Antileukemic effect of a synthetic vitamin D₃ analog, HY-11, with low potential to cause hypercalcemia

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Article contents

Abstract. 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is capable of inhibiting the proliferation of acute myelogenous leukemia (AML). However, toxicity of hypercalcemia has limited the use of 1,25(OH)₂D₃ in clinical trials. We have evaluated 11 synthesized vitamin D₃ analogs for their ability to inhibit clonal growth of HL-60 myeloid leukemic cells. Among the 11 vitamin D₃ analogs, HY-11 (code name) showed the most potent antileukemic activity with 2.5x10⁻⁶ M of IC₅₀, however, it did not affect the cellular growth of normal peripheral blood mononuclear cells until 10⁻⁶ M. Flow cytometric analysis indicated that HY-11 induced the G1 arrest in a dose-dependent manner, which was mediated via inactivation of CDK4 and CDK6 in association with up-regulation of CDKI (cyclin-dependent kinase inhibitor), p27 and Rb protein. Induction of apoptosis was mediated via caspase-3 pathway in HY-11-treated HL-60. In addition, HY-11 enhanced the expression of TGF-β₁, TGF-β receptor type I and II and vitamin D₃ receptor (VDR). VDR expression was increased by TGF-β₁, suggesting that TGF-β₁ might be involved in the antiproliferative effect of HY-11 on HL-60 cells by autocrine and paracrine regulation. Serum calcium levels were within normal limit when HY-11 was given intraperitoneally (i.p.) every other day for 5 weeks to BALB/c mice at the doses of 10⁻⁷, 10⁻⁶ and 10⁻⁵ M. HY-11 inhibited the growth of WEHI-3BD⁺ mouse leukemic cells in vitro, and syngeneic BALB/c mice that received WEHI-3BD⁺ mouse leukemic cells and HY-11 had a significantly longer survival without producing hypercalcemia compared to control group. In summary, HY-11 is a vitamin D₃ analog that inhibited the proliferation of human AML cell line, HL-60, through induction of cell cycle arrest, triggering apoptosis as well as modulation of TGF-β₁ and its receptors. In particular, HY-11 significantly increased the survival of mice that had myeloid leukemia without producing hypercalcemia.

Introduction

The seco-steroid hormone 1α,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] plays an important role in the maintenance of calcium homeostasis mediated through binding to specific intracellular receptor (1). The classic target organs of this hormone are the intestine, kidney and bone, but a number of other tissues not involved in mineral and bone metabolism also possess specific vitamin D₃ receptors (VDRs), including normal and neoplastic hematopoietic cells (2,3).

1,25(OH)₂D₃ induces differentiation of cells from selected acute myelogenous leukemia (AML) cell lines into macrophage-like cells in vitro (1,6,7). In addition, clonogenic blast cells from AML patients are inhibited in their proliferation by 1,25(OH)₂D₃ and many are induced to differentiate into macrophage-like cells (1,7). In contrast, normal human colony forming units granulocyte-macrophage (CFU-GM) are slightly stimulated in their clonal proliferation by 1,25(OH)₂D₃ (8,9).

AML arises from neoplastic transformation of a myeloid stem cell; these leukemic cells are unable to undergo cellular...
maturation at an early stage of development. High-dose chemotherapy shows improved survival of AML patients, but severe myelosuppression limits its use. An ideal alternative therapeutic strategy for these patients is to induce differentiation and/or inhibit clonal proliferation of their leukemic blasts without toxic effects on their normal hematopoietic stem cells. Studies in vivo suggest that 1,25(OH)2D3 is able to prolong the survival of mice injected with syngeneic leukemic cells (10). A trial of oral administration of 1,25(OH)2D3 to preleukemic patients was partially effective, perhaps because concentrations required to see activity in vitro could not be achieved in vivo unless hypercalcemia developed (11). Therefore, research activities have been directed at finding new 1,25(OH)2D3 analogs with a more favorable therapeutic profile.

In the present study, we report a new vitamin D3 analog (code name: HY-11) that can inhibit the clonal growth of human AML cells, HL-60, by cell cycle arrest, induction of apoptosis and modulation of TGF-β1. Interestingly, treatment of leukemic mice with HY-11 considerably prolonged their survival without hypercalcemia.

