Combination of a Potent 20-epi-Vitamin D₃ Analogue (KH 1060) with 9-cis-Retinoic Acid Irreversibly Inhibits Clonal Growth, Decreases bcl-2 Expression, and Induces Apoptosis in HL-60 Leukemic Cells

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ABSTRACT

All-trans retinoic acid (RA) is the first highly effective differentiation-inducing agent for remission induction in patients with acute promyelocytic leukemia. However, remissions are short-lived because the treatment fails to induce complete differentiation and fail to eradicate the malignant clone. To eliminate rapidly the malignant clone, in analogy with aggressive chemotherapy, the combination of potent differentiation- and apoptosis-inducing drugs working through different receptors and signal pathways may be useful. The active form of vitamin D₃ (1,25-dihydroxyvitamin D₃; 1,25(OH)₂D₃) inhibits proliferation and induces differentiation of myeloid leukemic cells. The 9-cis-RA, unlike all-trans-RA which binds only retinoic acid receptors, is a high affinity ligand for both retinoic acid receptors and retinoid X receptors. The aim of this study was to evaluate the therapeutic potential of combining a vitamin D₃ analogue, 20-epi-22-oxa-24a,26a,27a-tri-homo-1,25(OH)₂D₃ (KH 1060), which belongs to the family of potent 20-epi-1,25(OH)₂D₃ analogues, with 9-cis-RA by assessing their effects on the proliferation, differentiation, and apoptosis of the human leukemia cell line HL-60 in vitro. Our data show that KH 1060 alone is a very potent inhibitor of clonal proliferation of HL-60, but this effect is reversible, and that 9-cis-RA alone is a weak inhibitor of clonal proliferation of HL-60 cells. In contrast, the combination of KH 1060 and 9-cis-RA synergistically and irreversibly inhibited the clonal proliferation of HL-60 cells and induced apoptosis, as detected by morphological changes and DNA fragmentation. This combination also affected the expression of apoptosis-related genes. The bcl-2 protein became nearly undetectable, and expression of bax protein increased slightly (the bax:bcl-2 ratio was 14-fold higher than in untreated cells). Differentiation of treated HL-60 cells was assessed by their ability to produce superoxide, as measured by reduction of NBT, nitro blue tetrazolium; NSE, neutrophil-specific esterase. The ligand concentration helps discriminate between the signaling pathway involving either homo- or heterodimeric hormone receptors (12). The clinical use of 1,25(OH)₂D₃ for leukemia is, however, limited due not only to its calcemic side effects but also because 1,25(OH)₂D₃ alone fails to eliminate the malignant clone (13–15). KH 1060 belongs to the family of 20-epi-vitamin D₃ analogues. The analogues from this family are considerably more potent in vitro than 1,25(OH)₂D₃ as inhibitors of clonal growth of leukemic cells (16, 17) as well as cell lines from breast cancers (18) and prostate cancers. The induction of apoptosis should be an important goal of cancer therapy (19, 20). Therefore, the possibility to induce programmed cell death of leukemic cells during the early stages of induced myelocytic differentiation should be explored. Recently, evidence has accumulated that ligands that bind to RARs are sufficient to inhibit proliferation and induce myelocytic differentiation of HL-60 cells but cannot cause apoptosis. Induction of apoptosis of these cells requires ligand activation of RXR after they have been induced to begin their differentiation pathway (21). Our study evaluated the antileukemic potential of the combination of 9-cis-RA and KH 1060 on the HL60 cells in vitro. The two potent seco-steroids produced enhanced activity, per-

INTRODUCTION

All-trans-RA is the first highly effective differentiation-inducing agent for remission induction in patients with acute promyelocytic leukemia, but remissions are short-lived because the treatment fails to eliminate the malignant clone and clinical resistance develops rapidly (1). Another retinoic acid compound, 9-cis-RA, unlike all-trans-RA which binds only RARs, is a high affinity ligand for both RARs and RXRs. In the presence of 9-cis-RA, RXRs form RAR/RXR heterodimers and/or RXR/RXR homodimers (2, 3). 9-cis-RA was slightly more potent than all-trans-RA in inducing myeloid differentiation of HL-60 cells in vitro (4, 5), but alone it did not reverse clinically acquired retinoid resistance in acute promyelocytic leukemia cells (6).

