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19-Nor-1α,25-dihydroxyvitamin D_2 (paricalcitol) exerts anticancer activity against HL-60 cells in vitro at clinically achievable concentrations☆

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Abstract

19-Nor-1α,25-dihydroxyvitamin D_2 (paricalcitol) is an analogue of 1,25$(OH)_2D_3$ with reduced calcemic effects that is approved in the United States for the suppression of parathyroid hormone in chronic renal failure. Paricalcitol has anticancer activity in prostate cancer cells. We tested the effects of paricalcitol on the HL-60 leukemia cells, studying cellular differentiation, cell cycle changes, apoptosis and cellular proliferation. Paricalcitol at 10^{-8} M concentration induced the maturation of HL-60 cells in a time-dependent manner, as shown by increased expression of CD11b differentiation surface antigen. The ability of HL-60 cells to reduce nitroblue tetrazolium (NBT) was markedly increased after exposure to paricalcitol at 10^{-8} M for 72 h. Paricalcitol inhibited colony formation of HL-60 cells in a soft agar semisolid media after 10-day incubation (estimated IC_{50} of 5×10^{-9} M. Exposure to 10^{-8} M paricalcitol for 72 h increased the number of cells in G_0/G_1 phase, and decreased the number of cells in S phase, and significantly increased the number of HL-60 cells undergoing apoptosis. The concentration required to achieve inhibition of growth of HL-60 cells is comparable to clinically achievable levels. These findings support the clinical evaluation of paricalcitol as an antileukemia agent.
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Keywords: Paricalcitol; HL-60; Apoptosis; Differentiation; Experimental therapeutics

1. Introduction

19-Nor-1α,25-dihydroxyvitamin D_2 (paricalcitol, Zemplar, Abbott Laboratories, Chicago, IL) is an analogue of 1,25$(OH)_2D_3$ (calcitriol) that is approved in the United States for the treatment of secondary hyperparathyroidism in chronic renal failure (Fig. 1) [1].

Paricalcitol is approximately three times less calcemic than calcitriol in humans [2], and is as potent as calcitriol in suppressing parathormone secretion in patients on hemodialysis [3]. In addition, paricalcitol has been shown to inhibit the proliferation of LNCaP prostate cancer cells, of primary prostate cell cultures [4], NIH-929 myeloma cells, and SW837 colon cancer cells [5].

1,25$(OH)_2D_3$ inhibits the proliferation and induces the differentiation of normal and leukemic myeloid cells into monocytes [6]. The growth-inhibitory effects of 1,25$(OH)_2D_3$ are mediated by regulation of cell cycle progression [7]. The antiproliferative and differentiating effects of calcitriol suggest a therapeutic role for the drug in hematological malignancies but the problem of hypercalcemia has been dose-limiting [8–10].

We examined the in vitro effect of paricalcitol on cell maturation, inhibition of colony formation, cell cycle arrest, and apoptosis in the myeloid leukemia cell line, HL-60.

2. Methods

2.1. Cells and compounds

HL-60 were obtained from the American Type Culture Collection (Rockville, MD) and were maintained in RPMI 1640 with 10% fetal calf serum and 100 U/ml penicillin, 100 μg/ml streptomycin in a 37 °C incubator containing 5% CO_2. Paricalcitol was provided by Abbott Laboratories, Chicago, IL, 1,25$(OH)_2D_3$ (calcitriol) was purchased from Biomol Research Labs.