Materials and methods

Cells and cell culture. Human acute myeloid leukemia cell line HL-60 was purchased from the ATCC (Rockville, MD, USA) and maintained according to the recommendation of ATCC. The HL-60 cell was cultured in tissue flasks in RPMI-1640 medium (Gibco-BRL, Gaithersburg, MD, USA) supplemented with 10% (vol/vol) fetal bovine serum (FBS; Hyclone Labs, Inc., Logan, UT, USA), 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma Chemical, St. Louis, MO, USA). Cells were maintained in a humidified atmosphere, 5% CO2 at 37°C, culture medium was changed every 3-4 days.

Mouse myeloid leukemia cell line WEHI-3BD+ was a gift from Dr H.P. Koeffler (Cedars-Sinai Medical Center/University of California, Los Angeles, CA, USA), cultured with same condition as HL-60 cells except using modification of Isocove's modified Dulbecco's medium (IMDM; Gibco-BRL, CA, USA) as a culture medium.

Bone marrow from AML patients and peripheral blood from healthy volunteers after written consent were obtained. Mononuclear cells (MNC) from bone marrow and peripheral blood were collected by separation on Ficoll-Paque (Amersham Biosciences AB, Sweden) gradients at a density of 1.077, washed twice in phosphate-buffered saline (PBS), and suspended in IMDM (Gibco-BRL) containing 20% heat-inactivated FBS (Gibco-BRL).

Vitamin D3 compounds. Eleven analogs of 1,25(OH)2D3 used in this study were synthesized by one of the authors in this study (C.K. Cho, Hang Yang University, Seoul, Korea) and are shown in Fig. 1; their code names are indicated. All analogs were dissolved in absolute ethanol at 1x10⁻³ M as a stock solution; this was stored at -20°C and protected from light. Immediately before use, stock solutions were diluted in RPMI-1640 without FBS. The final alcohol concentration in the cultures did not exceed 0.1% and this concentration did not influence either clonal growth or growth of GM-CFC (Granulocyte-Macrophage colony forming cell) from normal individuals (data not shown).

Clonogenic assay. Cells were plated in tissue culture grade 35-mm petri dish in a volume of 1 ml RPMI-1640 containing 10% FBS, 10% bovine serum albumin (BSA;
Sigma Chemical), 1.2% methylcellulose (Eastman Kodak, Rochester, NY, USA), 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were plated at 2x10^4 (HL-60 and WEHI-3BD) or 1x10^5 (normal MNC and AML blasts) per dish, incubated in a humidified atmosphere, 5% CO2 at 37°C for 6-10 days. For CFU-GM of normal MNC, 200 pM of GM-CSF and 5x10^{-5} M of 82-mercaptoethanol (Sigma Chemical) were added. Colonies (>40 cells) were scored with an inverted microscope. The IC_{50} was defined as the drug concentration required inhibiting cell growth by 50%.

Cell cycle analysis. Cell cycle distribution was determined by staining DNA with PI (propidium iodide) (Sigma Chemical). Briefly, 1x10^6 cells were incubated with or without HY-11 for indicated times. Cells then were washed in PBS and fixed in methanol. Cells were again washed with PBS, then incubated with PI (10 μg) containing 50 μg/ml RNase A treatment at 37°C for 30 min. The percentage of cells in the different phases of the cell cycle was measured with a FACStar flow cytometer (Becton-Dickinson, San Jose, CA, USA), analyzed using Becton-Dickinson software (Lysis II, CellFit).

Evaluation of apoptosis. Apoptosis was determined by staining cells with Annexin V-FITC and PI labeling, because Annexin V can identify the externalization of phosphatidylserine during the apoptotic progression and, therefore, can detect early apoptotic cells (12). To quantify apoptosis of cells, prepared cells were washed twice with cold DPBS and were then resuspended in binding buffer (10 mM HEPES/NaOH, pH 7.4, 140 mM NaCl, 2.5 mM CaCl2) at a concentration of 1x10^6 cells/ml. One hundred microliters of this solution (1x10^5 cells) were transferred to a 5-ml culture tube with 5 μl of Annexin V-FITC (PharMingen, San Diego, CA, USA) and 10 μl of 20 μg/ml PI (Sigma). Cells were gently vortexed and incubated for 15 min at room temperature in the dark. After the addition of 400 μl of binding buffer, these cells were analyzed with a FACStar flow cytometer (Becton-Dickinson).