The active vitamin D₃ metabolite, 1,25(OH)₂D₃, is another differentiation-inducing agent and an important modulator of cellular proliferation in a number of malignant cell types. This biological response is mediated by binding to the nuclear receptors for 1,25(OH)₂D₃ (VDR), which belong to the same steroid receptor superfamily as RARs and RXRs (7). This ligand-inducible transcription factor mediates the 1,25(OH)₂D₃ signaling pathways by binding to specific response elements in the promoter region of genes regulated by 1,25(OH)₂D₃. The VDR can function as a homodimer (VDR-VDR) but probably more often heterodimerizes with RXR (8–11). The ligand concentration helps discriminate between the signaling pathway involving either homo- or heterodimeric hormone receptors (12). The clinical use of 1,25(OH)₂D₃ for leukemia is, however, limited due not only to its calcemic side effects but also because 1,25(OH)₂D₃ alone fails to eliminate the malignant clone (13–15). KH 1060 belongs to the family of 20-epi-vitamin D₃ analogues. The analogues from this family are considerably more potent in vivo than 1,25(OH)₂D₃ as inhibitors of clonal growth of leukemic cells (16, 17) as well as cell lines from breast cancers (18) and prostate cancers. The induction of apoptosis should be an important goal of cancer therapy (19, 20). Therefore, the possibility to induce programmed cell death of leukemic cells during the early stages of induced myelocytic differentiation should be explored. Recently, evidence has accumulated that ligands that bind to RARs are sufficient to inhibit proliferation and induce myelocytic differentiation of HL-60 cells but cannot cause apoptosis. Induction of apoptosis of these cells requires ligand activation of RXR after they have been induced to begin their differentiation pathway (21). Our study evaluated the antileukemic potential of the combination of 9-cis-RA and KH 1060 on the HL60 cells in vitro. The two potent seco-steroids produced enhanced activity, per-

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3 The abbreviations used are: RA, retinoic acid; RAR, RA receptor; RXR, retinoid X receptor; 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; VDR, vitamin D receptor; KH 1060, 20-epi-22-oxa-24a,26a,27a-tri-homo-1,25(OH)₂D₃; NBT, nitro blue tetrazolium; NSE, α-naphthyl acetate esterase; mAb, monoclonal antibody; Cl, combination index.

haps by mediating their effects by two independent but interrelated pathways.

MATERIALS AND METHODS

Cell Lines. HL-60 cells were established from a patient with acute myeloid leukemia (22). The cells were cultured according to American Type Culture Collection recommendations in culture flasks (Costar, Cambridge, MA) in RPMI 1640 (Sigma Chemical Co., St. Louis, Mo) with 10% FCS (Irvine Scientific, Santa Ana, CA).

Vitamin D<sub>3</sub> Analogues. KH 1060 (synthesized in the Department of Chemical Research, Leo Pharmaceutical Products, Ballerup, Denmark) was dissolved in isopropanol at 4 X 10<sup>-3</sup> M as a stock solution, which was stored at −20°C and protected from light. 9-cis-RA (a generous gift of Dr. H. Klaus, F. Hoffmann-La Roche, Basel, Switzerland) was dissolved in DMSO to 10<sup>-2</sup> M, stored at −80°C, and protected from light.

Studies of Induction of Differentiation. Differentiation of HL-60 cells was assessed by their abilities to produce superoxide as measured by reduction of NBT (23), by NSE staining (Sigma), by morphology as detected on cytopsin preparations stained with Diff-Quick Stain Set (Baxter Healthcare Corp., Miami, FL), and by analysis of membrane-bound differentiation markers with two-color immunofluorescence. The latter was performed as described (18). Briefly, cells were preincubated at 4°C for 60 min in 10% human AB serum (Sigma) and then with FITC-conjugated human mAbs to CD14 and phycoerythrin-conjugated mAb to CD11b (Becton Dickinson, Mountain View, CA) or with FITC-conjugated mouse IgGl isotype control (Becton Dickinson). Analysis of fluorescence was performed on a FACScan flow cytometer, using the LYSYS II program (Becton Dickinson).

