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# Inhibition of MG-63 cell cycle progression by synthetic vitamin $D_3$ analogs mediated by p27, Cdk2, cyclin E, and the retinoblastoma protein

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**Abstract**

Progression through eukaryotic cell division cycle is regulated by synergistic activities of both positive and negative regulatory factors. The active form of vitamin $D_3$ ($1\alpha,25(OH)_2D_3$, 1,25D) and a number of its synthetic analogs have been shown to arrest cells in the $G_1$ phase of the cell cycle. In the present study, $1\alpha,25(OH)_2D_3$ and the analogs KH1060, EB1089, and CB1093 were used to study the mechanism of the cell cycle arrest and to compare the effectiveness of these compounds in human MG-63 osteosarcoma cells. The 20-epi analogs KH1060 and CB1093, as well as the 20-normal analog EB1089, were found to be more potent than $1\alpha,25(OH)_2D_3$ in inhibiting cell proliferation and arresting the MG-63 cells in the $G_1$ phase. These analogs were more active than $1\alpha,25(OH)_2D_3$ in increasing the cyclin dependent kinase inhibitor p27 protein levels (approximately 2.3–2.5-fold compared to $1\alpha,25(OH)_2D_3$) by both increasing its formation and decreasing its degradation rate. The increased p27 formation was accompanied by stabilization of binding of nuclear proteins to the Sp1 + NF-Y responsive promoter region of the *p27* gene. The increase in p27 protein levels and the simultaneous decrease in cyclin E protein levels was accompanied by decreased Cdk2 kinase activity, retinoblastoma (Rb) protein hypophosphorylation and, finally, cell cycle arrest in the $G_1$ phase. In summary, the analogs KH1060, EB1089, and CB1093 keep Rb protein in its growth-suppressing, hypophosphorylated form and prevent cell cycle progression through the restriction point. Therefore, these synthetic vitamin $D_3$ analogs may be potential candidates for treating diseases, where cell cycle regulation is needed.


*Keywords:* Cdk2; Cell cycle; Cyclin E; Osteoblastic cells; p27; Rb; Vitamin D analogs

## 1. Introduction

Most of the biological actions of 1α,25-dihydroxyvitamin $D_3$ ($1\alpha,25(OH)_2D_3$, 1,25D) are mediated through the nuclear VDR, which is a member of structurally related steroid/thyroid hormone superfamily of ligand-dependent transcription factors [1]. VDR forms heterodimers with another member of this family, the retinoid X receptor, and regulates gene expression positively or negatively through binding to the vitamin D response elements (VDREs) in promoter regions of target genes. In addition to its role in mineral homeostasis, the active $1\alpha,25(OH)_2D_3$ affects growth and differentiation of different cell types, e.g., human breast, brain, colon, prostate, and skin cells [2–7]. The potentially beneficial use of the hormone in treatment of hyperproliferative diseases is, however, compromised by hypercalcemia and hypercalciuria developing at therapeutic doses. This has evoked an increased interest in synthesis and evaluation of new $1\alpha,25(OH)_2D_3$ analogs that would retain the properties of the parent compound on cellular proliferation and differentiation while having reduced calcemic activity [2,8,9]. New analogs could be potentially useful in the treatment of, e.g., several types of cancer and skin disorders [2,8]. One interesting group

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*Abbreviations:* 1,25D, ($1\alpha,25(OH)_2D_3$), 1α,25-dihydroxyvitamin $D_3$; CB1093, 1α,25-dihydroxy-20-epi-22-ethoxy-23-yne-24a,26a,27a-trihomo-vitamin $D_3$; EB1089, 1α,25-dihydroxy-22,24-diene-24a,26a,27a-trihomo-vitamin $D_3$; KH1060, 1α,25-dihydroxy-20-epi-22-oxa-24a,26a,27a-trihomo-vitamin $D_3$; Cdk, cyclin dependent kinase; CH, cycloheximide; CKI, cyclin dependent kinase inhibitor; CTRL, control; DMEM, Dulbecco's modified Eagle's medium; EMSA, electrophoretic mobility shift assay; FCS, fetal calf serum; GST-pRb, glutathione-*S*-transferase-hypophosphorylated retinoblastoma protein; pRb, hypophosphorylated retinoblastoma protein; ppRb, hyperphosphorylated retinoblastoma protein; VDR, Vitamin D receptor; VDRE, Vitamin D response element.

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among the side chain analogs of $1\alpha,25(OH)_2D_3$ is the 20-epi analogs. The prolonged effects of these analogs quite likely result from their resistance against cell metabolism further leading to increased stabilization of the analog–receptor complex [10,11].

Inhibition of cell proliferation is tightly linked to mechanisms that regulate cell cycle progression. Knowledge of mechanisms by which cell growth is arrested after exposure to $1\alpha,25(OH)_2D_3$ and its analogs is quite fragmentary and partly unclear. In most cases, the inhibition of human cell growth in response to $1\alpha,25(OH)_2D_3$ involves cell cycle arrest in the $G_1$ phase [12–14]. In dividing mammalian cells, cell cycle progression is regulated by sequential formation, activation, and subsequent inactivation of a series of cyclin dependent kinase (Cdk)–cyclin complexes. Cdk activities are regulated by phosphorylations and cyclin binding, as well as by the action of specific Cdk inhibitor (CKI) proteins ([15–18] and references therein). In mammalian cells, complexes such as cyclin D/Cdk4,6 and cyclin E-Cdk2 are catalytically active during the $G_1$ phase and the main function of these complexes is phosphorylation of the retinoblastoma (Rb) protein. Indeed, many of these events have been identified during $1\alpha,25(OH)_2D_3$-induced inhibition of human cell proliferation. In a variety of human cells, the $1\alpha,25(OH)_2D_3$-induced cell cycle arrest is mediated by CKIs including p21 (Cip1/Waf1) and p27 (Kip1) [14,19–21]. There are also studies showing that $1\alpha,25(OH)_2D_3$ signaling is mediated by the cell cycle regulatory proteins Rb and p57 (Kip2) and by the induction of apoptosis [22–24]. In addition to the inhibitory role of the Cip/Kip family proteins on cell cycle progression p21, and especially p27 can also be found in a stimulatory role in promoting activation of cyclin $D_1$-Cdk4,6 in proliferative cells [18,25].

