption of the 25(OH)D$_3$/DBP complex from the primary urine in the kidney (28). The reabsorbed 25(OH)D$_3$ was shown to be essential for the synthesis of 1,25(OH)$_2$D$_3$ in the proximal tubule cells. The interpretation of these results suggested that LRP2 is a novel membrane bound receptor for 25(OH)D$_3$. Since LRP2 is expressed also in parathyroid chief cells (26, 212) we hypothesized that these cells might endocytose and hydroxylate 25(OH)D$_3$ to 1,25(OH)$_2$D$_3$. A prerequisite was that parathyroid cells express the vitamin D activating enzyme 1α-hydroxylase.

In paper I and II we demonstrated by RT-PCR that all three vitamin D hydroxylating enzymes, 25-hydroxylase, 1α-hydroxylase and 24-hydroxylase were indeed expressed in normal tissue and tumors of the parathyroid gland. We confirmed these results with immunohistochemistry for 1α-hydroxylase and 25-hydroxylase (unpublished results). 24-hydroxylase has earlier been claimed to be expressed in the parathyroid gland (213). Furthermore, we performed real-time quantitative RT-PCR to investigate if the vitamin D hydroxylating enzymes were differentially expressed in parathyroid tumors versus normal tissue. The tumor specimens for the 1α-hydroxylase mRNA determination consisted of 5 normal parathyroid glands, 15 parathyroid adenomas, 10 secondary hyperplastic glands (8 nodular and 2 diffuse hyperplasia) and 5 parathyroid carcinomas (3 primary tumors and 2 metastases). Except for one adenoma and one secondary hyperplastic gland the same tissues were analyzed for 24- and 25-hydroxylase expression levels.
We found higher mRNA levels of 1α-hydroxylase in parathyroid adenomas (80.2 ± 18.0, p=0.03) and hyperplastic glands of uremic sHPT (49.3 ± 12.8, p=0.03) compared to normal glands (5.1 ± 1.9), while parathyroid carcinomas displayed reduced expression (0.4 ± 0.11, p=0.04).

In addition, compared to normal parathyroid tissue 24- and 25-hydroxylase mRNA levels were significantly reduced in parathyroid adenomas with 58% (p=0.01) and 79% (p=0.001), secondary hyperplastic glands with 89% (p=0.0001) and 88% (p=0.002) and parathyroid carcinomas with 96% (p=0.0003) and 96% (p=0.01). Only a small number of adenomas exhibited normal levels of 24-hydroxylase (3 adenomas) and 25-hydroxylase (1 adenoma). These four adenomas all overexpressed 1α-hydroxylase.

In 61% of the investigated primary and secondary parathyroid tumor specimens increased 1α-hydroxylase mRNA levels were combined with reduced 24-hydroxylase mRNA expression. These results may indicate high levels of locally produced 1,25(OH)₂D₃, not only because 1α-hydroxylase is overexpressed but also due to decreased degradation of both 25(OH)D₃ and 1,25(OH)₂D₃. Although the LRP2 receptor is generally underexpressed in parathyroid tumors (212), the endocytotic supply of 25(OH)D₃ or the intracellular storage of fat soluble vitamins may still be sufficient to locally produce 1,25(OH)₂D₃. Autocrine/paracrine action of increased or maintained local 1,25(OH)₂D₃ levels may have antiproliferative or prodifferentiative effects in these benign tumors, as has been described earlier in keratinocytes (119). The comparatively low expression level of 24-hydroxylase as well as the high level of 1α-hydroxylase could relate to the reduced VDR expression in parathyroid tumors (57, 58, 61).

CYP27A1/25-hydroxylase is not the only enzyme performing 25-hydroxylation of vitamin D (99). It is quite possible that additional enzymes exert 25-hydroxylation of vitamin D in the parathyroid gland as mentioned earlier in the Introduction.

