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ZK 156718, a Low Calcemic, Antiproliferative, and Prodifferentiating Vitamin D Analog

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The physiologically active form of vitamin D, 1,25-dihydroxyvitamin D_3, plays an important role not only in the establishment and maintenance of calcium metabolism, but also in regulating cell growth and differentiation. Because the clinical usefulness of 1,25-dihydroxyvitamin D_3 is limited by its tendency to cause hypercalcemia, new analogs with a better therapeutic profile have been synthesized, including ZK 156718. We compared the effects of 1,25-dihydroxyvitamin D_3 and ZK 156718 on growth, differentiation, and on $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ expression in human colon cancer cells (Caco-2). Whereas ZK 156718 at the concentration [10^{-8} M] was as potent as 10^{-6} M 1,25-dihydroxyvitamin D_3 in inducing differentiation and $p21^{Waf1/Cip1}$ expression, it was even more effective in inhibiting cell growth and stimulating $p27^{Kip1}$ expression than 1,25-dihydroxyvitamin D_3 itself. In summary, our study presents a new and potent vitamin D analog with a decreased metabolic stability, making it useful for the treatment of a diversity of clinical disorders.

Key Words: **Caco-2 cells; differentiation; proliferation; $p21^{Waf1/Cip1}$ expression; $p27^{Kip1}$ expression; vitamin D analog.**

In addition to its well recognized function in the regulation of calcium and phosphorus homeostasis, there is increasing evidence that 1,25-dihydroxyvitamin D_3 (1,25-$(OH)_2D_3$), the active form of vitamin D_3, has important physiological effects on growth and differentiation in a variety of malignant and nonmalignant cell lines (1). Its prodifferentiating and antiproliferative effects have been demonstrated in rapidly growing cells, including normal human bone cells (2), human monocytic cells (3, 4) and malignant cells derived from colon (5–7) and other tissues. In addition to its antiproliferative and differentiation-inducing effects, 1,25-$(OH)_2D_3$ has been found to induce apoptosis and to decrease the invasiveness of a number of different cancer cells *in vitro* (8). *In vivo,* inhibition of the development of metastases, regression of tumors, inhibition of angiogenesis, and prolongation of survival time have been observed in tumor-bearing animals treated with the hormone (1, 9).

The vitamin D receptor (VDR), which is present in classical vitamin D-responsive organs such as bone, kidney, and intestine, has also been localized in a variety of other normal tissues and several cancer cell lines (8, 10) including the Caco-2 cell line (7). Through binding to the VDR, induction of a conformational change of the VDR (12) and subsequent binding of the VDR-RXR complex to unique promoter sequences (response elements) of vitamin D target genes, 1,25-$(OH)_2D_3$ has been shown to regulate the transcription of numerous genes involved in calcium and phosphorus regulation, metabolism of vitamin D, DNA replication and differentiation (1, 10, 13).

The broad distribution of VDR in the human body and the fact that 1,25-$(OH)_2D_3$ is able to affect cell growth and differentiation make this hormone a potentially useful agent in the treatment of diseases such as cancer, psoriasis and immunological disorders. However, to inhibit cellular proliferation and induce differentiation, pharmacologic concentrations of vitamin D are frequently necessary (14). Therefore, the systemic or extensive topical application of 1,25-$(OH)_2D_3$ is limited because of the hypercalcemic side effects occuring after resorption of the hormone (14). Much effort has consequently been directed into identifying vitamin D analogs with potent cell regulatory effects but with weaker effects on the calcium metabolism than those of 1,25-$(OH)_2D_3$. A strategy to decrease the hypercalcemic

Abbreviations used: 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; AP, alkaline phosphatase; cdk, cyclin-dependent kinase; RXR, retinoid X receptor; VDR, vitamin D receptor; VDRE, vitamin D response element.