The 20-epi analogs, especially KH1060 and CB1093, as well as EB1089, an analog with a natural side chain orientation at carbon-20, have already shown their therapeutical potencies as antileukemic compounds and inhibitors of tumor cell growth as well as differentiation-inducing agents [23,26–29]. The present study was undertaken, first, to analyze several candidates of cell cycle controlling proteins possibly responsible for the $G_1$ arrest caused by $1\alpha,25(OH)_2D_3$ in human bone cells. Second, we were also interested in comparing these effects with those of selected $1\alpha,25(OH)_2D_3$ analogs. We used human MG-63 osteosarcoma cells which share many features of normal human osteoblasts and, therefore, should provide useful information relevant to osteoblastic proliferative events. Our results indicated that, in the osteoblastic cells, $1\alpha,25(OH)_2D_3$ signaling appears to be mediated through the Rb pathway, as cells treated with $1\alpha,25(OH)_2D_3$ showed decreased amounts of hyperphosphorylated Rb protein. Consistent with its effects on Rb protein, $1\alpha,25(OH)_2D_3$ reduced Cdk2 activity through increased p27, but not p21 protein levels. The selected analogs, KH1060, EB1089, and CB1093, were clearly more potent than the parent compound in their effects on these cell cycle control mechanisms.

## 2. Materials and methods

### 2.1. Chemicals and antibodies

$1\alpha,25$-Dihydroxyvitamin $D_3$ and the synthetic analogs $1\alpha,25$-dihydroxy-20-epi-22-oxa-24a,26a,27a-trihomo-vitamin $D_3$ (KH1060), $1\alpha,25$-dihydroxy-22,24-diene-24a,26a,27a-trihomo-vitamin $D_3$ (EB1089), and $1\alpha,25$-dihydroxy-20-epi-22-ethoxy-23-yne-24a,26a,27a-trihomo-vitamin $D_3$ (CB1093) (Fig. 1) were a kind gift from Leo Pharmaceutical Products Ltd. The vitamin $D_3$ compounds were dissolved in isopropanol at 4 mM concentration and diluted with ethanol. Radioactive labels [$\alpha$-$^{32}$P]dCTP and [$\gamma$-$^{32}$P]dATP were from Du Pont de Nemours. GST-pRb, the monoclonal antibodies specific for p21, cyclin E, and cyclin $D_1$, as well as the polyclonal antibodies against Cdk2, 4, 6, and p27 were purchased from Santa Cruz Biotechnology, Inc. Monoclonal antibody specific for

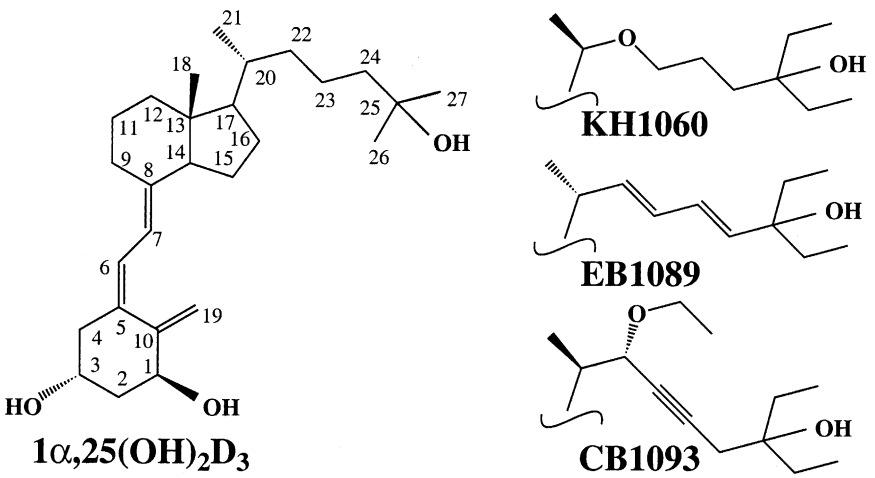


Fig. 1. Structure of $1\alpha,25(OH)_2D_3$ showing the location of the carbon atoms and the side chain structures of the analogs KH1060, EB1089, and CB1093.

pRb protein was purchased from Pharmingen, and the secondary antibodies, as well as protein synthesis inhibitor cycloheximide, were from Sigma–Aldrich. Hybridization probe for p21 was from American Type Culture Collection (ATCC) and the probe for p27 was from Dr. Joan Massague (Memorial Sloan Kettering Cancer Center and Howard Hughes Medical Institute).

### 2.2. Cell culture and nuclear extracts

MG-63 human osteosarcoma cells (ATCC) were cultured in DMEM supplemented with 7% FCS, 2 mM L-glutamine, 0.1 mg/mL streptomycin, and 100 units/mL penicillin at 37° in a humidified (95% air:5% $CO_2$) incubator. All experiments were performed in a medium containing 2% charcoal-treated FCS to eliminate endogenous steroid hormones.

Nuclear extracts for immunoblotting, kinase assay and EMSA were prepared as described in Hurst *et al*. [30] without the heat denaturation step. Protein concentrations were determined using Bio-Rad protein assay dye (Bio-Rad).

### 2.3. Proliferation assays

The MG-63 cells were seeded at $5 \times 10^4$ cells/well onto 6-well plates and incubated in DMEM containing 7% FCS for 24 hr. The medium was replaced by a medium containing 2% charcoal-treated FCS combined with 1 pM to 0.1 μM 1α,25$(OH)_2D_3$ or its analogs, and the cells were cultured for 3 days without further additions of hormones. The cells were trypsinized and quantified using a Coulter Counter. The concentration resulting in 50% of the maximal response was calculated ($IC_{50}$) by subtracting the cell numbers obtained after treatments with the different vitamin $D_3$ compounds from the respective control values. Maximal inhibition value was obtained with 0.1 μM KH1060 and arbitrarily set to 100%.

### 2.4. Cell cycle analysis by flow cytometry

The harvested MG-63 cell pellets were stained for 15 min with propidium iodide (0.05 mg/mL, containing 0.1 M sodium citrate and 0.03% NP40, v/v) at room temperature. RNase A (final concentration 0.2 mg/mL) was added and the cells were further incubated at room temperature for 15 min. Data were collected with FACScan flow cytometer (Becton Dickinson) and analyzed using RFIT program to obtain the portion of the cells in the different phases of the cell cycle.

