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## Original Paper

# Inhibition of Insulin- and Insulin-like Growth Factor-I-stimulated Growth of Human Breast Cancer Cells by 1,25-Dihydroxyvitamin $D_3$ and the Vitamin $D_3$ Analogue EB1089

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

**Key words: breast cancer, *C-FOS*, 1,25-dihyroxyvitamin $D_3$, EB1089, insulin, insulin-like growth factor-I, MCF-7, vitamin D analogue**

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## INTRODUCTION

Besides regulation of calcium and bone metabolism, the most active metabolite of vitamin D, 1,25-dihydroxyvitamin $D_3$ (1, 25-$(OH)_2D_3$), regulates growth and differentiation of a wide variety of cells and tissues [1, 2]. Both *in vitro* and *in vivo* studies have shown that 1,25-$(OH)_2D_3$ inhibits the growth of breast cancer cells [3–7] and these findings suggest a potential use of 1,25-$(OH)_2D_3$ for the treatment of breast cancer. A major drawback is that high doses are needed for tumour suppression. This may result in undesirable side-effects, such as the development of hypercalcaemia. To overcome these problems, a number of promising synthetic vitamin $D_3$ analogues have been developed, with more potent effects on growth and differentiation and a reduced *in vivo* calcaemic activity [8, 9].

Breast cancer growth is regulated by numerous factors, including steroid hormones and polypeptide growth factors [10]. Insulin, insulin-like growth factor-I (IGF-I) and insulin-like growth factor-II (IGF-II) are structurally related molecules which are believed to play an important role in promoting breast tumour growth [11–14]. The IGFs and insulin are derived from the circulation. The IGFs are also produced by breast tumour cells and by neighbouring stromal cells in the

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breast tumour, and can act in an autocrine or paracrine manner [11, 13, 15]. These three polypeptide hormones exert their biological activity by binding to distinct transmembrane receptors: the closely related insulin and type I IGF receptor, and the type II IGF receptor or mannose-6-phosphate receptor. Considerable crossreactivity exists between the various ligands and receptors [16]. In addition to the three receptors, the IGFs bind to other binding proteins which are found in serum and are produced by a wide variety of different cell types. To date, six IGF binding proteins (IGF-BPs) have been cloned [17]. Breast tumours and breast cancer cell lines express IGF-BP2 to IGF-BP6 in a heterogenous pattern, while little or no expression of IGF-BP1 is seen [18–20]. At the cellular level, IGF-BPs can either potentiate or suppress the activity of the IGFs [17, 18].