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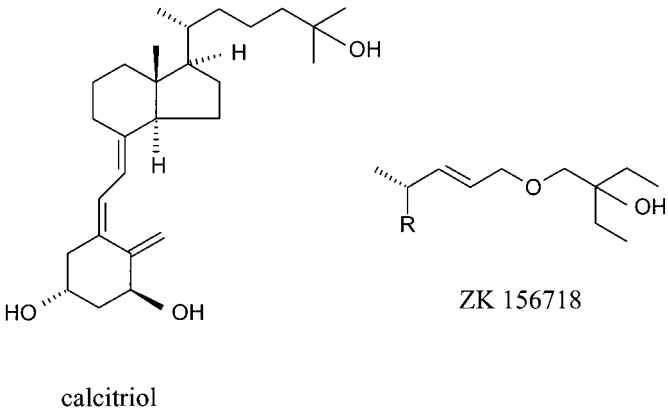

FIG. 1. Structure of 1,25-$(OH)_2D_3$ and the side-chain structure of ZK 156718.

effects of topically applied vitamin D analogs is the development of compounds with increased metabolic lability, which are more rapidly cleared than 1,25-$(OH)_2D_3$ after resorption. One of these new synthetic analogs is ZK 156718, which is characterized by an altered side chain structure exhibiting the 22-ene-25-oxa modification (Fig. 1). When injected into rats, the calcitriol analog ZK 156718 displayed a 100-fold lower hypercalcemic effect (15). The lower calcemic effect of some vitamin D analogs may be due, in part, to their lower binding affinity for the vitamin D binding protein (16) and their increased clearance, but other mechanisms such as tissue-specific differences in the binding affinities of 1,25-$(OH)_2D_3$ and the analogs for the VDR, and different signalling pathways have also been suggested to contribute (17, 18).

In the present work we used the human intestinal cell line Caco-2 to examine growth inhibition, differentiation and expression of the two cyclin-dependent kinase-inhibitors $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ induced by either 1,25-$(OH)_2D_3$ or by the vitamin D analog ZK 156718. Even though Caco-2 cells are derived from a colon adenocarcinoma, they have retained the ability for spontaneous redifferentiation in culture into enterocyte-like cells. It is already known that Caco-2 cells are not solely dependent on circulating 1,25-$(OH)_2D_3$ but can also produce their own source of the steroid hormone by 1α-hydroxylation from 25-$(OH)D_3$ (19).

Our study demonstrates that ZK 156718 is more effective at a lower concentration in inhibiting cell proliferation and inducing $p27^{Kip1}$ expression compared to 1,25-$(OH)_2D_3$. In contrast, in inducing cell differentiation and $p21^{Waf1/Cip1}$ expression, both agents, used in different concentrations, are comparable to each other.

MATERIALS AND METHODS

Chemicals and supplies. Disposable cell culture was purchased from Nalge Nunc International (Wiesbaden, Germany). Dulbecco's modified Eagle medium (DMEM), fetal calf serum (FCS), sodium pyruvate, nonessential amino acids and PBS were obtained from GIBCO BRL (Eggenstein, Germany). Penicillin/streptomycin was from Biochrom (Berlin, Germany). 1,25-$(OH)_2D_3$ and ZK 156718 (19) were supplied by the Department of Medicinal Chemistry at Schering AG (Berlin, Germany).

1,25-$(OH)_2D_3$ and ZK 156718 were dissolved in ethanol (final maximal concentration of ethanol in medium was 0.1% [v/v]) to yield a 10^{-2} mol/L stock solution which was stored at −20°C.

Cell culture. Caco-2 cells were obtained from the German Cancer Research Center (Heidelberg, Germany). The stock was maintained in 175-cm^2 flasks in a humidified incubator at 37°C in an atmosphere of 95% air and 5% CO_2. The medium consisted of DMEM, supplemented with 10% FCS, 1% penicillin/streptomycin, 1% sodium pyruvate and 1% nonessential amino acids. The cells were passaged weekly using Dulbecco's PBS containing 0.25% trypsin and 1% EDTA. The medium was changed three times per week. Passages 40–45 were used in all experiments. The cells were screened for possible contamination with mycoplasma at monthly intervals. For experiments, the cells were seeded onto plastic cell culture wells in serum-containing medium and allowed to attach for 24 h. Before treatment, the cells were synchronized in medium containing 1% FCS. To assess whether the solvent may influence the experimental conditions, control cells were treated with 0.1% (v/v) ethanol. No difference was observed.

Cell proliferation. Cell proliferation was assessed by cell counting after staining with crystal violet (19). For this assay, the cells were seeded onto 96-well plates (0.33 cm^2). After 24 h incubation with 1,25-$(OH)_2D_3$, the cells were carefully washed with PBS, fixed with 5% (v/v) formaldehyde, and stained with crystal violet (0.5% [w/v]). After washing with PBS and addition of acetic acid 33% (v/v), absorbance at 620 nm was measured using an ELISA plate reader.