Western blot analysis. Cells were washed in DPBS, suspended in lysis buffer containing 50 mM Tris (pH 7.5), 1% NP-40, 2 mM EDTA, 10 mM NaCl, 20 μg/ml aprotinin, 20 μg/ml leupeptin and 1 mM phenylmethylsulphonyl fluoride and were placed on ice for 20 min. After centrifugation for 1 h at 4°C at 15,000 x g, the supernatant was collected. Whole lystate (100 μg) was resolved on 12% SDS-PAGE gel, transferred onto a nitrocellulose membrane (Bio-Rad, Hercules, CA, USA) by electroblotting and was probed with monoclonal antibodies and a mouse anti-human α-tubulin monoclonal antibody. The blot was developed by using the enhanced chemiluminescence (ECL) kit (Amersham).

Immunoprecipitation and kinase assay. Cells were collected after HY-11 treatment and washed with PBS. They were then suspended in an extraction buffer [50 mM Tris (pH 7.5), 0.1% NP-40, 250 mM NaCl, 5 mM EDTA, 50 mM NaF, 0.1 mM NaVO_4, 20 μg/ml aprotinin, 20 μg/ml leupeptin, 1 mM phenylmethylsulphonyl fluoride] and incubated on ice for 15 min. After centrifugation at 13,000 rpm for 20 min, the supernatant was collected and protein concentrations were determined using a Bio-Rad assay kit. Two micrograms of antibody were added to 200 μg of each cell extract in 500 μl of extraction buffer and incubated for 4 h at 4°C with continuous agitation. To collect immune complexes, 30 μl of protein A/G agarose was added to the mixture, which was then incubated for 2 h. Immune complexes were centrifuged at 1,200 rpm for 2 min, and precipitates were washed three times with extraction buffer and twice with kinase reaction buffer [50 mM Tris-Cl (pH 7.5), 10 mM MgCl_2 and 1 mM DTT]. CDK4 and CDK6 kinase assays on Rb-c were performed by mixing the respective immune complexes with 2 μg of Rb-c and 5 μCi [γ-32P]-ATP in 35 μl of kinase reaction buffer. Kinase reaction was performed at 37°C for 30 min and was terminated with 2X SDS-PAGE loading buffer. The reaction mixtures were resolved by SDS-PAGE. The extent of phosphorylation was determined by autoradiography.

Measurement of serum calcium levels in mice. Six- to 8-week-old BALB/c male mice were maintained in accordance with institutional guidelines under pathogen-free conditions and fed with calcium-free food for 2 days before and during each experiment. The mice were divided into 3 groups of 10 mice each and maintained for 35 days. While control mice were given vehicle alone (DPBS), experimental mice were given HY-11 i.p. at the doses of 10^{-7}, 10^{-6} and 10^{-5} M as well as calcitriol [1,25(OH)_2D_3] as a reference every other day for 5 weeks: serum was collected twice a week and Ca^{2+} was measured by atomic absorption spectro-photometry (Perkin-Elmer 560).

Establishment of mouse leukemic model. Six- to 8-week-old BALB/c male mice were maintained in accordance with institutional guidelines under pathogen-free conditions and fed a standard laboratory diet. WEHI-3BD+ cells were washed twice with isotonic phosphate-buffered saline (PBS), counted with trypan blue to confirm viability >95%, and adjusted to appropriate cell concentration at 1.5x10^7, 1x10^6 and 5x10^5, respectively. Each group had 5 mice. BALB/c mice were injected i.p. with each dose of WEHI-3BD+ cells and observed for survival. Leukemia was confirmed by autopsy; bone marrow, spleen, liver and lung were stained by hematoxylin/eosin (H/E).

Antileukemic activity in BALB/c mice. Six- to 8-week-old BALB/c male mice were maintained in accordance with institutional guidelines under pathogen-free conditions and fed a standard laboratory diet. Animals were inoculated i.p. with 1x10^5 WEHI-3BD+ AML cells, which derived from the BALB/c mouse. The mice were divided into 3 groups (control group, HY-11-treated group and EB1089-treated group as a reference), each group had 10 mice, and maintained for 26 days. EB1089 is known to be one of the most potent synthetic vitamin D_3 analogs. Control mice were given vehicle alone (PBS) and experimental mice were given vitamin D_3 analogs i.p. 24 h after inoculation of leukemic cells, thereafter, every other day. HY-11 inhibited the proliferation of WEHI-3BD+ cells in vitro in a dose-dependent manner (data not shown).
Statistical analysis. Median survival analysis was evaluated by Kaplan-Meier analysis and was considered significant when the p-value was \( \leq 0.05 \).