### 2.5. Western blot analysis

Nuclear extracts (20 μg) were separated on SDS–PAGE gels and electrotransferred onto PVDF membranes (Boehringer Mannheim Gmbl). Immunodetection was performed using either mouse monoclonal antibodies against the human pRb protein, p21, cyclin E, or cyclin $D_1$, or rabbit polyclonal antibodies against the human Cdk2, 4, 6, or p27. The secondary antibodies were respective alkaline phosphatase-conjugated anti-mouse or anti-rabbit immunoglobulins. The protein patterns were detected with nitroblue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate (Sigma–Aldrich).

### 2.6. Immunoprecipitations and kinase assays

Nuclear extracts (70–80 μg) were diluted with buffer A [50 mM HEPES (pH 7.4), 150 mM NaCl, 10% glycerol (v/v), 1% Triton X-100, 1 mM EDTA, 2.5 mM EGTA, 5 mM sodium fluoride, 0.1 mM sodium orthovanadate, 5 μg/mL leupeptin, 10 μg/mL aprotinin, and 50 μg/mL phenylmethylsulfonyl fluoride]. The extracts were incubated with an antibody against Cdk2, 4, or 6 proteins at 4° overnight, followed by precipitation using protein-A Sepharose at 4° for 1 hr. For immunoprecipitations, the beads were washed three times with cold buffer A, twice with cold buffer A without Triton X-100. Protein precipitates were boiled in 1× SDS sample buffer, separated on SDS–PAGE gels, transferred onto PVDF membranes, and probed with primary antibody against p27 protein as described in Western blot methods. For kinase assays, the beads were washed three times with cold buffer A, twice with cold buffer A without Triton X-100 and once with kinase buffer (50 mM HEPES, pH 7.5, 1 mM DTT, 1 mM EGTA, 0.1 mM orthovanadate, 10 mM β-glycerophosphate, and 10 mM $MgCl_2$). The beads were resuspended in 30 μL of the kinase buffer containing 5 μCi of [γ-$^{32}$P]dATP, 20 μM ATP, and 1 μg of GST-pRb as the substrate. After 30 min at 30°, the reaction was terminated by the addition of 5× SDS sample buffer. The supernatant was separated on a SDS–PAGE gel and visualized by autoradiography.

### 2.7. Northern blot analysis

Total cellular RNA was isolated by the guanidine thiosyanate method of Chomczynski and Sacchi [31]. Denatured RNA samples (10 μg) were fractionated on 1% agarose-formaldehyde gels and transferred onto nylon filters. The filters were hybridized with the $^{32}$P-labeled probes for p21 or p27, washed twice with 5× SSC at 42°, once with 2× SSC + 0.1% SDS, and once with 0.1× SSC + 0.1% SDS at room temperature.

### 2.8. EMSA

The binding reactions were done as described previously [11]. The $^{32}$P-labeled double-stranded oligonucleotides from the p27 promoter were (the core binding motifs are shown in bold): Sp1 + NF-Y, 5′-AGC CTC GGC **GGG GCG G**CT **CCC GCC** GCC GCA **ACC AAT** GGA TCT CC -3′; Sp1, 5′- AGC CTC GGC G**GG**

**GCG GCT CCC GCC** GCC -3′; and NF-Y, 5′- CCG CCG CAA **CCA ATG** GAT CTC C -3′ [32]. Protein-bound DNA was separated from the free probe on a 7% polyacrylamide gel run in 25 mM Tris/borate, pH 8.3, 0.5 mM EDTA and the gels were dried and visualized by autoradiography.

### 2.9. Statistical analysis

The cell numbers were determined and intensities of the bands in immunoblots or autoradiographs were scanned. The mean and SEM values were calculated, the data were analyzed and the statistical significance was determined by Student's *t*-test using Statworks software (Cricket Software Inc.).

## 3. Results

### 3.1. Effects of 1α,25(OH)$_2$D$_3$ and its analogs on MG-63 cell growth and on cell cycle phase distribution

To establish conditions to investigate the mechanism of action of the vitamin $D_3$ compounds on inhibition of MG-63 cell growth, the cells were treated with the compounds (concentrations from 1 pM to 0.1 μM) and cell numbers were assessed after 72 hr. All the compounds studied reduced MG-63 cell numbers dose-dependently (Fig. 2). The concentration needed for 50% inhibition of proliferation in our study was calculated to be 120 pM (1α,25(OH)$_2$D$_3$), 3.5 pM (KH1060), 17 pM (EB1089) and 3.8 pM (CB1093), showing that the three analogs were significantly more potent (34×, 7×, 32×, respectively) than 1α,25(OH)$_2$D$_3$ in inhibiting MG-63 cell proliferation. No significant reduction in viability was seen with any of the compounds tested (data not shown). To study the mechanism of regulation of cell cycle progression, in subsequent studies 1 nM concentration was chosen to obtain maximal difference in the effects of vitamin $D_3$ and the analogs but still maintaining the vitamin D response.

To determine the cell cycle distribution of MG-63 cells, the cells were treated with 1 nM vitamin $D_3$ compounds and analyzed by FACS. Cell cycle analysis revealed that 1 nM concentration of the analogs caused accumulation of the cells in the $G_1$ phase already after 48 hr (85–88% of the cells vs. 64% in the control cultures) (Table 1). The effect was even more obvious after 72 hr (90–91% vs. 66%). In 1α,25(OH)$_2$D$_3$-treated cultures the maximal effect was obtained earlier but it was lower (74% in $G_1$ at 48 and 72 hr) than with the analogs. The duration of the cell cycle of MG-63 cells is around 27 hr (data not shown) and, thus, the effects of studied vitamin $D_3$ compounds on regulation of cell cycle can not be reliably seen during the first 24 hr of incubation. According to these results, the effects on different regulatory proteins were thought to take place between 24 and 72 hr.