The wide tissue distribution of vitamin D hydroxylases extend their role beyond the classical physiological functions related to calcium and phosphate homeostasis. Appropriate dosage of vitamin D or vitamin D analogues has already proved to interfere with sHPT and maintain normal proliferation and differentiating effects in several tissues including prostate, colon, breast, leukemia and skin (214). We suggest that local activity of 1,25(OH)₂D₃ may contribute to the benign feature of parathyroid adenomas and hyperplastic glands of sHPT. This local production can most likely be enhanced by treatment with 25(OH)D₃ or by non-1α-hydroxylated precursor vitamin D analogues, since expression of 1α-hydroxylase is enhanced in tumors of both primary and sHPT. In addition, this local production of 1,25(OH)₂D₃ or active vitamin D analogue may not only apply to colon, prostate and parathyroid tumors, but also to other tumors where 1α-hydroxylase is expressed. The present findings warrant further studies on hydroxylase expression in malignances and open new possibilities for designing vitamin D analogues.
Evaluation of five vitamin D analogues for future therapy of secondary HPT (Paper III)

Even though the clinically used vitamin D analogues, OCT, 19-norD₂, 1α(OH)D₂ and falecalcitriol have increased the dosage range and reduced the calcemic effects in secondary HPT patients compared to 1,25(OH)₂D₃, still there is need for less hypercalcemic analogues with growth controlling potential of the parathyroid gland.

Five vitamin D analogues, EB1089, GS1590, CB1393, MC1598 and HEP187 (Fig. 6) were chosen upon their low calcemic effect in normal rat, elevated VDR transcriptional activity and increased antiproliferative potential compared to 1,25(OH)₂D₃.

In cultured primary bovine parathyroid cells, the ability of the five vitamin D analogues to suppress PTH secretion as well as PTH mRNA level were compared to 1,25(OH)₂D₃, OCT, 19-norD₂, 1α(OH)D₂ (Fig. 5).

For all the examined vitamin D analogues, except for HEP187, the PTH secretion and the PTH mRNA levels significantly (p< 0.05) decreased by 23-36% at 10⁻⁸ M, 18-29% at 10⁻¹⁰ M and 5-24% at 10⁻¹¹ M. Notably, there was no significant effect of any analogue, at the concentration of 10⁻¹² M. Only GS1590 and OCT suppressed PTH secretion and/or PTH mRNA level by
20-30% at 10^{-11} \text{ M}, with no significant difference between the two analogues. When comparing all vitamin D analogues which had an effect, there were no significant differences between any of them, or between analogues and 1,25(OH)_{2}D_{3}. The results confirmed earlier studies where OCT and 19-norD_{2} showed equal effect on PTH secretion as 1,25(OH)_{2}D_{3} (146, 154). As previously observed, PTH secretion and PTH mRNA level were suppressed equally by the vitamin D compounds (215), indicating that the regulation is performed at transcriptional level.

In Table 1 (Paper III), different characteristics of the vitamin D analogues in comparison to 1,25(OH)_{2}D_{3} are presented. The calcemic effect in normal rat compared to 1,25(OH)_{2}D_{3} was reduced by 50% for EB1089, 84% for GS1590, 90% for CB1393, 83% for MC1598, 70% for OCT and by 59% for HEP187. The calcemic activity of 19-norD_{2} and 1\alpha(OH)D_{2} was not determined in these experiments.

Although the VDR binding affinity determined for all analogues except for 19-norD_{2} and 1\alpha(OH)D_{2} (Paper III) was less than for 1,25(OH)_{2}D_{3}, both the inhibition of cell proliferation and VDR transactivating potential in MCF-7 cells were increased several fold for EB1089, GS1590, MC1598, CB1393 and HEP187. However, the clinically used vitamin D analogues 19-norD_{2} and 1\alpha(OH)D_{2} exhibited decreased antiproliferative activity and reduced VDR transcriptional effect compared to 1,25(OH)_{2}D_{3}. The proliferation assay was performed in MCF-7 cells because bovine parathyroid cells grow poorly in vitro.