Cell Cycle Analysis. The cell cycle was analyzed by flow cytometry after 60 h of incubation of HL-60 cells either with or without analogues (10<sup>-5</sup> M) as described (18). Briefly, the cells were fixed in cold methanol and incubated for 30 min at 4°C in the dark with a solution of 50 µg/ml propidium iodide, 1 mg/ml RNase (Sigma), and 0.1% NP40 (Sigma). Analysis was performed immediately after staining using the CELLFIT program (Becton Dickinson) whereby the S-phase was calculated with a RFit model.

Clonogenic Assay in Soft Agar. HL-60 cells were cultured in a two-layer soft agar system for 10 days without adding any growth factors as described previously, and colonies were counted using an inverted microscope (24). The analogues were added to the agar upper layer on day 0. For analysis of the reversibility of inhibition of proliferation, the cells were cultured in suspension culture with and without analogues. After 60 h, the culture flasks were gently jarred to loosen adherent cells, the cells were washed twice in cultured medium containing 10% FCS to remove the test drugs, and then the clonogenic assay was performed.

Apoptosis. Apoptosis of HL-60 cells was assessed by changes in cell morphology and by measurement of DNA nicks using the Apop Tag Kit (Oncor, Gaithersburg, MD). Morphologically, HL-60 cells undergoing apoptosis possess many prominent features, such as intensely staining, highly condensed, and/or fragmented nuclear chromatin, a general decrease in overall cell size, and cells undergoing fragmentation into apoptotic bodies (25). These features make apoptotic cells relatively easy to distinguish from necrotic cells. These changes are detected on cytopsin preparations stained with Diff-Quick Stain Set. Apoptotic cells were enumerated in a total of about 300 cells by light microscopy.

For evaluation of apoptosis by flow cytometry, cells were fixed and permeabilized in 1% paraformaldehyde and ice-cold 70% ethanol. Digoxigenin-dUTP was incorporated at the 3'OH ends of the fragmented DNA in the presence of terminal deoxynucleotidyltransferase, and the cells were incubated with FITC-labeled anti-digoxigenin-dUTP and with propidium iodide. Green (apoptotic cells) and orange (total DNA) fluorescence were measured with a FACScan flow cytometer and analyzed with LYSIS II and CELLFIT programs. These results were periodically confirmed by fluorescence microscopy and by DNA fragmentation (26).

Immunostaining for bcl-2 and bax. To determine the bcl-2 cell content, cytosin slides of HL-60 cells, which had been grown in suspension culture with and without analogue (10<sup>-5</sup> M, 60 h), were fixed in cold buffered methanol/acetone and rinsed with normal saline for 10 min. Incubation with a murine monoclonal antihuman bcl-2 antibody (DAKO Corp., Carpinteria, CA) at 1:300 dilution for 30 min was followed by incubation with horseradish peroxidase-conjugated rabbit antimouse antibody and (DAKO) diluted 1:50 in PBS and with 3,3'-diaminobenzidine substrate. The slides were counterstained with methyl green. Control slides consisted of substitution of primary antibody by PBS.

Intracellular bax protein was detected with a rabbit polyclonal antihuman bax antibody (27). Briefly, cells were fixed and permeabilized in 1% paraformaldehyde and in ice-cold 70% ethanol. The pellet was preblocked with human AB serum (4%; Sigma), and rabbit antihuman bax polyclonal antibody (1:500) was added. The cells were stained using FITC-conjugated goat antirabbit IgG (1:100; Cappel, West Chester, PA). Control slides were incubated initially with PBS instead of primary antibody and then exposed to FITC-conjugated goat antirabbit IgG.

Phagocytosis. The HL-60 cells, after exposure to analogues, were washed twice with PBS, cultured in regular medium for 1 day, and then tested for their ability to phagocytose. The method developed for studying phagocytosis has been described in detail by Territo and Cline (28). Briefly, Candida albicans was opsonized in 10% human AB serum. A 5:1 ratio of Candida to leukemic cells was incubated at 37°C for 30 min. The cells were stained with Diff-Quick Stain Set, and the percentage of cells completely ingesting one or more yeast was determined microscopically.

Statistical Analysis. Isobologram analysis was used to evaluate the effects of combinations of drugs on leukemic cells (29). Dose-dependent effects were determined separately for each compound, and then the effects were determined for the combination of one compound held at a fixed concentration and the other at different dilutions. The interaction of two compounds was quantified by determining the CI according to the classic isobologram equation (30): \( CI = (D_1/D_2)_{(D_1)(D_2)} \), where \( D_1 \) is the dose of one compound alone required to produce an effect and \( D_1 \) and \( D_2 \) are the dose of both compounds that produce the same effect. From this analysis, the combined effects of two drugs can be assessed as either summation (additive or zero interaction) indicated as \( CI = 1 \), synergism indicated as \( CI < 1 \), or antagonism indicated as \( CI > 1 \). Other statistical data were handled using Student's t test.