### 3.2. Characterization of cell cycle regulatory proteins involved in analog-induced $G_1$ arrest

Protein levels of selected cell cycle regulatory proteins involved in $G_1$ progression were studied by immunoblots (Fig. 3). Both the untreated and 1α,25(OH)$_2$D$_3$-treated

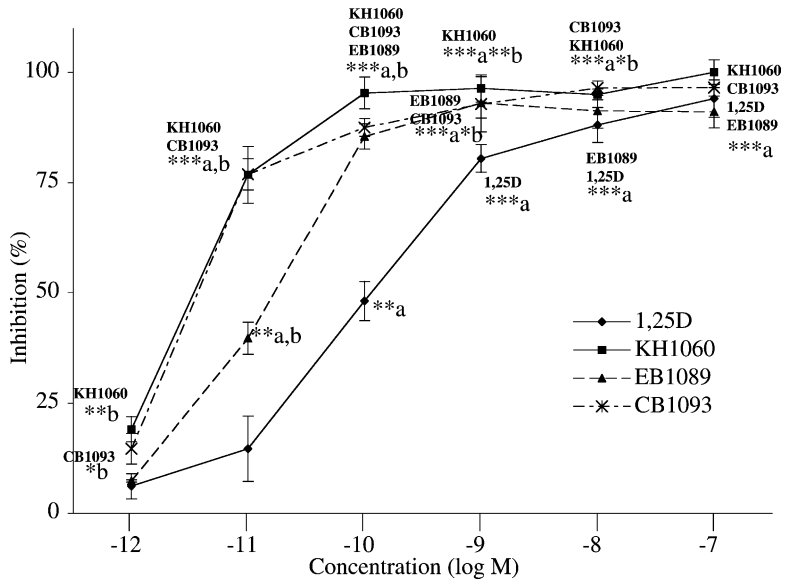


Fig. 2. Inhibition of MG-63 cell growth by different concentrations of 1α,25(OH)$_2$D$_3$ and the synthetic analogs. The cells were treated with one addition of 1 pM to 0.1 μM 1α,25(OH)$_2$D$_3$ (1,25D), the analogs KH1060, EB1089, and CB1093, or the vehicle for 3 days before determination of cell numbers. The concentration resulting in 50% of the maximal response was calculated ($IC_{50}$) by subtracting the cell numbers obtained after treatments with different vitamin $D_3$ compounds from the respective control value. Maximal inhibition value was obtained with 0.1 μM KH1060 and arbitrarily set to 100%. The values are means ± SD (N = 6). Statistical significance for the difference between control vs. different treatments (a) and 1α,25(OH)$_2$D$_3$ vs. analog treatments (b); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

Table 1
Cell cycle phase distribution of the MG-63 cells after treatment with the vitamin $D_3$ compounds for different periods of time

| Treatment[#] | | Percentage (%) in | | |
|---|---|---|---|---|
| | | $G_1$ | S | $G_2$/M |
| 24 hr | CTRL | 64.8 ± 2.8 | 20.5 ± 1.8 | 14.7 ± 1.3 |
| | 1,25D | 68.8 ± 0.7 | 15.8 ± 1.0 | 15.4 ± 1.3 |
| | KH1060 | 68.9 ± 1.8 | 16.9 ± 1.3 | 14.2 ± 1.0 |
| | EB1089 | 69.3 ± 3.9 | 16.5 ± 2.6 | 14.2 ± 2.6 |
| | CB1093 | 67.8 ± 0.0 | 18.3 ± 0.4 | 13.9 ± 0.2 |
| 48 hr | CTRL | 63.5 ± 0.8 | 23.6 ± 1.6 | 12.9 ± 2.4 |
| | 1,25D | 74.1 ± 1.6[*] a | 17.7 ± 1.2[*] a | 8.2 ± 0.3[*] a |
| | KH1060 | 85.3 ± 0.6[***,**] a, b | 7.8 ± 0.9[**,**] a, b | 6.9 ± 0.6[*,*] a, b |
| | EB1089 | 85.0 ± 2.3[**,*] a, b | 8.4 ± 1.0[**,**] a, b | 6.6 ± 1.3[*] a |
| | CB1093 | 88.0 ± 1.4[**,*] a, b | 6.7 ± 0.1[**,**] a, b | 5.3 ± 1.1[*,*] a, b |
| 72 hr | CTRL | 66.0 ± 5.3 | 21.1 ± 1.8 | 12.9 ± 2.6 |
| | 1,25D | 74.3 ± 2.7 | 16.5 ± 5.6 | 9.2 ± 2.6 |
| | KH1060 | 90.4 ± 3.3[*,**] a, b | 5.6 ± 1.5[***,*] a, b | 4.0 ± 1.2[**,*] a, b |
| | EB1089 | 90.5 ± 1.6[*,**] a, b | 5.3 ± 0.3[**] a | 4.2 ± 1.2[**,*] a, b |
| | CB1093 | 91.4 ± 1.9[*,**] a, b | 5.4 ± 1.7[**] a | 3.2 ± 0.1[**,*] a, b |

Data from FACScan (Becton Dickenson) were analyzed using the RFIT program to obtain data for cell cycle phase distribution. The values are means ± SD (N = 3). Statistical significance for the difference between control vs. different treatments (a) and 1α,25$(OH)_2D_3$ vs. analog treatments (b). Abbreviations: CTRL, control; and 1,25D, 1α,25$(OH)_2D_3$.

[#] Cells were grown in the absence (control) or presence of 1 nM test compounds for up to 72 hr.

[*] $P < 0.05$; [**] $P < 0.01$; [***] $P < 0.001$.

MG-63 cells expressed the hypophosphorylated (pRb; approximately 110 kDa) and hyperphosphorylated (ppRb; approximately 114 kDa) Rb protein at all time points (24–72 hr). In the analog-treated cells, however, the Rb protein was predominantly in its hypophosphorylated form from 48 hr on.

The Cdks mainly involved in $G_1$ phase are Cdk2, 4, and 6. The phosphorylation level of the Cdk2 protein did not change in the control cells during the 72-hr incubation, but treatment with 1α,25$(OH)_2D_3$ resulted in a slight decrease in phosphorylation status of Cdk2. In the analog-treated cells, however, the Cdk2 protein levels were downregulated and, after 48 hr, the protein was mainly in its hypophosphorylated form (slower migrating band in SDS–PAGE [33,34]). These treatments had no effect on the expression or phosphorylation levels of the proteins Cdk4 or 6.