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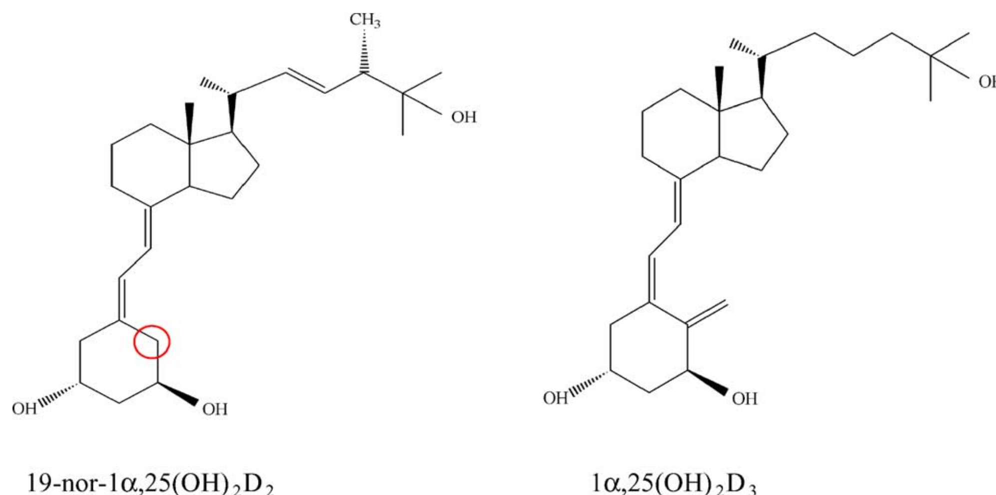


Fig. 1. Chemical structure of 19-nor-1α,25$(OH)_2$ Vitamin D_2 (paricalcitol) and 1α,25$(OH)_2$ Vitamin D_3 (calcitriol).

2.2. Flow cytometry analysis of maturation antigens

For analysis of cellular differentiation, expression of cell surface antigens was determined using immunofluorescence staining. Cells were incubated with paricalcitol at 10^{-8} M final concentration for 1, 2, 4, 24 and 72 h in a 24-well tissue culture plate (Costar, Corning Inc., Corning, NY) in RPMI-1640 supplemented with 10% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin in a humidified atmosphere, 5% CO_2 at 37 °C. Control cells were incubated for 72 h with vehicle added. After drug exposure, cells were harvested, washed twice and resuspended in fresh media for additional incubation for a total of 72 h (including the time of drug exposure). After harvesting, the cells were incubated for 10 min with bovine serum albumin as a blocking agent and then stained using R-phycoerythrin (RPE)-conjugated mouse anti-human CD11b (DAKO, Carpinteria, CA). Control studies were performed with mouse RPE-conjugated IgG_1 isotype antibodies (DAKO, Carpinteria, CA). Cells were analyzed using a FACSTAR Plus flow cytometer (Becton Dickinson).

2.3. Analysis of differentiation

Differentiated HL-60 cells produce superoxide anions ($O^-{}_2$) when stimulated with 12-O-tetra-decanoyl-phorbol-acetate (TPA). After 4-day incubation, 1×10^6 cells were harvested. Half of the cell suspension was used to make cytospin slides for morphological assessment and comparison to the NBT exposed cells. The other half was mixed 1:1 with a solution of Dulbecco's phosphate buffered saline (DPBS) containing reagents to achieve a final concentration of nitroblue tetrazolium (NBT) (Sigma, St. Louis, MO) 0.5 mg/ml, 12-O-tetradecanoylphorbol-13-acetate (TPA) (Sigma) 162 nM, FBS 10% and 0.5×10^6 cells/ml. After 25 min in humidified air at 37 °C in a CO_2 (5%) incubator, the reaction was stopped by placing the tubes on ice. Cytospin slides were made for each condition and the slides were stained with Wright stain (Sigma, St. Louis, MO). A minimum of 100 cells was counted and the percentage of NBT-positive cells was assessed under light microscopy for each experimental point.

2.4. Apoptosis analysis

Annexin V (human) (recombinant) FITC labeled was used to detect apoptotic cells. Briefly, cells were washed in PBS, resuspended in a binding buffer (10 mM Hepes/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM $CaCl_2$), incubated with annexin-FITC for 10 min in the dark, and propidium iodide was added prior to two-color flow cytometry analysis. Statistical differences between groups were analyzed by *t*-test for independent samples.