In summary, we have shown that four of the five examined vitamin D analogues are equipotent to OCT, 19-norD_{2} and 1\alpha(OH)D_{2} in inhibiting PTH secretion and reducing PTH mRNA level in bovine parathyroid cells. In combination with low calcemic activity, high transcriptional potential and impressive inhibition of proliferation the vitamin D analogues EB1089, GS1590 and CB1393 are of great interest for further evaluation, as candidate compounds for treatment of sHPT and will hopefully be suitable for clinical use.
25-hydroxyvitamin D₃ 1α-hydroxylase expression in breast cancer and use of non-1α-hydroxylated vitamin D analogue

1α-hydroxylase is expressed in several malignances such as colon, prostate and ovarian cancer (116, 216-220). In our previous studies we have demonstrated that 1α-hydroxylase is highly expressed in parathyroid tumors. We also presented the idea that expression of 1α-hydroxylase could be used therapeutically to locally convert a precursor vitamin D analogue to an active drug and in that way avoid side effects such as hypercalcemia (Paper I, II).

Since it was not possible to test this idea in a parathyroid tumor cell line we turned to breast cancer where vitamin D analogues, such as EB1089, has well-established anti-tumorigenic effects. Our first aim was to determine the 1α-hydroxylase mRNA levels in breast carcinomas and the second aim was to investigate whether a non-1α-hydroxylated precursor to EB1089, EB1285 (Fig. 7), displayed transcriptional activity in a 1α-hydroxylase-expressing breast cancer cell line.

![Figure 7. 1α-hydroxylase converts EB1285 into EB1089.](image)

First we determined the mRNA levels of 1α-hydroxylase, VDR and 24-hydroxylase relative to the mRNA levels of 28S rRNA in 19 randomly chosen breast cancer specimens (17 ductal and 2 lobular breast carcinomas) and in 10 normal breast tissue lesions. The semi-quantitative RT-PCR analysis revealed that the breast cancer specimens had significantly lower 1α-hydroxylase/28SrRNA ratio (0,7±0,05, p<0,001) than normal breast tissue (1±0,07). The VDR/28SrRNA ratio (1,4±0,12, p<0,05) and the 24-hydroxylase/28SrRNA ratio (2,1±0,2, p<0,001) were significantly higher in the breast cancer tissue than normal breast tissue (1±0,09) and (1±0,08) respectively.
In addition, the protein expression of 1α-hydroxylase and VDR was demonstrated by immunohistochemical analysis using polyclonal peptide antibodies. All 10 normal breast and 15 out of 19 breast cancer specimens exhibited a positive 1α-hydroxylase staining pattern. VDR protein expression was detected in 95% of all breast tissue specimens. Only one normal breast tissue and one ductal breast cancer biopsy showed no staining for VDR. Notably, also MCF-7 breast cancer cells showed expression of the 1α-hydroxylase protein. Importantly, both 1α-hydroxylase and VDR immunohistochemical staining pattern were consistent with the mRNA levels of 1α-hydroxylase and VDR.

Even though the 1α-hydroxylase expression was generally somewhat lowered in breast carcinoma it was not by far to the extent as in parathyroid carcinoma and in a few parathyroid tumors of primary and secondary HPT (Paper I). In our breast cancer specimens 95% stained positively for VDR in comparison to 80-90 % in earlier studies (172, 173). In addition, VDR has previously been shown to be upregulated at the protein level, when compared to normal breast tissue. Only about 68% of normal breast tissue in that study expressed detectable levels of VDR (221).

Interestingly, the breast carcinomas displayed a two-fold increase in 24-hydroxylase mRNA level supporting the proto-oncogene hypothesis for 24-hydroxylase (222).