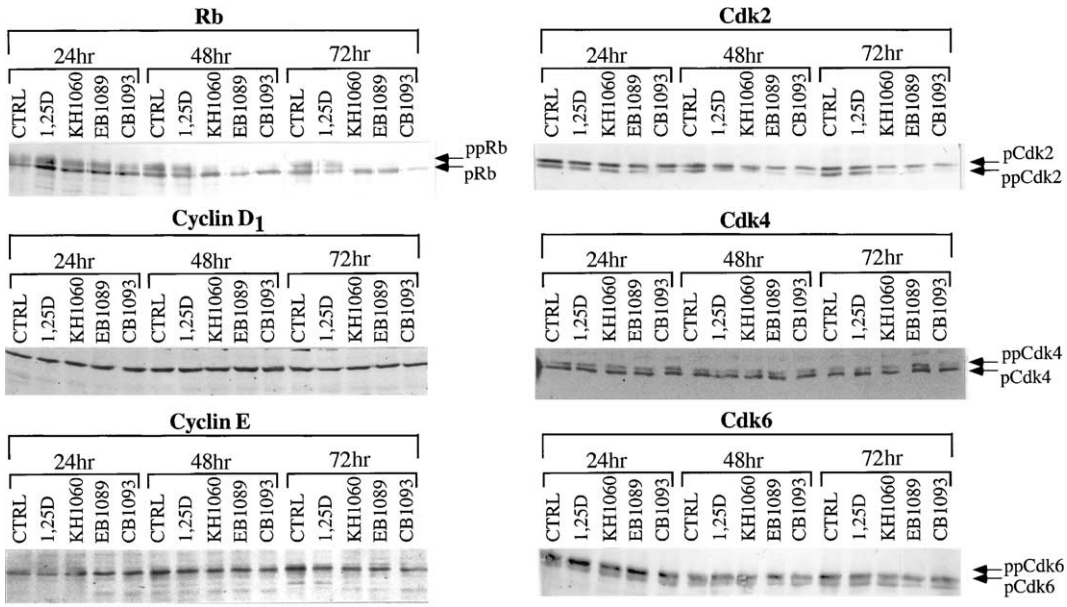


Fig. 3. Effects of the vitamin $D_3$ compounds on Rb protein, cyclin $D_1$, cyclin E, Cdk2, 4, and 6 protein levels. Extracts from MG-63 cells treated for 72 hr with 1 nM 1α,25$(OH)_2D_3$ (1,25D), KH1060, EB1089, and CB1093, or the vehicle for, were analyzed by SDS–PAGE, and immunoblotted as indicated in Section 2. Abbreviations: ppRb, hyperphosphorylated Rb; pRb, hypophosphorylated Rb; pCdk2, 4, 6, hypophosphorylated Cdk2, 4, 6; and ppCdk2, 4, 6, hyperphosphorylated Cdk2, 4, 6. The results shown are representative of 3–5 independent experiments.

From the cyclins, cyclin $D_1$ and cyclin E were chosen for further studies, because they have been reported to be essential for $G_1$ phase progression in cells with a functional pRb [35,36]. According to the immunoblot analyses (Fig. 3), after 24 hr cyclin E levels varied in 1α,25$(OH)_2D_3$-treated cells (85%) and analog-treated cells (103–105%) compared to control, whereas in later time points cyclin E was constantly more abundant in control cells than in cells treated with 1α,25$(OH)_2D_3$ (80% of the control level at 48 hr, and 67% at 72 hr) or the analogs (53–64% of the control level at 48 hr, and 42–64% at 72 hr). On the other hand, there were no alterations in the expression of cyclin $D_1$ protein levels during the 72 hr treatment.

### 3.3. Measurement of activities of Cdk2, 4 and 6

Western blot analysis showed that the analogs induced changes in the amount and phosphorylation status of the Cdk2 protein, but these treatments did not alter the levels of Cdk4 and 6. We next questioned whether the activities of immunoprecipitated Cdks were changed. The Cdk2, 4, and 6 immunoprecipitates were prepared from cells treated with 1 nM 1α,25$(OH)_2D_3$ or the analogs and subjected to *in vitro* kinase assays using GST-pRb fusion protein (46 kDa) as a substrate. In control cells, Cdk2 activity remained constant for up to 48 hr (Fig. 4). 1α,25$(OH)_2D_3$ reduced the Cdk2 activity by about 19% at 24 hr, and by 45% at 48 hr compared with the control cells. Treatment with the analogs reduced the Cdk2 activity by about 20% of the control level at 24 hr, and by about 95% at 48 hr. There was no detectable change in the phosphorylation of pRb by Cdk4 or 6 (data not shown).

### 3.4. Effect of 1α,25$(OH)_2D_3$ and its analogs on p21 and p27

Potential mediators for the $G_1$ phase block are Cdk inhibitors p21 and p27. The p27 protein levels were low in untreated MG-63 cells (Fig. 5A). In the analog-treated cells, the p27 protein levels peaked at 48 hr (2.6–2.9-fold compared with control). In the 1α,25$(OH)_2D_3$-treated cells, the p27 levels slightly increased (approximately 1.2-fold compared with control) at 48 hr. The p21 protein levels responded only minimally to the treatments with the vitamin $D_3$ compounds at 24 hr whereas at 48 hr all these compounds clearly reduced p21 protein levels (Fig. 5A).

The proteins responsible for the cell cycle progression can be regulated not only at the level of activity, but also at the level of transcription and translation. The p27 mRNA levels were examined from the cells treated with 1 nM 1α,25$(OH)_2D_3$ or the analogs for 48 hr using Northern analysis (Fig. 5B). The expression of p27 mRNA was slightly increased during the first 24 hr (1.1–1.2-fold increase at 8 hr and 1.2–1.6-fold increase at 24 hr) and reached highest values at 48 hr (1.6–2.2-fold).