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

## MATERIALS AND METHODS

*Chemicals*

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

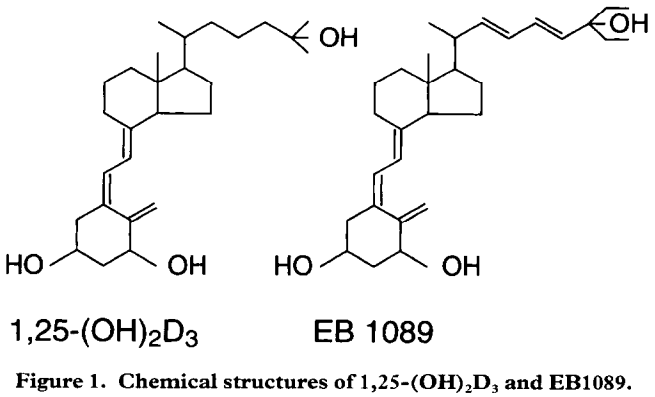


**Figure 1. Chemical structures of 1,25-$(OH)_2D_3$ and EB1089.**

*Cell culture*

MCF-7 cells were generously provided by Dr J.A. Foekens, Dr Daniel den Hoed Cancer Centre (Rotterdam, The Netherlands). MCF-7 cells were routinely maintained in phenol red-free RPMI-1640 medium supplemented with 2 mM glutamine, 100 U/ml penicillin, 100 μg/ml streptomycin, 24 mM sodium bicarbonate (basal RPMI medium) plus 10% FCS and 10 μg/ml insulin. Prior to the start of the experiments, the cells were deprived of insulin and serum as follows: the cells were trypsinised and seeded in phenol red-free basal RPMI medium supplemented with 2% charcoal-treated FCS (25000 cells/cm$^2$). The FCS was added for the attachment of the cells. After 24 h, the medium was changed to serum-free basal RPMI medium supplemented with 2 mg/ml BSA, 10 μg/ml transferrin and 30 nM sodium selenite (SFM). The medium was replaced every 2–3 days. After 1 week, the cells had grown to subconfluence and the procedure was repeated. For the experiments, cells that were cultured in SFM for several weeks were used. The experiments were started by seeding the cells in basal RPMI medium supplemented with 2% charcoal-treated FCS. After 24 h, the medium was changed to SFM and, after another 24 h, the SFM was refreshed and the agents to be tested were added. For long-term experiments, the medium and test agents were replaced every 3 days.

*Proliferation experiments*

MCF-7 cells were seeded into 24-well dishes at a density of 4000 cells/cm$^2$ and incubated with the compounds to be tested as described above. After 8 days of incubation, DNA content was measured using the ethidium bromide method as described previously [7].

*Insulin and IGF-I binding*

The insulin and IGF-I binding assay was performed as described by Maassen and associates [28]. For short-term incubations, MCF-7 cells were grown to subconfluence in SFM in six-well dishes and were subsequently exposed to the vitamin $D_3$ compounds for 24 or 48 h. For long-term incubations (8 days), the cells were exposed to the vitamin $D_3$ compounds from the start of the experiment as described above. At the end of both the short- and long-term incubations, the cells were washed twice in PBS (pH 7.8) plus 10 mg/ml BSA. Subsequently, the cells were incubated with $3 \times 10^{-11}$ M [$^{125}$I]insulin in PBS (pH 7.8) plus 10 mg/ml BSA for 2.5 h at room temperature or with $3 \times 10^{-11}$ M [$^{125}$I]IGF-I in PBS (pH 7.8) plus 10 mg/ml BSA and 2 mg/ml bacitracin for 3.5 h at 4°C. Non-specific binding was assessed by incubating the cells with labelled insulin in the presence of 1 μM unlabelled insulin or with labelled IGF-I in the presence

of 0.1 μM unlabelled IGF-I. After the incubation, the cells were washed 5 times with ice-cold PBS plus 1 mg/ml BSA and solubilised in 0.1 M NaOH, 0.1% SDS. The amount of bound [$^{125}$I]insulin or [$^{125}$I]IGF-I was determined in a gamma counter (NE1600, NE Technology Ltd, Reading, U.K.) and corrected for the absorption at 260 nm, which was used as a measure for the cell number.

*Assay of MAP kinase activity*

MCF-7 cells were seeded ($2 \times 10^4$ cells/cm$^2$) and incubated with the compounds to be tested for 5–30 min as described above. Upon activation, MAP kinase is phosphorylated. Activation of MAP kinase was detected by immunoblotting of cell homogenates as described by Van den Berghe and associates [29]. The method is based on a shift in gel electrophoretic migration owing to phosphorylation of MAP kinase.

*RNA isolation and hybridisation*

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

## RESULTS

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

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

The growth stimulation by 10 μg/ml insulin could be dose-dependently inhibited both by 1,25-$(OH)_2D_3$ and the vitamin $D_3$ analogue EB1089 (Figure 3). Based on the median effective concentration, EB1089 was approximately 50 times more potent than 1,25-$(OH)_2D_3$ in the inhibition of insulin-stimulated growth. Both vitamin $D_3$ compounds not only reversed the growth stimulation by insulin, but also inhibited part of the autonomous growth of MCF-7 cells.