Results

Effect of 1,25(OH)\(_2\)D\(_3\) analogs on clonal growth of HL-60 cells. We examined the effect of 1,25(OH)\(_2\)D\(_3\) (code name: C) and its analogs on clonal proliferation of the myeloid leukemic cell line HL-60. Among 11 vitamin D\(_3\) analogs examined, HY-11 revealed the most potent antiproliferative activity in HL-60 cells (Fig. 2). HY-11 demonstrated a dose-dependent inhibition of cell growth of HL-60. The HY-11 at \( 1 \times 10^{-6} \) M caused >50% inhibition of clonal growth of HL-60 and the calculated concentration that inhibited 50% growth (IC\(_{50}\)) was \( 2.5 \times 10^{-6} \) M. The IC\(_{50}\) of reference compound C was almost the same as that of HY-11.

Effect of HY-11 on clonal proliferation of normal and human leukemic myeloid clonogenic cells. The normal committed myeloid stem cells, CFU-GM and committed erythroid stem cells, BFU-E, were not significantly inhibited until \( 1 \times 10^{-6} \) M of HY-11, although total inhibition was observed at \( 1 \times 10^{-5} \) M (Table I). We examined the effect of HY-11 on the clonal

Table I. Effect of HY-11 in normal hematopoietic cells and blasts from AML patients.

<table>
<thead>
<tr>
<th>Normal hematopoietic cells</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HY-11 Con (M)</td>
<td>BFU-E</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>( 10^{-5} ) M</td>
<td>105</td>
</tr>
<tr>
<td>( 10^{-7} ) M</td>
<td>86</td>
</tr>
<tr>
<td>( 10^{-6} ) M</td>
<td>93</td>
</tr>
<tr>
<td>( 10^{-5} ) M</td>
<td>0</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Blasts from AML patients</th>
<th>% of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>HY-11 Con (M)</td>
<td>BFU-E</td>
</tr>
<tr>
<td>Control</td>
<td>100</td>
</tr>
<tr>
<td>( 1 \times 10^{-6} ) M</td>
<td>97</td>
</tr>
<tr>
<td>( 5 \times 10^{-6} ) M</td>
<td>42</td>
</tr>
<tr>
<td>( 1 \times 10^{-5} ) M</td>
<td>0</td>
</tr>
</tbody>
</table>

*ND, not detected.

Table II. Effect of HY-11 on cell cycle in HL-60 cells.

<table>
<thead>
<tr>
<th>0 h</th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
<th>30 h</th>
<th>36 h</th>
<th>48 h</th>
<th>72 h</th>
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</thead>
<tbody>
<tr>
<td>G0-G1</td>
<td>46.39</td>
<td>48.77</td>
<td>52.23</td>
<td>50.81</td>
<td>47.8</td>
<td>51.84</td>
<td>58.85</td>
<td>95.58</td>
</tr>
<tr>
<td>G2-M</td>
<td>11.74</td>
<td>18.67</td>
<td>20.64</td>
<td>22.99</td>
<td>20.51</td>
<td>25.36</td>
<td>41.15</td>
<td>3.88</td>
</tr>
<tr>
<td>S</td>
<td>41.87</td>
<td>32.56</td>
<td>27.13</td>
<td>26.2</td>
<td>32.42</td>
<td>22.81</td>
<td>0</td>
<td>0.54</td>
</tr>
</tbody>
</table>

Cell cycle in HL-60 cells without HY-11.

<table>
<thead>
<tr>
<th>0 h</th>
<th>6 h</th>
<th>12 h</th>
<th>18 h</th>
<th>24 h</th>
<th>30 h</th>
<th>36 h</th>
<th>48 h</th>
<th>72 h</th>
</tr>
</thead>
<tbody>
<tr>
<td>G0-G1</td>
<td>46.39</td>
<td>48.77</td>
<td>48.59</td>
<td>46.05</td>
<td>48.65</td>
<td>48.91</td>
<td>45.21</td>
<td>47.77</td>
</tr>
<tr>
<td>G2-M</td>
<td>11.74</td>
<td>15.67</td>
<td>15.92</td>
<td>15.69</td>
<td>13.05</td>
<td>14.53</td>
<td>15.15</td>
<td>13.42</td>
</tr>
<tr>
<td>S</td>
<td>41.87</td>
<td>35.56</td>
<td>36.49</td>
<td>38.26</td>
<td>38.30</td>
<td>36.56</td>
<td>39.64</td>
<td>38.81</td>
</tr>
</tbody>
</table>
growth of leukemic blasts from patients with AML. HY-11 compound inhibited the proliferation of leukemic blast colonies (CFU-L) in a dose-dependent manner with an IC50 of 4x10^-6 M (Table I). At 10^-5 M of HY-11, leukemic colony was absolutely suppressed.