Cytotoxicity. Cytotoxicity assay was done by measuring lactate dehydrogenase activity in cell culture medium, using a commercial kit (LDH kit, Merck, Darmstadt, Germany).

Cell differentiation. Alkaline phosphatase (AP) activity was used to assess differentiation of Caco-2 cells. For the assay, the cells were washed with cold PBS, scraped, sonicated (2 × 5 s) and centrifuged at 1500*g* for 10 min. AP activity in the supernatant was measured by hydrolysis of *p*-nitrophenyl phosphate at pH 9.8 and 25°C (Ecoline Alkaline Phosphatase Assay, Merck, Darmstadt, Germany). Cellular protein was determined by Coomassie blue assay using a commercial kit (Bio-Rad Laboratories GmbH, Munich, Germany). Enzyme activity was expressed as milliunits per milligram of protein, one unit representing the enzyme activity hydrolyzing 1 μmol of substrate/min.

SDS–polyacrylamide gel electrophoresis and immunoblot analysis. Cells were washed twice with ice-cold PBS and lysed in protein lysis buffer (Biolabs, Beverly, MA) containing protease inhibitors (Boehringer Mannheim, Germany). Protein extracts were obtained after sonication of cell lysates 2 × 5 s and centrifugation at 10,000 rpm at +4°C. Protein content was again quantified with the Bio-Rad protein colorimetric assay. After addition of sample buffer to the whole cellular extract and boiling samples at 95°C for 15 min 40 μg of total protein lysate were separated on a 15% SDS–polyacrylamide gel. Proteins were transferred onto nitrocellulose membrane (Schleicher & Schuell, Dassel, Germany) and the membrane was blocked overnight at +4°C with 3% (w/v) skim milk in Tris-buffered saline containing 0.05% (v/v) Tween 20 (TBS-T). Next, blots were washed and incubated 1 h in TBS-T with a 1:500 dilution of primary antibody for $p21^{Waf1/Cip1}$ (Oncogene, Cambridge, UK) or in 3% skim milk in TBS-T a 1:1000 dilution of primary antibody for $p27^{Kip1}$ (Santa Cruz Biotechnologies, Santa Cruz, CA). The secondary, horseradish peroxidase-conjugated antibodies (Vector-Lab., Burlingame, CA) were diluted at 1:2000 and incubated with the membrane for another 30 min. After chemoluminescence reaction (ECL, Amersham Phar-

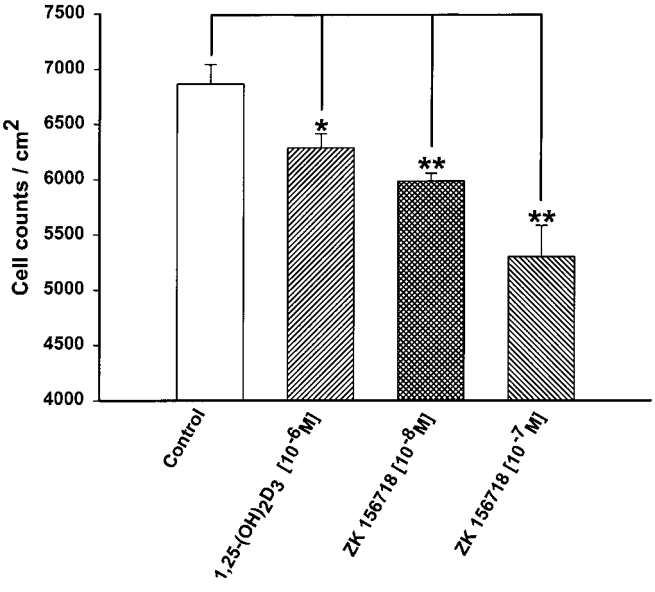

FIG. 2. Effect of 1,25-$(OH)_2D_3$ and the VDR-agonist ZK 156718 on Caco-2 cell growth. Cells were treated with medium supplemented with 1,25-$(OH)_2D_3$ [10^{-6} M] or with ZK 156718 [10^{-7} and 10^{-8} M] for 24 h. Cell counts were calculated by staining with crystal violet. Values are means ± SD, $n = 8$. *$P < 0.05$; **$P < 0.01$.

macia Biotech, Buckinghamshire, UK), bands were detected after exposure to Hyperfilm-MP (Amersham International Plc, Buckinghamshire, UK). Blots were reprobed with actin antibody (Santa Cruz Biotechnologies, Santa Cruz, CA). For quantitative analysis, bands were detected and evaluated densitometrically by ProViDoc system (Desaga, Wiesloch, Germany), normalized for the density of β-actin.