Since insulin binds not only the insulin receptor but also the IGF type I receptor with low affinity [16], it is possible that the observed stimulation by supraphysiological concentrations of insulin is mediated via the type I IGF receptor. To test this possibility, we studied whether the insulin-stimulated

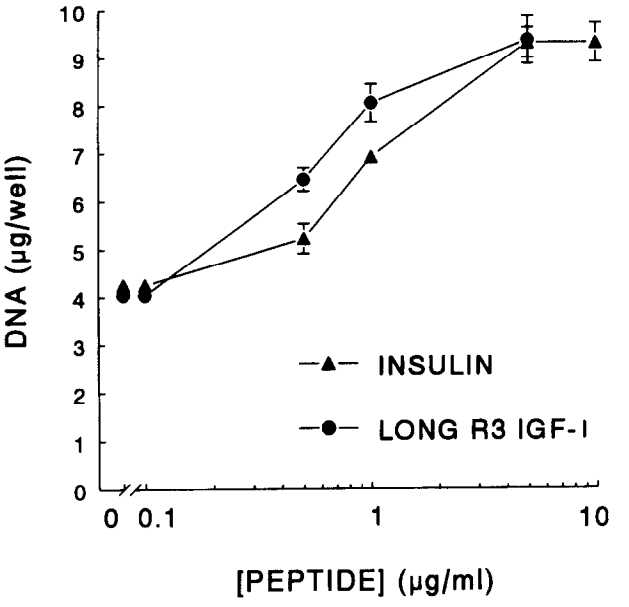


**Figure 2. Growth stimulation of MCF-7 cells by insulin and long R$^3$ IGF-I. Cells were cultured for 8 days in SFM, as described in Materials and Methods, with various concentrations of insulin or long R$^3$ IGF-I. Subsequently, DNA content was measured. Each point represents mean ± S.D. of duplicate wells.**

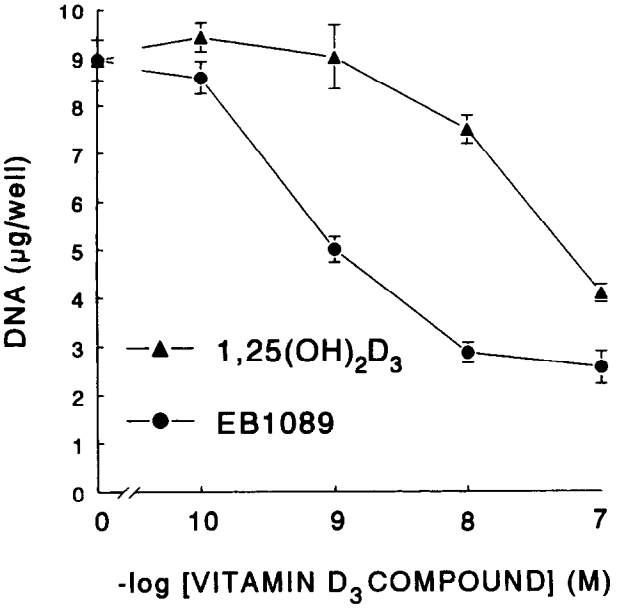


**Figure 3. Inhibition of insulin-stimulated growth of MCF-7 cells by 1,25-$(OH)_2D_3$ and EB1089. Cells were cultured for 8 days in SFM supplemented with 10 μg/ml insulin and the indicated concentrations of the vitamin $D_3$ compounds or vehicle (0.1% ethanol) as described in Materials and Methods. Each point represents mean ± S.D. of duplicate wells.**

growth could be inhibited by the type I IGF receptor blocking antibody αIR3. The antibody inhibited the insulin-stimulated growth by 24% and IGF-I-stimulated growth by about 30%. A small inhibition of the autonomous growth (9%) was observed (data not shown).