2.5. Cell cycle analysis

Cell cycle analysis was performed on HL-60 cells treated with paricalcitol for 72 h. 1×10^6 cells were harvested, washed twice with PBS, and stained with propidium iodide 50 and 37 μg/ml RNase, and 0.6% NP40 in a 3.6 mM citrate buffer. Cell cycle analysis was performed from the list mode data of the FACSTAR plus flow cytometer using a modeling program (MODFIT™ Verity Software House). The results were analyzed by multivariate analysis of variance (ANOVA).

2.6. Colony formation in agar

HL-60 cells were seeded in a two-layer soft agar system. The lower layer consisted of 0.6% agar (Seaplaque agarose, FMC Bioproducts, Rockland, ME) in which the test substances were mixed; the upper layer was 0.3% agar in which 10^3 HL-60 cells were suspended. The underlayer was

plated in tissue culture grade 35 mm Petri dish in a volume of 1 ml RPMI-1640 (GIBCO, BRL Grand Island, NY) containing 10% FBS (Sigma, St. Louis, MO), 100 U/ml penicillin, 100 μg/ml streptomycin, 0.6% agar, 0.1% ETOH and test substance in a final concentration ranging from 10^{-7} to 10^{-9} M. All experiments using triplicates were incubated in a humidified atmosphere, 5% CO_2 at 37 °C for 10 days. Colonies (>40 cells) were scored with an inverted microscope.

3. Results

3.1. Effect of paricalcitol on maturation of HL-60

CD11b, one of the β2-integrins, is the α subunit of a heterodimeric surface glycoprotein with a role in inflammatory and phagocytic responses. CD11b is expressed mainly on mature monocytes, macrophages, most polymorphonuclear leukocytes, and on a minor subset of B-lymphocytes. Exposure of the HL-60 cells to paricalcitol at 10^{-8} M for 1, 2, 4, 24, and 72 h produced a time-dependent increase in the expression of this maturation antigen. This maturation effect requires prolonged drug exposure, and the cells reached the maximum observed maturation after 24 h exposure (Fig. 2).

Differentiated HL-60 cells, similarly to normal monocytes are able to produce superoxide and reduce nitroblue tetrazolium. After 72 h treatment of HL-60 cells with paricalcitol at 10^{-9}, 10^{-8} and 10^{-7} M, the percentage of cells able to reduce NBT rose from 5.5 to 31, 83 and 93%, showing a concentration-dependent maturation effect.

3.2. Effect of paricalcitol on induction of apoptosis on HL-60

Annexin V has high affinity for the membrane phospholipid phosphatidylserine (PS), which is translocated from the inner to the outer leaflet of the plasma membrane in apoptotic cells. Propidium iodide (PI) is used as a viability probe. Cells staining positive for annexin V-FITC and