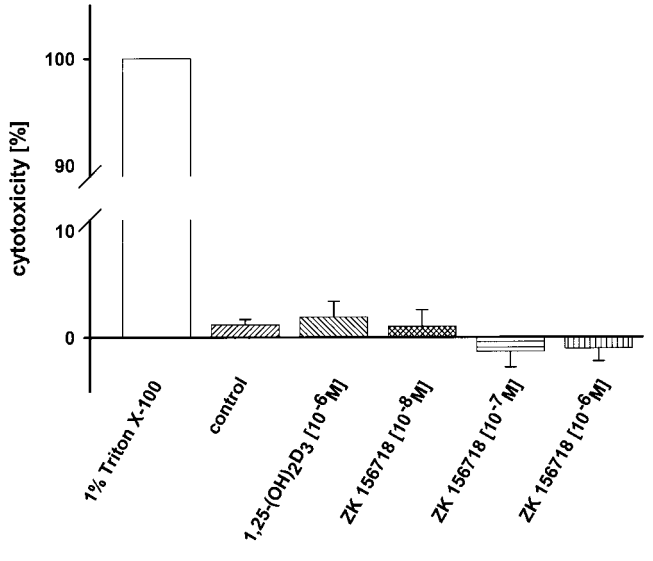

FIG. 3. Cytotoxicity assay. Caco-2 cells were treated with Triton X-100 (1%) as a positive control, ethanol (0.1%), 1,25-$(OH)_2D_3$ [10^{-6} M] or ZK 156718 [10^{-8} to 10^{-6} M] for 24 h. Values are means ± SD, $n = 8$. Differences between control cells and cells treated with 1,25-$(OH)_2D_3$ or ZK 156718 were not statistically significant; differences between cells treated with Triton X-100 and cells treated with ethanol, 1,25-$(OH)_2D_3$ or ZK 156718 were extremely significant ($P < 0.001$).

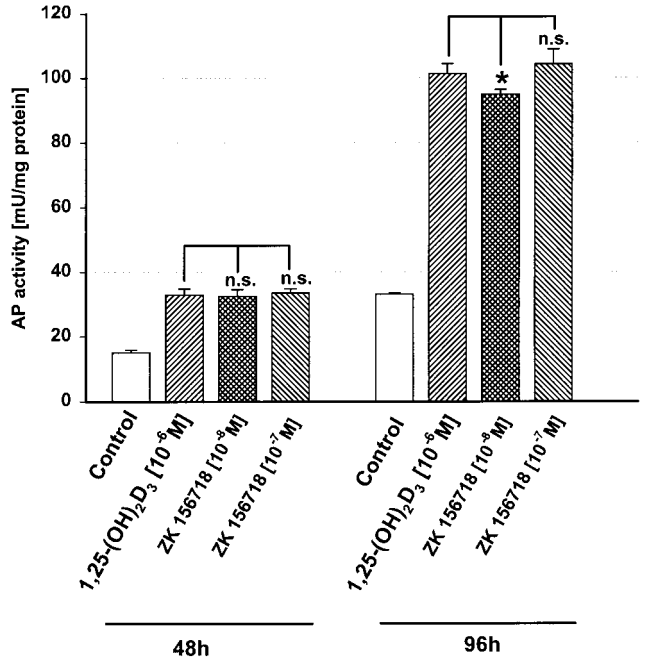

FIG. 4. Effect of 1,25-$(OH)_2D_3$ [10^{-6} M] and ZK 156718 [10^{-7} or 10^{-8} M] on cell differentiation, as assessed by AP activity. Caco-2 cells were treated daily with medium supplemented with 1,25-$(OH)_2D_3$ or with ZK 156718 and harvested after 48 and 96 h incubation. Values are expressed in milliunits of AP activity per milligram cellular protein, and are means ± SD, $n = 3$. *$P < 0.05$; n.s., not significant.

Statistics. All data presented in this paper are mean values from 3 to 8 different experiments ±SD. One-way ANOVA was used to compare means; $P < 0.05$ was considered to be significant.