**Cell cycle analysis.** The effect of HY-11 on the cell cycle was determined in HL-60 by FACS analysis. HL-60 cells treated with 2.5x10^-6 M (IC50) of HY-11 exhibited increased numbers of cells in G1 phase in a time-dependent manner, indicating that HY-11 inhibited the cellular proliferation of HL-60 cells via G1 arrest (Table II). At 72-h exposure of HY-11, cell fraction of G1 phase could not be detected probably due to apoptotic process of HL-60 cells.

Because HY-11 induced a G1 arrest in HL-60 cells, we determined the protein levels of CDKIs (cyclin-dependent kinase inhibitors), p21 and p27, in HL-60 cells exposed to 2.5x10^-6 M of HY-11. The level of p27 protein was increased in a time-dependent manner, however, little detectable change in p21 protein was noted (Fig. 3). In vertebrate cells, the G1 progression and G1/S transition are also regulated by D-type cyclins that bind to and activate CDK4 and CDK6 and cyclin E and cyclin A, which activates CDK2. The treatment of HL-60 cells with 2.5x10^-6 M of HY-11 resulted in down-regulation of CDK4 protein and cyclin A, whereas the levels of CDK6, cyclin D and cyclin E were up-regulated and those of CDK2 and cyclin B1 were not altered (Fig. 3).

Since HY-11 induced a marked accumulation of CDKI, p27 protein, we next questioned whether the p27 protein induced by HY-11 (2.5x10^-6 M) could be detected in complex with CDKs in the cell cycle. As shown in Fig. 4A, the complexes immunoprecipitated with anti-CDK4 and anti-CDK6 antibodies exhibited higher amounts of immunodetectable p27 protein from HY-11-treated cells in a time-dependent manner. In addition, the p27-cyclin D1 complex form was also increased. However, the complex immunoprecipitated with anti-CDK2 antibody was not induced by HY-11 exposure (Fig. 4A).

**Effect of HY-11 on CDK-associated kinase activity.** To determine whether the increased CDKI and the changed cell cycle-regulatory proteins result in the inhibition of CDK activity in HY-11-treated cells, we performed in vitro CDK activity assay on Rb-c substrate and histone H1 in immunoprecipitates with anti-CDK2, -CDK4 and -CDK6 antibodies. Reduction of CDK4- and CDK6-associated kinase activity on Rb-c substrate was observed in HL-60 cells treated with HY-11 (2.5x10^-6 M) at 24 h (Fig. 4B). In addition, the decrease in CDK-4 and CDK6-associated kinase activity was associated with the up-regulation of Rb protein (Fig. 4B). Taken together, these results suggest that p27 protein could play a critical role in G1 arrest via its increased binding to CDK4, CDK6 and cyclin D1 proteins, and subsequently the reducing activities of CDK4- and CDK6-associated kinase in HY-11-treated HL-60 cells.

**Induction of apoptosis by HY-11 in HL-60 cells.** In order to determine whether HY-11 treatment could induce apoptosis in HL-60 cells, in vitro apoptosis detection assay was performed using FACS analysis. As shown in Fig. 5A, treatment of HL-60 cells with HY-11 (2.5x10^-6 M) exhibited a slight increase of the sub-G1 population at 12 h, and then marked increase in a time-dependent manner, demonstrating that HY-11 induced apoptotic cell death in HL-60 cells. To confirm and evaluate the induction of apoptosis, cells were stained with Annexin V and propidium iodide (PI), analyzed by FACS (Fig. 5B). As with the finding of the sub-G1 group by FACS, the proportion of Annexin V-staining cells (the lower right quadrant) was dramatically increased in a time-dependent manner following treatment of HY-11 (2.5x10^-6 M).