In a recent study, 1α-hydroxylase as well as 24-hydroxylase and VDR mRNAs, were reported to be highly overexpressed in a number of individual breast tumor samples compared to matched normal samples (223). This is not consistent with our results. The authors, however, attributed the overexpression of 1α-hydroxylase to the presence of infiltrating macrophages. The breast cancer samples that we used for the immunohistochemical and mRNA analysis consisted of consecutive 6 μm tissue cryosections, essentially free of infiltrating immune cells. Thus, these circumstances could explain the inconsistent results.

The noncalcemic prohormone 25(OH)D₃ has been demonstrated to exert an antiproliferative activity in 1α-hydroxylase-expressing prostate cancer cells (219). Unfortunately, a huge disadvantage of 25(OH)D₃ as therapeutic alternative is the efficient inactivation of 25(OH)D₃ by 24-hydroxylation. However, in keratinocytes 25(OH)D₃ combined with a specific 24-hydroxylase inhibitor showed promising growth inhibitory effects (94). In theory, the best therapeutic precursor vitamin D analogue would be a non-calcemic 24-hydroxylase resistant compound hydroxylated into a fully active drug. In our study we used the non-1α-hydroxylated precursor of EB1089, named EB1285. The identical side chains of EB1285 and EB1089 are elongated and polyunsaturated, to avoid 24-hydroxylation. The calcemic effect of EB1285 is low compared to 1,25(OH)₂D₃ in normal rat (Kaae Holm, unpublished). To study whether MCF-7 cells express a functional 1α-hydroxylase enzyme and if EB1285 could convert to an active vitamin D analogue we
examined the transcriptional activity of EB1285 in MCF-7 cells transfected with a VDRE-luciferase reporter gene construct and a VDR expression vector. The transfected MCF-7 cells were exposed to EB1089, EB1285 alone or in combination with the unspecific CYP inhibitor ketoconazole. Notably, only partial inhibition of enzymatic activity for both 1α-hydroxylase and 24-hydroxylase is obtained in vitro by ketoconazole (224).

EB1089, showed the same potent transcriptional activity whether ketoconazole was present or not, likely due to the inherent resistance to 24-hydroxylation (225, 226). Transcription was activated 14-fold by EB1285 at 10 nM and in combination with ketoconazole the activity was suppressed by approximately 50%. Thus, the ketoconazole-sensitive transcriptional activity induced by EB1285 strongly indicated presence of 1α-hydroxylase activity in these breast cancer cells. The transcriptional potential of EB1285 was approximately 20% when compared to 1,25(OH)2D3 and about 0.2% when compared to EB1089 (data not shown).

Although the activity of EB1285 was much lower than EB1089, all these results together suggested that activation by hydroxylation of a non-1α-hydroxylated precursor vitamin D analogue can occur in vitro, supporting our original idea. The future perspectives include design and testing of other precursor vitamin D analogue compounds, which hopefully could be used therapeutically in diseases where 1α-hydroxylase is expressed.

Paper V

Potentiating inhibitory effects of the non-1α-hydroxylated precursor vitamin D analogue of EB1089 (EB1285) and ketoconazole in parathyroid cells

In the previous study (Paper IV) we demonstrated transcriptional activity of the precursor vitamin D analogue EB1285 in MCF-7 cells. In this study the primary aim was to investigate effects on cellular proliferation and PTH secretion in primary human pathological- and bovine parathyroid cells, respectively. The secondary aim was to investigate whether the unspecific CYP inhibitor ketoconazole could potentiate the inhibitory effect on the PTH secretion of 1,25(OH)2D3 and earlier selected vitamin D analogues EB1089 and GS1590 (Paper III). In other studies ketoconazole and liarozole, another unspecific CYP inhibitor, were shown to increase the antiproliferative effect of 1,25(OH)2D3 and EB1089 in cultured prostate cancer cells (224, 227). In addition, a specific 24-hydroxylase inhibitor, VID400, clearly enhanced the antiproliferative effect of 25(OH)D3 in keratinocytes, increasing stabilization and/or production of 1,25(OH)2D3 (94). Thus, one reason for the potentiating effect of CYP inhibitors is inhibition of 24-hydroxylase activity, stabilizing 1,25(OH)2D3 and vitamin D analogues. In the present study the impact of 25(OH)D3 and EB1285 on PTH secretion/PTH mRNA level was determined
together with ketoconazole, in lack of a specific CYP inhibitor, which would have been more suitable since ketoconazole partially inhibits 1α-hydroxylase activity (224). In addition, we determined the expression level of 24-hydroxylase mRNA as an independent control of 1,25(OH)2D3 and vitamin D analogue activity.