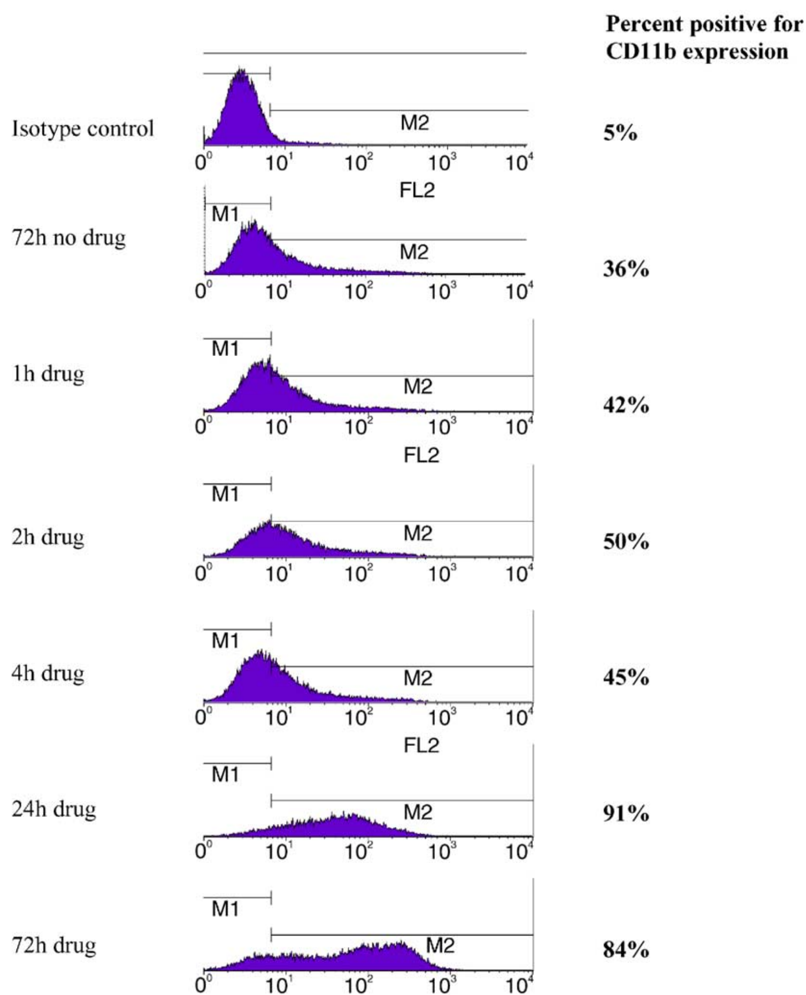


Fig. 2. Expression of CD11b antigen on HL-60 cells after exposure to 19-nor-1α,25$(OH)_2$ Vitamin D_2 (paricalcitol) for a defined time. Cells were incubated with 10^{-8} M paricalcitol in a liquid culture the resuspended in fresh drug-free media for a total of 72 h. Flow cytometry analysis for the expression of CD11b surface antigen was performed using R-phycoerythrin (RPE)-conjugated mouse anti-human CD11b monoclonal antibody.

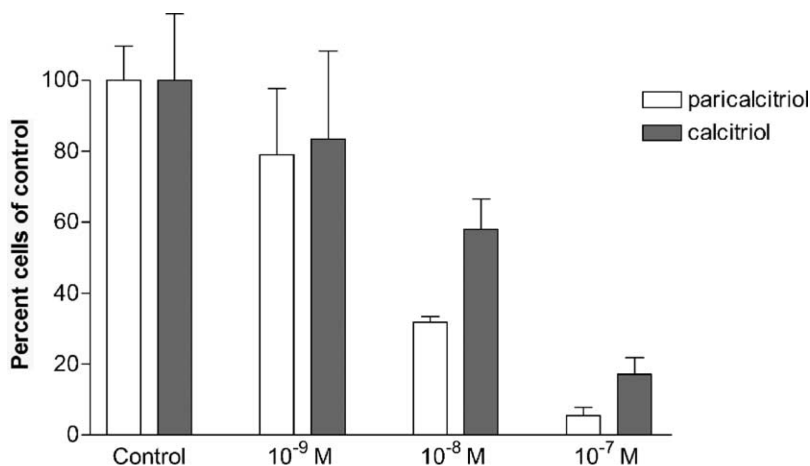
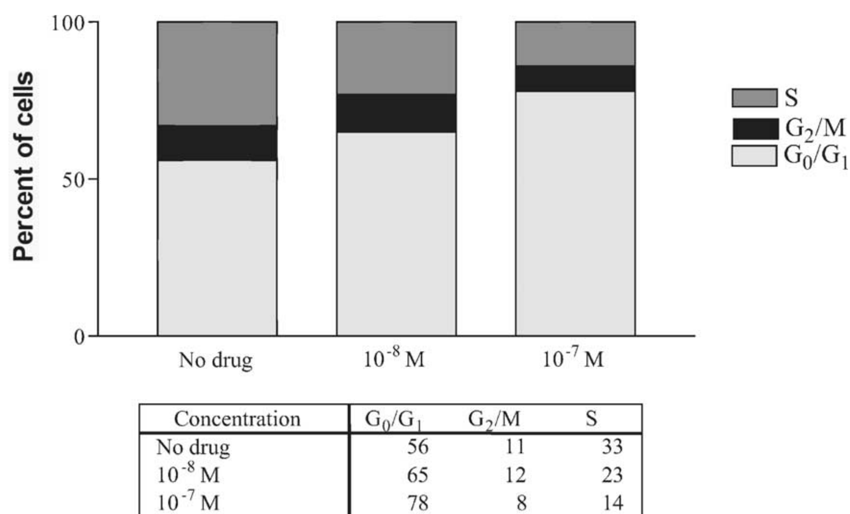