Concerning the relationship between Bcl-2 and Bax regulation during apoptosis, Bax protein was up-regulated with a peak at 12 h under HY-11 (2.5x10^-6 M); in contrast, Bcl-2 protein was down-regulated in a time-dependent manner (Fig. 6). The level of Bax protein was not altered by HY-11. In addition, expression of cytochrome C, one of the mitochondrial intermembrane space proteins, was increased in a time-dependent manner, implying that apoptotic pathway is mediated through mitochondria (Fig. 6). Next, we
determined whether caspases might be activated during the induction of apoptosis by HY-11, because cell death can be

Figure 5. (A) Antiproliferative effect of HY-11 involve cell cycle arrest at G1 and apoptosis in human leukemia cells. HL-60 cells were treated with HY-11 (2.5x10^{-6} M) for the indicated time periods and then collected for analysis. Cells were fixed with methanol, stained with propidium iodide and subjected to FACS analysis. (B) The externalization of phosphatidylserine during HY-11 induced apoptosis. Apoptosis was determined by staining cells with Annexin V-FITC and PI labeling. PI was added before analyzing HL-60 in order to distinguish between apoptotic (right lower quadrant on each plot) and necrotic cells (right upper quadrant on each plot). Annexin V staining combined with PI staining was performed in control cells or cells treated with HY-11 for the indicated time periods and then analyzed by flow cytometry. The percentage of cells in each panel is indicated.

Figure 6. Effect of HY-11 on apoptotic-related proteins. HL-60 cells were treated with 2.5x10^{-6} M of HY-11 for the indicated times. Equal amounts of cell extracts (100 μg) were analyzed by 12% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with the indicated antibodies. For the determination of release of cytochrome C from mitochondria to cytosol, mitochondria was separated from cytosol after incubating cells with HY-11 for the indicated times and the level of cytochrome C was determined by Western blot. α-tubulin was used as an internal control.

Figure 7. Expression of TGF-β1, TGF-β receptors and VDR by HY-11 and TGF-β1 in HL-60 cells. Total protein from HL-60 cells was treated with 2.5x10^{-6} M of HY-11 and TGF-β1 (2.5 ng/ml) for the indicated times. Aliquots of 30 μg of protein extracts were analyzed by 10% SDS-PAGE, transferred to a nitrocellulose membrane and immunoblotted with specific antibodies: TGF-β1, TGF-β receptor type I, TGF-β receptor type II and VDR. α-tubulin was used as an internal control.
completed through caspase activation after external stimuli. Expressions of initiator caspase-8 and -9 and effector caspase-3 were down-regulated in a time-dependent manner, and especially, the cleaved products of caspase-8 (p44/45) were detected in accordance with decrease of procaspase-8. (Fig. 6). The intact 116-kDa moiety of poly-(ADP-ribose) polymerase (PARP), substrate for active caspase-3 and hallmark of apoptosis, was degraded, as evidenced by increased 85 kDa cleavage products in a time-dependent manner (Fig. 6). These results indicate conclusively that HY-11-induced apoptosis of HL-60 cells was accompanied by caspase-3 activation via mitochondrial pathway.

**Induction of expression of TGF-β receptors, TGF-β1 and VDR by HY-11.** Since we previously reported that vitamin D₃ analog (EB1089) induced the expression of TGF-β1 and had synergistic effect on proliferation of HL-60 cells when...
combined with TGF-β1 (13), the expressions of TGF-β1 and TGF-β receptors were examined (Fig. 7). Treatment of HL-60 cells with 2.5x10^6 M of HY-11 enhanced expressions of TGF-β receptor type I and TGF-β receptor type II in a time-dependent manner. Neither structural changes nor amplification of TGF-β receptor type I and II genes were seen in HL-60 cells by Southern blot analysis (data not shown). Next, we wanted to determine whether HY-11 could induce the expression of TGF-β1. It was noted that HY-11 enhanced the expression of TGF-β1 protein level in a time-dependent manner. As expected, the expression of VDR (vitamin D receptor) was induced by treatment of HY-11 as well as TGF-β1 in a time-dependent manner (Fig. 7).

Effect of HY-11 on serum calcium level in mouse. BALB/c mice were injected intraperitoneally every other day with 1.25(OH)2D3 or HY-11, and their serum calcium levels were measured twice a week for 5 weeks (Fig. 8). The serum calcium levels remained within normal limit at all doses examined (10^-5 to 10^-7 M), similar to control group. Notably, after the second week, the mice receiving 1.25(OH)2D3 (10^-7 M) showed extreme hypercalcemia (up to 14 mg/dl), were very ill (hyperpyrexia, ruffled fur and lethargy) and eventually died at 25 days.