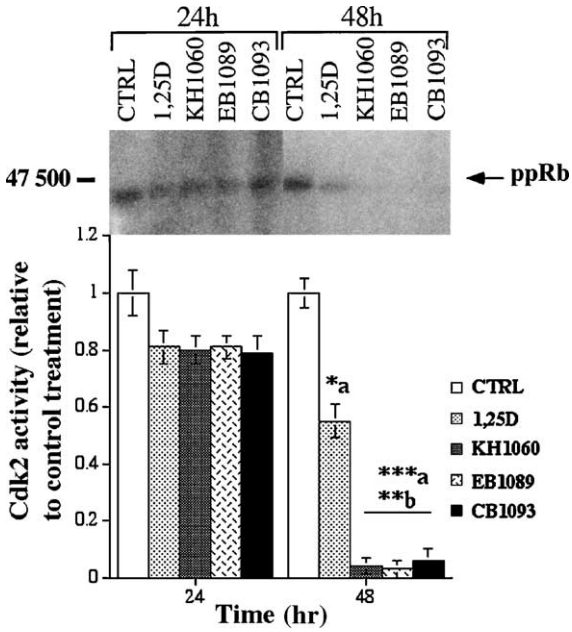


Fig. 4. Effects of vitamin $D_3$ compounds on Cdk2 kinase activity. Cdk2 immunoprecipitates were prepared from extracts derived from MG-63 cells treated with vehicle, 1 nM 1α,25$(OH)_2D_3$ (1,25D) or the analogs for 48 hr. Cdk2 associated kinase activity was determined using [$^{32}$P]ATP and Rb fusion protein as a substrate. Labeled Rb was detected by SDS–PAGE and autoradiography. Cdk2 activity is expressed in relation to the control treatment. The values are means ± SEM (N = 3). Statistical significance for the difference between control vs. different treatments (a) and 1α,25$(OH)_2D_3$ vs. analog treatments (b). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

The effects of 1α,25$(OH)_2D_3$ and its analogs on p27 degradation were studied in cycloheximide-treated MG-63 cells. After inhibition of new protein synthesis by cycloheximide for 24 hr, the p27 protein levels stayed clearly higher in the analog-treated cells (1.6–1.8-fold) than in the control or 1α,25$(OH)_2D_3$-treated cells (Fig. 5C) indicating that degradation of p27 is inhibited in the presence of vitamin $D_3$ analogs.

Since the p27 levels were increased, and the Cdk2 phosphorylation and the kinase activity were markedly decreased after treatment of MG-63 cells with the vitamin $D_3$ compounds, we examined whether the changes in p27 protein levels could be detected in the complex with Cdk2. There was only a small increase (about 5%) in the association of p27 with Cdk2 after a 48-hr 1α,25$(OH)_2D_3$ treatment (Fig. 5D). After treatment with analogs KH1060, EB1089, and CB1093, however, increased association of p27 with Cdk2 (about 1.3-fold higher than in control cells) was observed.

### 3.5. Protein binding to p27 promoter region

The expression of p27 is regulated by 1α,25$(OH)_2D_3$ through a complex element, which binds two ubiquitous transcription factors, namely Sp1 and NF-Y [32]. In this study, binding reactions with this element and the nuclear extracts from MG-63 cells resulted in the formation of