RESULTS

Cell growth. To compare the antiproliferative effects of 1,25-$(OH)_2D_3$ and ZK 156718 in colon cancer cells, Caco-2 cells were exposed to 10^{-6} M 1,25-$(OH)_2D_3$, 10^{-8} or 10^{-7} M ZK 156718 over time. As soon as after 24 h treatment, both 10^{-8} and 10^{-7} M ZK 156718 appeared to be a more potent inhibitor of cell growth than 10^{-6} M 1,25-$(OH)_2D_3$ itself (Fig. 2): while 1,25-$(OH)_2D_3$ reduced growth of Caco-2 cells to 91.5 ± 1.9% ($P < 0.05$ vs control, mean ± SD, $n = 8$) after 24 h incubation, ZK 156718 decreased growth to 87.1 ± 1.1% ($P < 0.01$ vs control) and 77.2 ± 4.1% ($P < 0.01$ vs control) at a concentration of 10^{-8} and 10^{-7} M, respectively. As we did not observe any significant antiproliferative effect of 1,25-$(OH)_2D_3$ at [10^{-8} M] and [10^{-7} M] (data not shown), we only used 1,25-$(OH)_2D_3$ at the concentration 10^{-6} M for further investigation.

Both 1,25-$(OH)_2D_3$ and ZK 156718 were found to be nontoxic at the used concentrations in Caco-2 cells (Fig. 3).

Cell differentiation. Figure 4 shows the effect of 1,25-$(OH)_2D_3$ and ZK 156718 on the activity of the

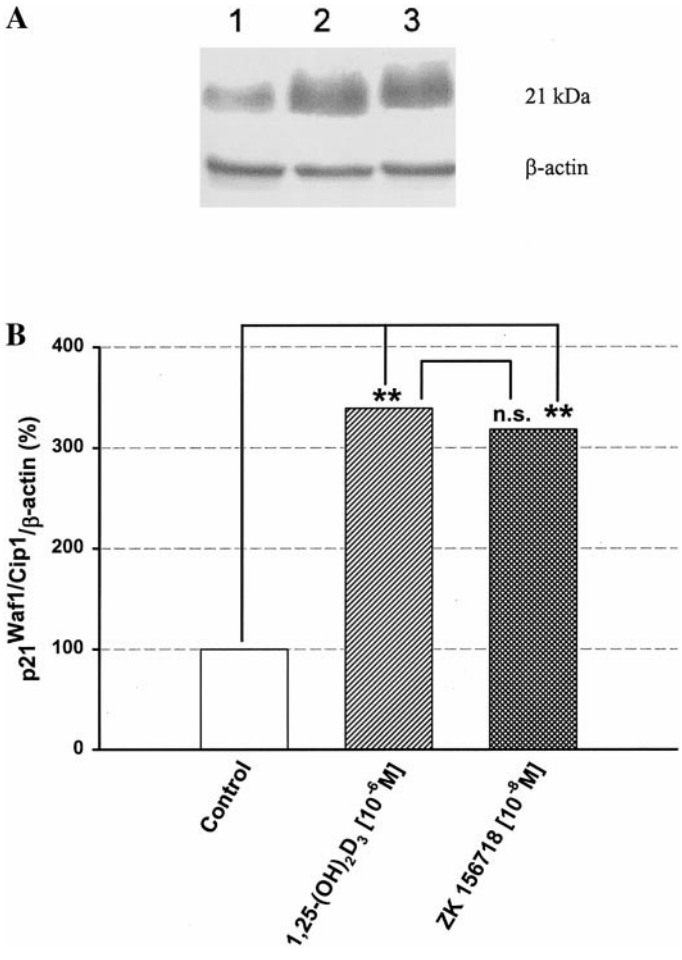

FIG. 5. (A) $p21^{Waf1/Cip1}$ expression in Caco-2 cells treated with 1,25-$(OH)_2D_3$ [10^{-6} M] (lane 2) or ZK 156718 [10^{-8} M] (lane 3) for 24 h. Control cells (lane 1) were treated with ethanol only. The band at 21 kDa corresponds to the $p21^{Waf1/Cip1}$ protein. (B) Ratio of $p21^{Waf1/Cip1}/\beta$-actin protein. $*P < 0.05$; $**P < 0.01$; n.s., not significant.

differentiation marker enzyme alkaline phosphatase (AP) in Caco-2 cells. Both 1,25-$(OH)_2D_3$ [10^{-6} M] and ZK 156718 at 10^{-8} and 10^{-7} M stimulated differentiation to a similar extent. In cells treated with 1,25-$(OH)_2D_3$ or with the analog, AP activity was approximately 2.2- and 3-fold higher than that in control cells after 48 and 96 h incubation, respectively.