Next, we examined the mitogenic effect of IGF-I on MCF-7 cells. To avoid interference with IGF-BPs secreted by MCF-

7 cells, we tested two analogues of IGF-I, long $R^3$ IGF-I and des(1–3)IGF-I, which have reduced binding to IGF-BPs [26, 27]. Figure 2 demonstrates that MCF-7 cells respond to long $R^3$ IGF-I with a similar increase of DNA content as obtained with insulin. The median effective concentration was 0.5 μg/ml long $R^3$ IGF-I. Also, for des(1–3)IGF-I relatively high concentrations were needed to elicit a proliferative response. Des(1–3)IGF-I was not active at concentrations below 1 μg/ml (data not shown). Several studies have shown a synergistic action of insulin–IGF-I and oestradiol [32–34]. However, in our culture system the mitogenic effects of insulin and the IGF-I analogues could not be sensitised by co-treatment with oestradiol (data not shown).

Similar to the inhibition of insulin-stimulated growth, the growth stimulation by long $R^3$ IGF-I was inhibited by 1,25-$(OH)_2D_3$ and the analogue EB1089 (Figure 4). At $10^{-9}$ M, the differences in potency between 1,25-$(OH)_2D_3$ and EB1089 were clearly demonstrated. In addition, Figure 4 demonstrates that autonomous growth was also potently inhibited by 1,25-$(OH)_2D_3$ and EB1089.

*Insulin and IGF-I binding*

Insulin binding was maximal after 2 h and non-specific binding was 9% of the total binding. For IGF-I, the non-specific binding was 22% of the total binding. Competitive binding studies of [$^{125}$I]insulin showed that insulin competed at approximately 300 times lower concentrations than IGF-I (Figure 5).

We also studied whether the vitamin $D_3$ compounds could exert their antiproliferative action on insulin- and IGF-I-stimulated growth via regulation of insulin or IGF-I binding.

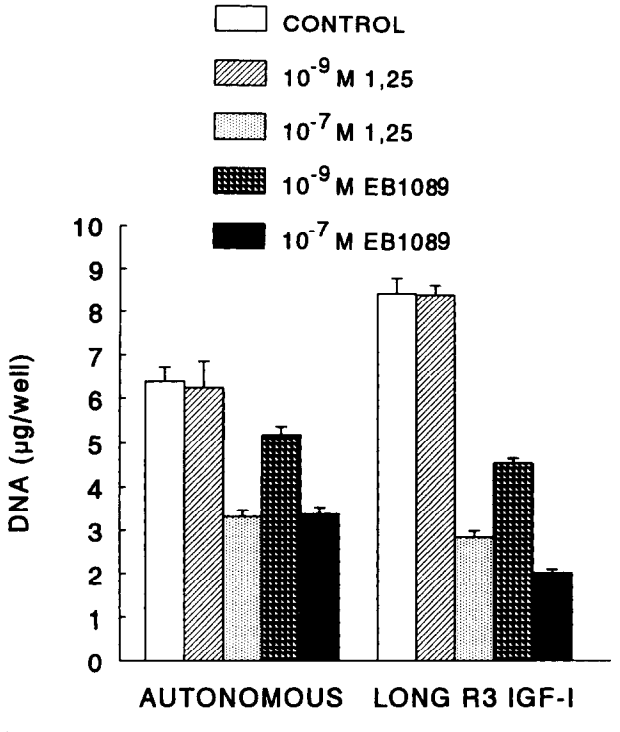


**Figure 4. Inhibition of autonomous and long $R^3$ IGF-I-stimulated growth of MCF-7 cells by 1,25-$(OH)_2D_3$ and EB1089. Cells were cultured for 8 days in SFM (autonomous growth), or SFM supplemented with 1 μg/ml long $R^3$ IGF-I. The cells were treated with vehicle (0.1% ethanol), 1,25-$(OH)_2D_3$ (1,25) or EB1089 as indicated in the figure. Data are presented as mean ± S.D. of duplicate wells.**