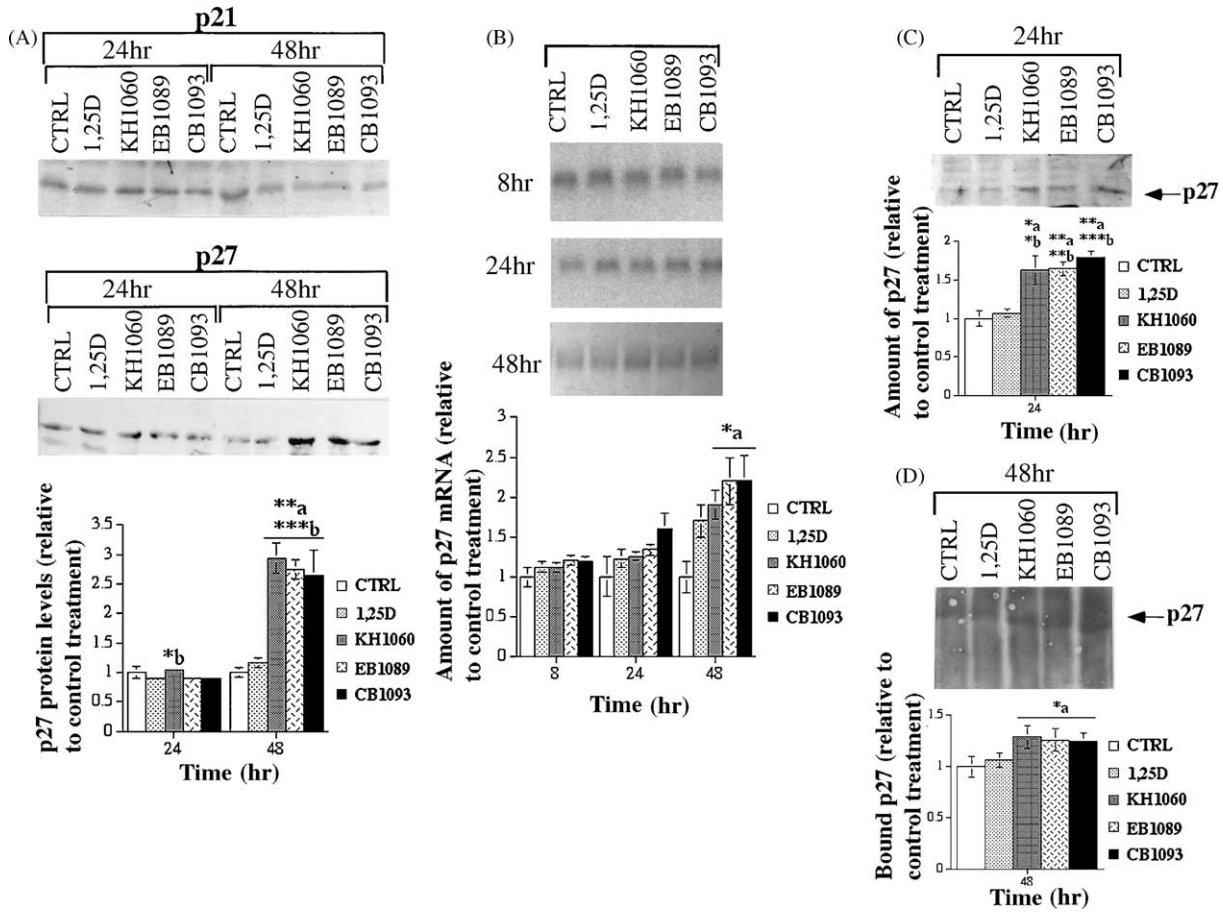


Fig. 5. Effects of vitamin $D_3$ compounds on p21 and p27 expression. (A) Protein levels of p21 and p27. Extracts from MG-63 cells treated for up to 48 hr with 1 nM vitamin $D_3$ compounds or the vehicle were analyzed by SDS–PAGE and immunoblotted as indicated in Section 2. The bands showing p27 protein levels were scanned and the results are shown in relation to the respective control treatment. (B) Nylon membranes containing 10 μg of total RNA in each lane were hybridized with the [α-$^{32}$P]p27 probe. The p27 mRNA intensities were corrected against loading of ribosomal RNA (data not shown) and the results from different time points are shown in relation to the respective control values. (C) p27 degradation in the presence of vitamin $D_3$ compounds. MG-63 cells were treated with the protein synthesis inhibitor cycloheximide (CH, 10 μg/ml) together with vehicle or the vitamin $D_3$ compounds for 24 hr. Nuclear extracts and Western immunoblotting were performed as indicated in Section 2. The p27 bands were scanned and shown in relation to the respective control treatment. (D) Binding activity of p27 to Cdk2. The binding activity of p27 to Cdk2 at 48 hr was determined by separating the Cdk2 immunoprecipitates on SDS–PAGE, transferring onto PVDF membranes, and probing with anti-p27 antibody as indicated in Section 2. The values in (A–D) are means ± SEM (N = 3). Statistical significance for the difference between control vs. different treatments (a) and 1α,25(OH)$_2$D$_3$ vs. analog treatments (b). $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

three differentially migrating protein–DNA complexes (Fig. 6A; I–III). Two of these complexes (II and III) bound to the Sp1 half site of the element. No complexes were obtained with the pure NF-Y half site of this region. For the formation of complex I, however, the NF-Y half site was also required (Fig. 6A). These results indicate that complexes II and III contain Sp1 and that NF-Y is needed for the formation of complex I. The composition of these complexes was further verified by EMSA reactions performed with Sp1 and NF-Y antibodies and competition experiments using the labeled Sp1 + NF-Y element and an excess of unlabelled Sp1 or NF-Y half site (data not shown). After a 24-hr treatment, all vitamin D compounds resulted in equal binding of protein to the Sp1 half site of the element (Fig. 6B). Dissociation of the proteins was, however, slower in cells treated with the analogs than in cells treated with 1α,25(OH)$_2$D$_3$ (1.4–1.5-fold difference at 48 hr) (Fig. 6B and C).

## 4. Discussion

Progression of cell cycle from the $G_1$ phase to the S phase is one of the most critical steps in the control of cell proliferation and also the step most often altered in cancer cells. In cancer therapy, much attention has been focused on the inhibition of cell proliferation and regulation of the $G_1$ phase cyclin-Cdk functions [17,37]. The antiproliferative effects of 1α,25(OH)$_2$D$_3$ and its synthetic analogs may be useful, e.g., in cancer treatment, and therefore a more detailed understanding of their molecular mechanisms of action as prospective anticancer agents is important.

Inhibition of MG-63 cell growth and accumulation of the osteoblastic cells in the $G_1$ phase was obvious after treatment with 1α,25(OH)$_2$D$_3$ and, especially, after treatment with the analogs KH1060, EB1089, and CB1093. This $G_1$ phase arrest occurring in human osteosarcoma cells and also in other cells treated with the vitamin $D_3$

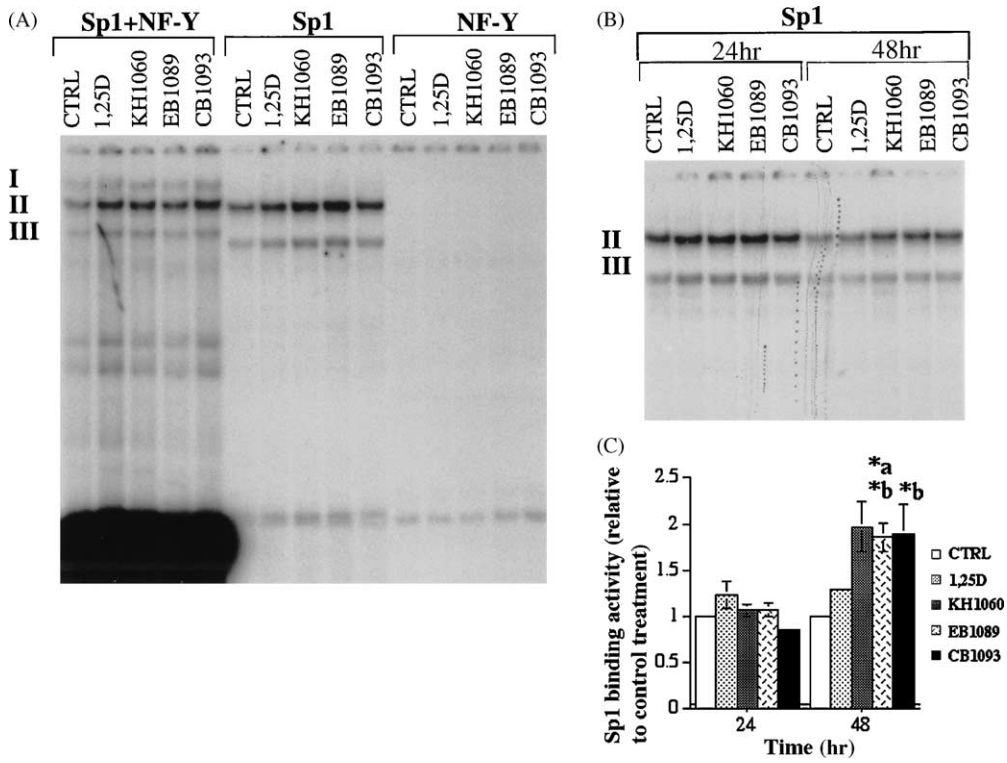


Fig. 6. Binding of nuclear proteins to the Sp1 + NF-Y region of the *p27* gene. (A) MG-63 cells were treated with $1\alpha,25(OH)_2D_3$ or its synthetic analogs (10 nM) for 48 hr and the binding of nuclear proteins to the Sp1 + NF-Y region of the *p27* gene was verified by EMSA. Free probe is shown only for the Sp1 + NF-Y probe because both the Sp1 and NF-Y probes are smaller and run from the gel. (B) The cells were treated for different time periods as indicated and nuclear proteins were used in EMSA with the Sp1 probe. Only the complexes are shown. (C) Intensity of Sp1 binding to the Sp1 probe. The complexes shown in (B) were scanned and the results from different time points are shown in relation to the respective control treatment. The values are means ± SEM (N = 3). Statistical significance for the difference between control vs. different treatments (a) and $1\alpha,25(OH)_2D_3$ vs. analog treatments (b); $^{*}P < 0.05$, $^{**}P < 0.01$, $^{***}P < 0.001$.