$p21^{Waf1/Cip1}$ expression. To examine the effect on the cell cycle inhibitor $p21^{Waf1/Cip1}$, Caco-2 cells were incubated with 1,25-$(OH)_2D_3$ [10^{-6} M] or ZK 156718 [10^{-8} M]. Western blot analysis (Fig. 5) revealed that 1,25-$(OH)_2D_3$ and ZK 156718 at a 100-fold lower concentration caused a similar significant ($P < 0.01$ vs control) 3.3-fold increase in the cyclin-dependent kinase (cdk) inhibitor $p21^{Waf1/Cip1}$ after 24 h incubation.

$p27^{Kip1}$ expression. To investigate the effect on $p27^{Kip1}$, Caco-2 cells were grown under standard conditions or incubated with medium containing 1,25-$(OH)_2D_3$ [10^{-6} M] or ZK 156718 [10^{-8} or 10^{-7} M], and cells were harvested after 24 h (Fig. 6). Western blots demonstrated that 1,25-$(OH)_2D_3$ caused a slight, 1.4-fold increase in $p27^{Kip1}$ after 24 h, whereas ZK 156718 treatment of Caco-2 cells resulted in a more prominent, 1.9-fold (at [10^{-8} M]; $P < 0.05$ vs control) or 2.1-fold (at [10^{-7} M]; $P < 0.01$ vs control) increase.

DISCUSSION

In Caco-2 cells (20) as well as in other cell lines (21, 22), analogs of 1,25-$(OH)_2D_3$ have been shown to induce growth arrest in the G1 stage of the cell cycle. This cell cycle arrest was accompanied by an increase in the expression of two key regulators in the cell cycle, the cdk-inhibitor $p21^{Waf1/Cip1}$ and $p27^{Kip1}$. It has already been shown by Liu *et al.* (23) that $p21^{Waf1/Cip1}$ contains a VDRE and is transcriptionally induced by 1,25-$(OH)_2D_3$. No VDRE has yet been identified for $p27^{Kip1}$, but an upregulation of both $p27^{Kip1}$ mRNA and protein

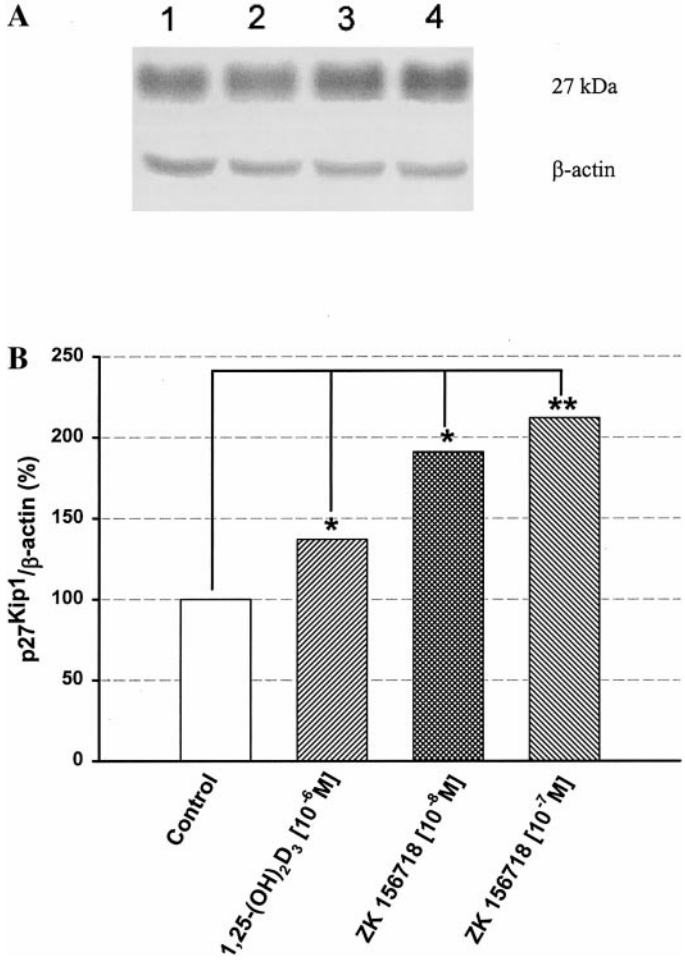

FIG. 6. (A) $p27^{Kip1}$ expression in Caco-2 cells treated with 1,25-$(OH)_2D_3$ [10^{-6} M] (lane 2) or ZK 156718 [10^{-8} M, lane 3, or 10^{-7} M, lane 4] for 24 h. Control cells (lane 1) were treated with the solvent only. The band at 27 kDa corresponds to the $p27^{Kip1}$ protein. (B) Ratio of $p27^{Kip1}/\beta$-actin protein. $*P < 0.05$; $**P < 0.01$.