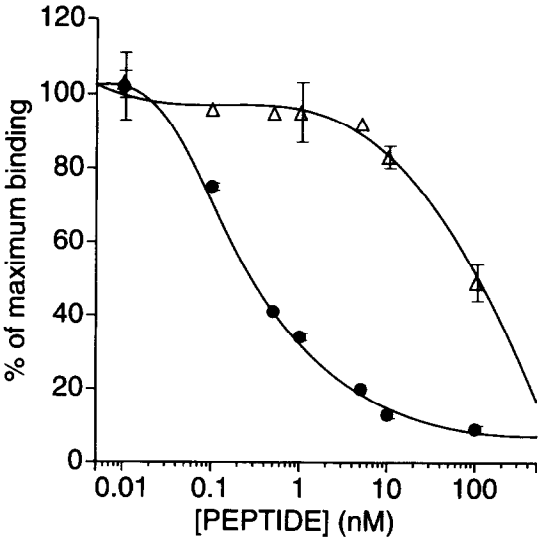


**Figure 5. Binding of insulin to monolayers of MCF-7 cells. MCF-7 cells were grown to confluence in SFM and incubated with $3 \times 10^{-11}$ M [$^{125}$I]insulin for 2.5 h in the presence of the indicated concentrations of unlabelled insulin (solid circles) or long IGF-I (triangles). Bound [$^{125}$I]insulin was measured as described in Materials and Methods. Data are expressed as mean ± S.D. of duplicate wells.**

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

*Effect of the vitamin $D_3$ compounds on intracellular mediators of the insulin–IGF-I signal*: C-FOS *mRNA expression and MAP kinase*

Induction of *C-FOS* expression is believed to play a central role in the transduction of mitogenic signals in the nucleus [22, 23]. *C-FOS* mRNA expression was rapidly induced by insulin with a peak at 30–60 min and a gradual return towards prestimulation level in 4 h (Figure 6). Figure 7 shows that the rapid induction of *C-FOS* mRNA by insulin was inhibited by treatment with 1,25-$(OH)_2D_3$. Basal *C-FOS* mRNA expression was not affected by 1,25-$(OH)_2D_3$ (not shown).

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

## DISCUSSION

This study shows that 1,25-$(OH)_2D_3$ and the vitamin $D_3$ analogue EB1089 are able to reverse the mitogenic effect of insulin and an IGF-I analogue (long $R^3$ IGF-I) on MCF-7 breast cancer cells. The vitamin $D_3$ analogue EB1089 was

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

| | Short-term incubation (48 h) | | | |
|---|---|---|---|---|
| | Insulin binding | | IGF-I binding | |
| | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| Control | 1.00 ± 0.13 | 1.00 ± 0.05 | 1.00 ± 0.02 | 1.00 ± 0.07 |
| $10^{-9}$ M 1,25-$(OH)_2D_3$ | 1.03 ± 0.09 | 0.99 ± 0.01 | N.D. | N.D. |
| $10^{-7}$ M 1,25-$(OH)_2D_3$ | 1.22 ± 0.11 | 1.25 ± 0.33 | 1.35 ± 0.03 | 1.06 ±0.05 |
| $10^{-9}$ M EB1089 | 1.29 ± 0.23 | 0.98 ± 0.02 | N.D. | N.D. |
| $10^{-7}$ M EB 1089 | 1.12 ± 0.00 | 0.85 ± 0.10 | 1.20 ± 0.08 | 1.01 ±0.08 |
| | Long-term incubation (8 days) | | | |
| | Insulin binding | | IGF-I binding | |
| | Exp. 1 | Exp. 2 | Exp. 1 | Exp. 2 |
| Control | 1.00 ± 0.20 | 1.00 ± 0.09 | 1.00 ± 0.02 | 1.00 ± 0.04 |
| $10^{-9}$ M 1,25-$(OH)_2D_3$ | 0.98 ± 0.02 | 1.25 ± 0.26 | N.D. | N.D. |
| $10^{-7}$ M 1,25-$(OH)_2D_3$ | 1.53 ± 0.18 | 1.65 ± 0.11 | 2.68 ± 0.10 | 3.92 ± 0.01 |
| $10^{-9}$ M EB1089 | 1.35 ± 0.13 | 2.39 ± 0.46 | N.D. | N.D. |
| $10^{-7}$ M EB1089 | 1.98 ± 0.49 | 2.30 ± 0.45 | 2.74 ± 0.01 | 2.63 ± 0.35 |