compounds suggests that these compounds can regulate one or more factors important in blockage of the $G_1$/S progression [14,20,38–40]. The major Cdk/cyclin complexes involved in cell cycle progression from the $G_1$ to the S phase are cyclin $D_1$-Cdk4,6 and cyclin E-Cdk2 [17]. These Cdks can sequentially phosphorylate Rb during the cell cycle. The cyclin $D_1$-Cdk4,6 complex partially phosphorylates Rb, which begins to release E2F transcription factors. This leads to elevated expression of the E2F-regulated genes, for example those of cyclins E and A [17], which are required for S phase entry. The cyclin E-Cdk2 complex then further fully phosphorylates Rb, which releases more E2F, and the cells transit to the S phase [41]. In MG-63 cells, the analogs efficiently prevented hyperphosphorylation of the Rb protein. This was not due, however, to decreased production of cyclin $D_1$, Cdk4, or 6. Cyclin E levels were, however, clearly reduced in the analog-treated cells. This may be explained by elevated levels of the p27 protein, which apparently causes increased formation of inactive cyclin E-Cdk2-p27 complex and reduced activity of Cdk2. This in turn prevents the hyperphosphorylation of Rb, further the release of E2F and, finally, the production of cyclin E.

Cdk inhibitors (CKIs) serve as main regulators of the various cyclin/Cdk complexes of the Rb pathway. Malfunction of CKIs can cause critical proliferative errors in cells leading to cancer development. Low expression of the p27 protein, for example, occurs frequently in many types of human tumors. CKIs are classified into two families on the basis of their structures and Cdk targets. The INK4 family (p16, p15, p18, and p19) specifically inhibits the catalytic subunits of Cdk4/Cdk6, whereas the Cip/Kip family (p21, p27, and p57) more broadly inhibits different kinds of cell cycle proteins, such as cyclin E and A dependent kinases ([18], and references therein). $1\alpha,25(OH)_2D_3$ has both transcriptional and translational effects on the expression of different CKIs (mainly to p21 and p27) leading to growth inhibition of different cell lines [19,21,42–45]. In MG-63 cells, the decreased activity of cyclin E-Cdk2 complex seems to be mainly controlled by the increased level of p27 as no difference was observed in p21 expression after a 24-hr treatment and a clear reduction after 48 hr. This effect of vitamin $D_3$ compounds on p21 is totally different from the effects reported in other cell lines [19,44]. As the degradation of vitamin $D_3$ analogs is slower than that of $1\alpha,25(OH)_2D_3$ itself, this would explain the higher effectivity of the analogs in p27 expression. In addition, the vitamin $D_3$ analogs seems to be able to stabilize the p27 protein in MG-63 cells. In fact, phosphorylation of p27 by cyclin E-Cdk2 has been shown to trigger

ubiquitin-mediated degradation of p27 via the proteasome [46]. Recently Liu *et al*. [47] showed that the growth of thyroid carcinoma cells was inhibited by 1α,25(OH)$_2$D$_3$ and EB1089 as a result of reduced p27 phosphorylation and diminished degradation of p27 [47].

1α,25-Dihydroxyvitamin D$_3$ is able to modulate the transcription of the *p27* gene although it lacks the classical VDRE. This was reported by Inoue *et al*. [32], who showed that the transcription factors Sp1 and NF-Y mediate the 1α,25(OH)$_2$D$_3$-stimulated expression of the *p27* gene through a composite Sp1 + NF-Y element. In our study, the mRNA expression of p27 was increased in the MG-63 cells, especially after treatment of the cells with the vitamin D$_3$ analogs. We show here that the Sp1 half site bound two differentially migrating complexes (II and III) whereas the NF-Y half site alone was not able to bind any proteins (Fig. 6A). Nevertheless, there is a NF-Y containing complex (designated as complex I in Fig. 6A) formed with the Sp1 + NF-Y element, suggesting that Sp1 binding, or at least the Sp1 half site of the element, is required for the binding of NF-Y to this region. Previous studies have shown that 1α,25(OH)$_2$D$_3$ slightly increases the expression of Sp1 for the first 36 hr [32] and that it also modifies Sp1 post-translationally [48,49]. On the other hand, 1α,25(OH)$_2$D$_3$ downregulates the expression of NF-Y, the critical component for the DNA binding of the trimeric NF-Y protein [32]. The time course of the increase in Sp1 binding after the 1α,25(OH)$_2$D$_3$-treatment obtained here (around 24 hr) is in good correlation with the 36 hr obtained by Inoue *et al*. [32]. The slow dissociation rate of the proteins from the Sp1 element reflects the prolonged stimulation of p27 expression and inhibition of cell cycle progression in the presence of the analogs. Although the *p21* gene contains a VDRE within its promoter region [19], we, and also other investigators using other types of malignant cells [50], did not find any changes in p21 protein or mRNA levels after the 1α,25(OH)$_2$D$_3$-treatment.

In summary, a sequence of events was defined for the effects of selected transcriptionally active vitamin D$_3$ analogs for the cell cycle arrest in human MG-63 osteosarcoma cells. The following deductions were made. The antiproliferative effect of the vitamin D$_3$ compounds in these cells is mediated by increased p27 levels, which results in marked reduction or total loss of Cdk2 activity. The decreased Cdk2 activity is also accompanied by downregulated Cdk2 and cyclin E protein levels, but not those of cyclin D$_1$. These factors apparently keep Rb protein in its hypophosphorylated form which also results in the arrest of the MG-63 cells in the G$_1$ phase. The results shown here and the ones reported elsewhere [20,21,43–45] suggest that regulation of the cell division cycle uses a complex network of regulatory processes differentially regulated by vitamin D in different cell lines. This further delineates the importance of cell-specific factors all of which are probably not recognized yet.

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