levels has been observed in a variety of different cancer cell types (1).

In our study, the vitamin D analog ZK 156718 has been found to be more potent than 1,25-$(OH)_2D_3$ itself with respect to inhibition of cell proliferation (Fig. 2), even at a 100-fold lower concentration, although this antiproliferative effect remained rather moderate. As also demonstrated by Scaglione-Sewell *et al.* (20), we observed that Caco-2 cells were rather resistant to antiproliferative interventions. The stronger antiproliferative effect of ZK 156718 may be due, at least in part, to its more pronounced effect on the cell cycle regulator $p27^{Kip1}$ (Fig. 6). However, in inducing cell differentiation (Fig. 4) and $p21^{Waf1/Cip1}$ (Fig. 5), ZK 156718 at the concentrations 10^{-8} and 10^{-7} M is comparable to 1,25-$(OH)_2D_3$ [10^{-6} M]. These results are coincident with data recently published by us (5, 24) showing that upregulation of $p27^{Kip1}$ may rather be related to the induction of apoptosis than to differentiation in colon cancer cells, whereas $p21^{Waf1/Cip1}$ is clearly involved in spontaneous and induced differentiation of Caco-2 cells.

The expression of $p27^{Kip1}$ is regulated mainly at the post-translational level via ubiquitin-proteasome-mediated proteolysis (25). Furthermore, it has been shown that the increase of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ was due to the inhibition of their ubiquitin-proteasome mediated proteolysis by the inhibitor of the enzyme 3-hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) reductase, lovastatin, resulting in an accumulation of $p21^{Waf1/Cip1}$ and $p27^{Kip1}$ and a subsequent G1 arrest (26). It is possible that the same mechanism is responsible for the increase of these cdk-inhibitors in Caco-2 cells by 1,25-$(OH)_2D_3$ or its analogs.

Our results in Caco-2 cells are in agreement with previous data of Werz *et al.* (15) in Mono Mac 6 cells showing that, compared with calcitriol, ZK 156718 was 10-fold more active in the induction of 5-lipoxygenase and in the inhibition of proliferation.

In the study of Werz *et al.* (15) the analog showed a decreased metabolic stability in rat liver homogenates and had a 50-fold lower affinity for the vitamin D binding protein than calcitriol. As the binding affinity of vitamin D analogs to the vitamin D binding protein correlates with their serum levels or the volume of distribution, ZK 156718 is metabolized and cleared more rapidly than calcitriol after systemic application. Werz *et al.* (15) also demonstrated that ZK 156718 binded with a 50% lower affinity to VDR than 1,25-$(OH)_2D_3$, but nevertheless was more active than calcitriol. The discrepancy between receptor binding and biological activity was also reported for other analogs (8, 16) and may be attributed to changes in VDR conformation, modified interaction of the analogs with the VDR or with coactivators, differential ability to form receptor-dimer complexes, increased half-life times of VDR-ligand complexes and to different preferences for specific VDRE types (8, 15). For example, higher efficiency of EB 1089 in stimulating gene activity has been demonstrated to be due to the enhanced stability of the occupied receptor, the stimulated heterodimerization of VDR with the retinoid X receptor (RXR), and/or to the stronger and longer binding of the VDR-RXR complex to VDRE (8). Other investigations even have suggested that EB 1089 preferentially activates a certain type of VDRE, which seems to be more closely related to the regulation of cell growth than to the classical actions of vitamin D, i.e., the effects on calcium metabolism (27, 28).

Thus, in the future, the use of vitamin D analogs, which possess lower risk/benefit ratios, may hopefully extend beyond its classical role in bone diseases, to encompass new areas in the field of the treatment or prevention of neoplastic diseases either as a monotherapy or in combination with other anticancer agents, e.g. butyrate (6).

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