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

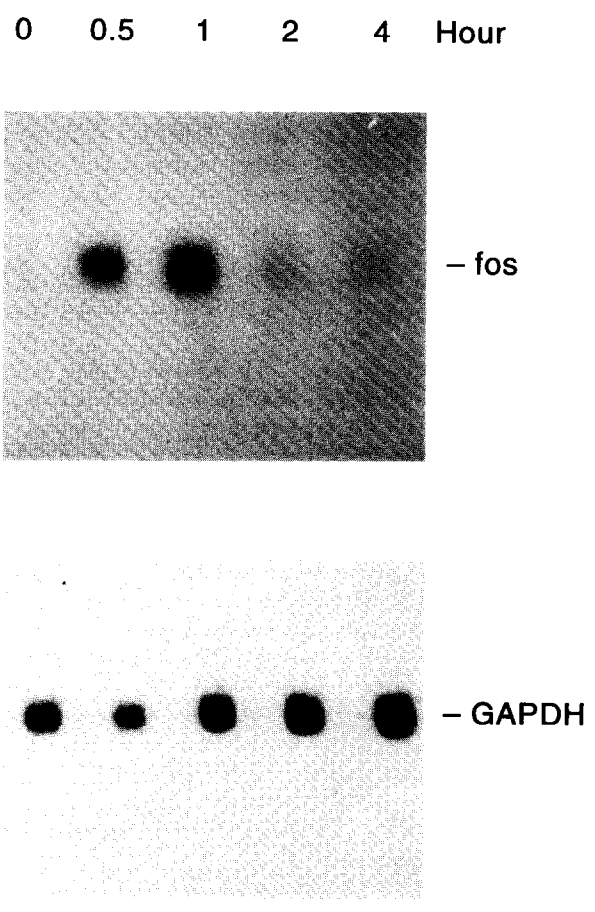


**Figure 6. Insulin-induced *C-FOS* mRNA expression over time. MCF-7 cells were incubated with 10 μg/ml insulin for 0, 0.5, 1, 2 or 4 h. RNA was isolated and Northern blots were prepared as described in Materials and Methods and the blots were hybridised with $^{32}$P-labelled fos and GAPDH probes.**

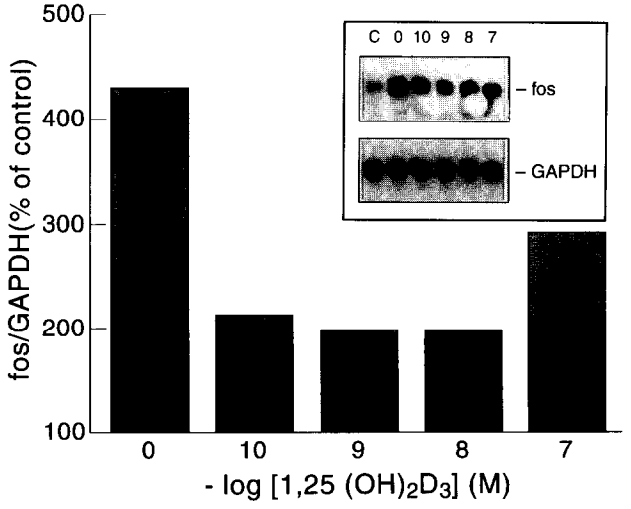


**Figure 7. Inhibition of insulin-induced *C-FOS* mRNA by 1,25-$(OH)_2D_3$. MCF-7 cells were treated without insulin (C) or with 10 μg/ml insulin and $10^{-10}$–$10^{-7}$ M, 1,25-$(OH)_2D_3$ (lanes 10–7) or vehicle (0) for 30 min. The autoradiograph shown was quantitated densitometrically. The *C-FOS* signal was divided by the *GAPDH* signal to correct for equal loading of the lanes. Data presented are from a representative experiment which was repeated twice with similar results.**