Experiments were performed by exposing bovine parathyroid cell cultures to vehicles, 1,25(OH)2D3 (10 nM), GS1590 (0.01 nM), EB1089 (0.1 nM), EB1285 (10 nM) and 25(OH)D3 (10 nM) with or without ketoconazole (5, 10 µM).

Treatment with ketoconazole alone reduced PTH secretion/PTH mRNA levels, possibly indicating an impact on endogenous degradation/production of 1,25(OH)2D3 in the bovine parathyroid cells. The 24-hydroxylase mRNA expression level simultaneously increased, supporting this view. We suggested that these effects could be explained by hydroxylation of intracellularly stored 25(OH)D3 to active vitamin D. In prostate cancer cells ketoconazole induced growth regression in vitro (224), whereas in human skin ketoconazole had no effect (228). The reason for this could be differences in the selectivity of ketoconazole for 1α-hydroxylase and 24-hydroxylase (94, 229) between tissues.

Combined treatments with vitamin D compounds and ketoconazole, including the non-1α-hydroxylated analogue EB1285, displayed significantly higher suppressive additive effect on PTH secretion/PTH mRNA level as well as increased 24-hydroxylase mRNA expression, when compared to treatment with single compounds. The reduced PTH secretion/PTH mRNA level obtained by 1,25(OH)2D3, GS1590 and EB1089 confirmed previous results (Paper III).

25(OH)D3 had no effects unless combined with ketoconazole and then with higher PTH suppressive activity/increased 24-hydroxylase expression, when compared to treatment with ketoconazole alone. This suggested a net increase of 1,25(OH)2D3, likely due to inhibition of 24-hydroxylase activity, which normally results in degradation of both the substrate 25(OH)D3 and 1,25(OH)2D3.

Interestingly, the PTH suppressive effect of EB1089 was additively increased in the presence of ketoconazole and also the 24-hydroxylase mRNA level was increased, even though EB1089 has been claimed not to be degraded by the 24-hydroxylase pathway, but rather by 26-hydroxylation (225). In prostate cancer cells it has been reported that ketoconazole also enhances the growth inhibiting effect of EB1089 (224). On the other hand, no additional increase in transcription activation by VDR was seen in MCF-7 cells treated with EB1089/ketoconazole and ketoconazole alone displayed no activity in this assay (Paper IV). As suggested above, the presence of ketoconazole could result in increased net synthesis of 1,25(OH)2D3 by hydroxylation of stored 25(OH)D3 in the bovine parathyroid cells. This ex-
plies the additive effect of ketoconazole observed with the combined treatments.

Human parathyroid cells obtained from patients with parathyroid tumors proliferate and retain functionality in a defined serum-free low calcium containing medium (211). The impact on cell proliferation by EB1089 (10 nM) and EB1285 (10 nM) was determined by $^3$H-thymidine incorporation in parathyroid tumor cell cultures from 5 pHPT and 2 sHPT patients. The effect of ketoconazole was not investigated due to shortage of cells. EB1089 and the non-1α-hydroxylated precursor analogue EB1285 significantly decreased cell proliferation by 22-53% respectively 10-41%, except for one adenoma cell culture. All tumors from which the cell cultures were prepared stained positively for 1α-hydroxylase and VDR. The antiproliferative activity of EB1285 in human pathological parathyroid cells indicated the presence of an active and potent 1α-hydroxylase enzyme.