Establishment of mouse leukemic model. We developed the leukemia model of mouse by intraperitoneal injection of WEHI-3BD+ leukemic cells. Mice (Balb/c) that received 1.5x10^4 leukemic cells showed the shortest survival, 1x10^4 cells, intermediate survival and 5x10^3 cells, longer survival, respectively (data not shown). Twenty-six days after inoculation of WEHI-3BD+ cells, all mice died, and leukemic cell infiltration was confirmed in bone marrow, spleen, liver and lung by autopsy (Fig. 9A).

Effect of HY-11 on the survival rate of mice inoculated with WEHI-3BD+ leukemic cells. For in vivo antileukemic effect of HY-11, leukemic BALB/c mice were treated with HY-11 (10^-7 M) i.p. every other day for 26 days. Mice treated with HY-11 showed survival of 50%, those treated with EB1089, 30% and control mice, 10% at day 26 (Fig. 9B). Collectively, survival of leukemic BALB/c mice treated with HY-11 was longer than the control group (p<0.05).

Discussion

In this study, we examined the biologic profiles of 11 analogs of 1,25(OH)2D3 synthesized by one of the authors, and identified that 2-aza-cyclohexene vitamin D3 analog, HY-11, had the most potent antileukemic effect on AML cells in vitro and in vivo. HY-11 exhibited a dose-dependent inhibition of cellular proliferation in HL-60 cells with an IC50 of 2.5x10^-6 M which was almost the same as that of 1,25(OH)2D3. In addition, HY-11 inhibited the growth of leukemic blasts isolated from patients with AML with an IC50 of 4x10^-9 M. In contrast to the effect on leukemic cells, HY-11 did not significantly suppress the clonal growth of normal human myeloid committed stem cells (CFU-GM) at the concentration of IC50.

Our cell cycle analysis revealed that HY-11 was able to prominently induce G1 growth arrest in HL-60 cells after 48-h exposure, and this was accompanied by a dramatic decrease in the proportion of S and G2-M phases. This result was consistent with those of other investigators who showed that the antiproliferative action of vitamin D3 analogs was linked to G1 phase arrest in normal and malignant cells (14,15). A family of cyclin-dependent kinase inhibitors (CDKIs) plays a major role in the negative regulation of cyclin-dependent kinases (CDKs) and is involved in the G1 phase arrest of cell cycle. Previously, we demonstrated that vitamin D3 analog, EB1089, induced G1 arrest via p27 in HL-60 cells (16). In the present study, treatment of HY-11 showed a marked up-regulation of p27 protein in HL-60 cells. CDKs, the heart of the eukaryotic cell cycle engine, are a family of serine/threonine kinases. Among CDKs that regulate cell cycle, CDK4 and CDK6 are activated in association with D-type cyclins during G1 progression, whereas CDK2 is activated primarily in association with cyclin E in the late G1 phase and cyclin A in the G1/S transition and during S phase progression (17,18). We found that expressions of CDK4 and cyclin A were down-regulated in a time-dependent manner by HY-11. In addition, accumulation of p27 protein in association with G1 arrest was detected largely in complexes with CDK4 and CDK6. The increased forms of p27-CDK4 and p27-CDK6 complexes support the notion that HY-11 decreased CDK4- and CDK6-associated kinase activity in HL-60 cells. Furthermore, reduced kinase activities of CDK4 and CDK6 were accompanied by increased expression of Rb protein, which is known to sequester the transcription factor E2F. Rb-bound E2F suppresses a number of key genes needed for S-phase progression including cyclin A, which is required in both S-phase and G2/M progression (19). Therefore, the decreased cyclin A caused by HY-11 might be mediated via E2F sequestered by activated Rb in this study. Taken together, the blocking of G1 from entry into S-phase by HY-11 seems to be mediated by down-regulation of CDK4 and CDK6-associated kinase activity in association with induction of CDKI, p27.