active at 50-fold lower concentrations than the native compound 1,25-$(OH)_2D_3$. This is in line with other studies on the antiproliferative action of EB1089 on breast cancer cells [21, 35, 36]. *In vivo* studies with rats have shown that EB1089 has a decreased ability to affect calcium metabolism compared to 1,25-$(OH)_2D_3$ [36]. Moreover, Colston and associates showed that EB1089 suppressed tumour growth in rats with chemically induced breast cancer [35]. Thus, EB1089 appears to be a promising analogue for the treatment of breast cancer.

IGF-I and insulin are believed to play an important role in

the development and growth of breast tumours [11–14]. In oestrogen-responsive cells, insulin and the IGFs can contribute to oestrogen-induced growth [32–34]. Thus, interruption of insulin–IGF-induced growth by the vitamin $D_3$ compounds may affect both oestrogen-independent and oestrogen-dependent breast tumours. This may provide an explanation for the observation that vitamin $D_3$ compounds inhibit the growth of breast cancer cells and tumours irrespective of the presence of the oestrogen receptor [4, 37].

Although most attention has focused on the tumour promoting role of the IGFs, insulin may also play a role in the growth and development of breast tumours, acting through its own receptor [12]. The high doses of insulin needed for growth stimulation of MCF-7 cells ($EC_{50}$ = 1 μg/ml) suggests that insulin was acting through the type I IGF receptor, which has a low affinity for insulin, and mimicked the effect of IGF-I. However, the small inhibition of insulin-stimulated growth by αIR3 (24% inhibition) suggests that insulin acted predominantly via the insulin receptor. In addition, the studies of Milazzo and colleagues [12] and Cullen and associates [14] with αIR3 suggested an involvement of the insulin receptor in the transmission of the mitogenic effect of insulin in breast cancer cells. The antibody was tested at a concentration 100-fold higher than the $K_D$ of αIR3 for binding to the type I IGF receptor in MCF-7 cells [38]. In view of the high concentrations of IGF-I needed to stimulate growth, this αIR3 concentration could still be too low, which may explain its small effect on IGF-I-stimulated growth.

The presence of specific insulin binding sites on MCF-7 cells was demonstrated by binding studies with [$^{125}$I]insulin. Half-maximal displacement of [$^{125}$I]insulin was achieved with insulin at 300 times lower concentrations than with IGF-I. Other reports have also demonstrated the presence of functional insulin receptors on several breast cancer cell lines, including MCF-7 [12]. In addition, a role of the insulin receptor in breast cancer initiation and/or progression is supported by the observations that the insulin receptor expression is increased in human breast cancer specimens and that the insulin receptor content is positively correlated with tumour grade and size [39]. Lastly, an insulin analogue with enhanced mitogenic activity increased the incidence of mammary tumours in female rats [40].

Besides insulin, the IGF-I analogues, long $R^3$ IGF-I and des(1–3)IGF-I, were also needed in high concentrations (0.5 μg/ml or more) to stimulate the growth of MCF-7 cells, whereas previous studies have shown that IGF-I and the IGF analogues can elicit a proliferative response at concentrations ranging from 1 to 50 ng/ml [13, 14, 26]. There are several possible explanations for the low sensitivity of the MCF-7 cells to insulin and IGF-I, regardless of the receptor activation pathway. First, we used serum-free culture conditions, and possibly additional serum-derived factors play a synergistic role in the growth stimulation by insulin and IGF-I [32–34]. Secondly, the cells could be less responsive because of the high basal growth rate. This could be due to the fact that MCF-7 cells produce autocrine acting growth factors [10].