The ability to inhibit cell proliferation, suppress PTH secretion/PTH mRNA level and increase 24-hydroxylase mRNA expression by the precursor analogue EB1285 supports our idea that a non-1α-hydroxylated analogue could perform vitamin D effects in 1α-hydroxylase expressing cells. Further studies will elucidate whether vitamin D precursor analogues as well as 24-hydroxylase inhibitors present a therapeutic option in patients with pHPT and sHPT.
General discussion

Still today, therapeutics for cancer remain rather generalized and possibly improved cure may be achieved by individual therapy based on specific biological features of each tumor. Individual treatment may not only provide optimal therapeutic effect, but also minimize side effects. Vitamin D analogues have been shown already to have an antitumorigenic potential in sHPT patients as well as in breast cancer animal models. However, hypercalcemia is still a problematic side effect. In this thesis, three novel low-calcemic vitamin D analogues have been identified for future potential treatment of HPT.

In both parathyroid and breast tumors we have identified a new biological property, the expression of a functional 1α-hydroxylase enzyme, CYP27B1. In the future, non-1α-hydroxylated precursor vitamin D analogues could present a therapeutic alternative, when transformed into potent drugs in the tumor and thereby avoiding hypercalcemic side effects.

Parathyroidectomy is an efficient cure for pHPT and sHPT. However, use of less calcemic vitamin D analogues or non-1α-hydroxylated precursor vitamin D analogues could prevent the disease from progressing, especially in uremic sHPT patients or in pHPT patients with mildly deficient renal function (230). In contrast to HPT, breast cancer is a heterogeneous and malignant disease and even if the medical and surgical therapies today are based on prognostic and predictive factors, the mortality remains high.

Eventually, vitamin D analogues or precursor vitamin D analogues could be effective by themselves or in combination with CYP inhibitors, chemo- or radiotherapy, to achieve favourable results of treatment in HPT, breast cancer and other malignances.
Acknowledgements

A lot of people have contributed to my work with this thesis and I would like to express my sincere gratitude to:

**Gunnar Westin**, my main-supervisor, for his enthusiasm, optimism, encouragement and great knowledge. To me he is the ultimate researcher, with tons of ideas and curiosity.

**Per Hellman**, my supervisor, for always having the time and never-ending interest in the research, even though he is an occupied clinician. I am very grateful that you initiated the contacts and co-operation with Leo Pharmaceuticals.

**Göran Äkerström**, my supervisor, for understanding the huge advantages with having a mixed research group with both clinicians and molecular biologists. It has meant a great deal for me as a non-clinician to feel the strong support from you.

**Pamela Correa**, my co-author and lab partner, for being the most efficient and hard-working person I have ever met and for your true interest in molecular biology.

**Birgitta Bondeson** and **Peter Lillhager** for being the core in the research. Your knowledge and working skills are invaluable.

**Lise Binderup**, for supporting and appreciating our research and for her true enthusiasm.

**Pernille Kaae Holm**, for her great interest and support and for always giving me extensive answers on my never-ending questions.

**Eva Szabo**, for being one of my best friends and for talking about everything else than research during lunches and breaks. I wish you all the best.

Jenny Redelius, Åsa Häkansson, Annica Jacobson, Maria Branting, Stina Johansson, Helena Brändström, Teresa Karlsson, Elin Grundberg, Gunilla Englund, Juan Ramon Lopez-Egido, Janet Cunningham, Dhana Aronsson, Malin Grönberg, Nasim Farrokhnia, My Quach, Pernilla Wahlgqvist, Åsa Hallgren, Kerstin Ahlgren and Anna-Stina Sahlqvist.

My parents, Krister and Yvonne, my brothers, Conny and Dan and all other relatives and friends for their great support and love.

My husband Per and my children Daniel and Joel for giving me a rich life outside the lab and preventing me from turning into a nurdy researcher. Love to all three of you.
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