Induction of apoptosis by vitamin D3 analogs has been documented in many cancer cell lines (20). Our data showed that HY-11 markedly induced apoptosis in HL-60 cells. The induction of apoptosis was accompanied by up-regulation of Bax protein for 24 h, although the expression of Bax protein was decreased slightly at 48 h. The expression of Bcl-2 protein was decreased in a time-dependent manner. Activated Bax translocates from the cytosol to the mitochondrion and inserts into the mitochondrial outer membrane, where its oligomerization and permeabilization of the mitochondrial outer membrane culminate in release of proteins, especially cytochrome C (22). In our data, as expected, expression of cytochrome C was increased in a time-dependent manner, suggesting that apoptotic process might be mediated through mitochondria. Cytochrome C in cytosol forms an apoptosome that is composed of Apaf-1 and procaspase-9, resulting in activation of initiator caspase-9. Caspase-9 activates the effector procaspases, including procaspase-3, to perform the process of apoptosis. In this study, HY-11 decreased the expression
of procaspase-9 and -3, although their cleavage forms could not be detected. Furthermore, PARP protein, a major substrate for active caspase-3 enzymatic protein was degraded by HY-11, as shown by the increase of its cleavage products. Caspase-8, which is associated with death receptors, cleaves Bid protein, rendering it to translocate to the mitochondrial membrane and ultimately to activate Bax (23). Recently, it has been reported that Fas-induced apoptosis is mediated via caspase-8 activation of caspase-3 and -7 directly in type I cells (mitochondria-independent apoptosis pathway), in contrast, type II cells require a mitochondrial amplification loop in which cleavage and activation of Bid are needed (24). In this study, the cleaved products of caspase-8 (p44/45) were detected in accordance with decrease of procaspase-8. In this regard, HY-11 also induced activation of caspase-3 through mitochondria-independent apoptosis pathway, since the level of Bid was not changed by HY-11 in our data. Taken together, these results provide strong evidence that the activation of caspase-3 might be one of critical steps in HY-11-induced apoptosis through mitochondrial pathway using caspase-8 as well as mitochondrial-independent pathway using caspase-8.

Transforming growth factor-ß (TGF-ß) is a multifunctional cytokine that regulates the proliferation, phenotype and differentiation of various of cell types. There are 3 different types of TGF-ß, TGF-ß1, TGF-ß2 and TGF-ß3. TGF-ß1 has been shown to be a potent inhibitor of primitive myeloid and erythroid progenitors, whereas it has little or no effect on more committed cells. Antiproliferative effect of TGF-ß1 is mediated via apoptosis or G1 cell cycle arrest resulting from induction of CDK1 and suppression of cyclin/CDK complex. We previously showed that combined treatment of TGF-ß1 and vitamin D3 analog resulted in a significant inhibition compared to either individual ligand (24). In the present study, treatment of HL-60 cells with HY-11 up-regulated the expression of TGF-ß1, TGF-ß receptors type I and II, and vitamin D3 receptor (VDR). Furthermore, TGF-ß1 increased the expression of VDR in HL-60 cells. Therefore, one possible mechanism of antiproliferative activity by HY-11 may be modulation of TGF-ß1 and TGF-ß1 receptor expression with autocrine and paracrine regulation in HL-60 cells.

Therapeutic trials of 1,25(OH)D3 in malignancy have been limited by the side effect of hypercalcemia. Therefore, development of potent vitamin D3 analogs without producing hypercalcemia is critical for the successful application of this type of therapy. We observed that intraperitoneal injection of HY-11 produced no hypercalcemia by high concentration of $10^{-5}$ M, in contrast, 1,25(OH)2D3 ($10^{-7}$ M) developed hypercalcemia in BALB/c mice from 5 days after treatment, eventually, all mice treated with 1,25(OH)D3 were dead at 25 days. In order to investigate the antileukemic effect of HY-11 in vivo, we developed BALB/c mouse leukemic model by intraperitoneal inoculation of WEHI-3BD+ cells. All animals were dead around day 25, and autopsy showed that all tissue sections (bone marrow, liver, spleen and lung) exhibited WEHI-3BD+ leukemic cell infiltration, as evidenced by H/E staining. When given every other day to BALB/c mice, HY-11 could prolong their survival as compared with those received either vehicle (DPBS) or EB1089 which is known to have most significant anti-leukemic effect (9).

In summary, HY-11, the 2-aza-cyclohexene vitamin D3, inhibited the cell proliferation of human AML cell line, HL-60, not only by induction of cell cycle arrest via p27, but also by triggering apoptosis. In addition, modulations of TGF-ß1 and its receptors were involved in HY-11-induced apoptosis. Furthermore, HY-11 inhibited the growth of WEHI-3BD+ cells in vitro as well as in vivo, and ultimately, prolonged the survival of HY-11-treated mice without producing hypercalcemia. Finally, development of more potent vitamin D3 analogs without producing hypercalcemia, like our HY-11, is needed for a novel investigational drug in AML patients by analysis of the structure-activity relationship.

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References