Fig. 3. Colony inhibition of HL-60 cells paricalcitol and calcitriol. Leukemic cells were cultured in triplicate in a soft agar semisolid media and colonies ($\geq$40 cells) were counted after 10 days. The results are expressed as percentage of colonies of control.



Concentration	G_0/G_1	G_2/M	S
No drug	56	11	33
10^{-8} M	65	12	23
10^{-7} M	78	8	14

Fig. 4. Cell cycle distribution in HL-60 cells after treatment with 19-nor-1α,25(OH)$_2$ Vitamin D_2 (paricalcitol).

negative for PI are undergoing apoptosis. Paricalcitol significantly increased the percentage of cells undergoing apoptosis in HL-60 cells ($P < 0.05$). After 60 h of incubation in a liquid media at 10^{-8} M concentration the number of apoptotic cells was 21% compared to 11.5% of the controls.

3.3. Inhibition of colony formation

Paricalcitol inhibited colony formation of HL-60 cells in semisolid media after a 10-day incubation. This inhibition was concentration-dependent. For paricalcitol, the estimated 50% inhibitory concentration (IC_{50}) was 5×10^{-9} M for HL-60 cells (Fig. 3).

3.4. Analysis of cell cycle

Calcitriol causes a decrease in HL-60 in S phase and an increase in cells in the G_0/G_1 phase [11]. Similarly, paricalcitol induced a decrease in the number of cells in S-phase and a simultaneous increase of cells in the G_0/G_1 phase ($P = 0.001$) after 72 h exposure (Fig. 4).

4. Discussion

Although calcitriol can inhibit the proliferation of leukemia cells in vitro, supraphysiological levels are needed (10^{-8} to 10^{-7} M) [12]. These levels are difficult to achieve in vivo without hypercalcemia. Intermittent administration of calcitriol leads to better tolerance and higher serum peak levels. Smith et al. gave subcutaneous calcitriol every other day to cancer patients in a Phase I trial. The peak concentration of calcitriol was found to be $0.77 \pm 0.09 \times 10^{-9}$ M [13]. Beer et al. have shown that weekly administration of oral calcitriol permitted substantial dose escalation, but the serum peak levels of calcitriol still remained lower than

the levels needed in vitro to inhibit leukemia cell growth (3.9×10^{-9} M [14]).

In patients with chronic renal failure the peak concentration of paricalcitol was $4.4 \pm 1.6 \times 10^{-9}$ M after an intravenous dose of 0.24 μg/kg [15]. This dose does not result in hypercalcemia in most patients and it is the currently recommended maximum dose in the treatment of secondary hyperparathyroidism. No pharmacokinetic data available with higher doses of paricalcitol but it is likely that higher plasma levels can be reached with further dose escalation.

In this study, we have shown that paricalcitol exerts antiproliferative, apoptotic and differentiating effect of HL-60 leukemia cells in vitro. The concentrations required to achieve these biological effects (5×10^{-9} to 10^{-8} M) in vitro can be achieved with intravenous administration of paricalcitol with minimal toxicity. Whether these biological effects observed in vitro can be reproduced in vivo in human subjects with myeloid malignancies warrants clinical investigation.

Acknowledgements

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