To gain insight into the mechanism of the antigrowth factor activity of 1,25-$(OH)_2D_3$ and EB1089, we studied several aspects of the insulin and IGF-I signal transduction pathway. First, it was shown that 1,25-$(OH)_2D_3$ and EB1089 did not suppress the insulin and IGF-I binding. The vitamin $D_3$ compounds had no direct effect on insulin and IGF-I binding after 48 h of treatment. At time points where the growth inhibition by the vitamin $D_3$ compounds was observed (8 days), an increase of insulin and IGF-I binding was observed with treatment with 1,25-$(OH)_2D_3$ and EB1089. The physiological implications of these findings are not clear, but a similar inverse relation between receptor regulation and growth inhibition by 1,25-$(OH)_2D_3$ was observed for the epidermal growth factor receptor in BT-20 breast cancer cells [41]. Possibly, the stimulation of the insulin and IGF-I binding is a secondary effect to 1,25-$(OH)_2D_3$ and EB1089 treatment, which is not linked to the inhibitory effect on cell growth. A role of IGF-BPs in the growth inhibition by 1,25-$(OH)_2D_3$ and EB1089 is unlikely because of the use of insulin and analogues of IGF-I which reduced binding to IGF-BPs.

We also studied two intracellular responses that can be activated by insulin and IGF-I: *C-FOS* mRNA expression [22, 23] and MAP kinase activity [24]. The rapid induction of *C-FOS* mRNA expression by insulin was inhibited by 1,25-$(OH)_2D_3$, indicating that *C-FOS* mRNA expression is a possible target for 1,25-$(OH)_2D_3$ and analogues in the growth inhibition of these breast cancer cells. It should be noted that the inhibition of insulin-induced *C-FOS* mRNA expression could already be observed at lower concentrations than the inhibition of proliferation, indicating that inhibition of only *C-FOS* expression is not sufficient to inhibit insulin-induced cell growth. This is supported by the observation that the vitamin $D_3$ concentration which most potently inhibited the proliferation had the weakest effect on *C-FOS* expression. Furthermore, a stimulation of *C-FOS* mRNA levels by 1,25-$(OH)_2D_3$ was associated with inhibition of cell proliferation in the MCF-7 cell line [36] and in the HL-60 leukaemic cell line [42]. Finally, we studied whether MAP kinase is involved in growth inhibition by 1,25-$(OH)_2D_3$ and EB1089. MAP kinase has been detected in a wide variety of cells and tissues and is activated by a number of growth factors (insulin, IGF-I and epidermal growth factor), hormones and other extracellular signals. Activated MAP kinase in its turn phosphorylates other proteins, including the proto-oncogene products of myc and jun, which act as transcription factors [24]. However, it appeared that MAP kinase phosphorylation was not detected, which may imply that MAP kinase is not involved in the growth stimulation by insulin and IGF-I in the MCF-7 breast cancer cells.

In conclusion, it was shown that 1,25-$(OH)_2D_3$ and the more potent analogue EB1089 block the mitogenic effect of insulin and IGF-I on MCF-7 breast cancer cells. It is questionable whether the observed growth inhibition of breast cancer cells by 1,25-$(OH)_2D_3$ and EB1089 is specific for insulin- and IGF-I-stimulated growth. Previously, we have shown that 1,25-$(OH)_2D_3$ also inhibits EGF-stimulated as well as oestradiol-stimulated growth [7, 21]. This led to the hypothesis that vitamin $D_3$ compounds interfere with growth stimulatory agents at a late, common stage in the signal cascades leading stimulation of cell proliferation. The present observations that vitamin $D_3$ compounds also inhibit insulin- and IGF-I-stimulated growth supports this hypothesis.

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