RESULTS

Effect of Analogues on Clonal Proliferation of HL-60 Cells. HL-60 cells were cloned in soft agar in the presence of either KH 1060 (10<sup>-14</sup>–10<sup>-6</sup> M), 9-cis-RA (10<sup>-12</sup>–10<sup>-6</sup> M), or the combination of 9-cis-RA (10<sup>-8</sup> M) with KH 1060 (10<sup>-14</sup>–10<sup>-6</sup> M) (Fig. 1). KH 1060 inhibited clonal proliferation of HL-60 cells by 50% at 10<sup>-10</sup> M and 90% at 10<sup>-9</sup> M. The 9-cis-RA at 10<sup>-8</sup> M inhibited approximately...
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Induction of Differentiation of HL-60 Cells. The NBT test becomes positive as cells undergo either monocytic or granulocytic differentiation (Fig. 3). A 60-h exposure of HL-60 to 9-cis-RA ($10^{-8}$ M) resulted in only 7% NBT-positive HL-60 cells. KH 1060 ($10^{-8}$ M) is a more potent inducer of differentiation than 9-cis-RA under the same conditions (44% NBT-positive cells). The combination of both KH 1060 ($10^{-8}$ M) and 9-cis-RA ($10^{-8}$ M) additively ($Cl = 1$) enhanced the percentage of NBT-positive cells (60% positive cells).

NSE is a monocytic-specific enzyme (Fig. 3). The wild-type HL-60 cells do not express NSE, and less than 1% of HL-60 cells were NSE positive after incubation with 9-cis-RA ($10^{-8}$ M; 60 h). The NSE-positive cells increased to 38% after incubation with KH 1060 ($10^{-8}$ M; 60 h) but decreased to 22% after the cells were cultured with the combination of KH 1060 and 9-cis-RA under the same conditions. Wild-type HL-60 cells and these cells, after incubation with 9-cis-RA ($10^{-8}$ M; 60 h), continued to have predominantly the morphology of blastic leukemia cells with large spherical nuclei surrounded by a thin shell of basophilic cytoplasm. KH 1060 ($10^{-8}$ M; 60 h) induced about 50% of HL-60 cells to differentiate to myelomonocyte-like cells. After culture with the combination of both analogues ($10^{-8}$ M; 60 h), about 50% of HL-60 cells had the condensed, lobulated nuclei char-

Fig. 2. Clonal inhibition of HL-60 cells after pulse-exposure (24, 48, and 60 h) to KH 1060, 9-cis-RA, or both compounds. HL-60 cells were exposed in liquid culture to either KH 1060, 9-cis-RA, or both ($10^{-8}$ M), washed, and plated in soft agar; resulting colonies were counted. Each point represents a mean of at least three experiments, with each experimental triplicate having triplicate dishes; bars, SD.

50% clonal proliferation of HL-60 cells. The combination of 9-cis-RA ($10^{-8}$ M) with KH 1060 ($10^{-14}$, $10^{-12}$ M) had no subadditive effects on inhibition of clonal proliferation of HL-60 cells, but the combination of 9-cis-RA ($10^{-8}$ M) with KH 1060 ($10^{-11}$–$10^{-10}$ M) produced synergistic effects ($Cl < 1$) on inhibition of clonal growth (90 and 100% inhibition, respectively; Fig. 1).

To examine if the clonogenic growth arrest of the HL-60 cells by the seco-steroids was reversible, the cells were cultured in liquid medium with $10^{-8}$ M of either KH 1060, 9-cis-RA, or both for different durations, washed extensively, and then plated in soft agar in the absence of the seco-steroids (Fig. 2). Inhibition of clonal proliferation was 30% at 48 h and about 50% at 60 h of pulse incubation with KH 1060 ($10^{-8}$ M). Pulse-exposure to 9-cis-RA ($10^{-8}$ M; 60 h) inhibited 30% of clonogenic growth. In contrast, when the cells were cultured in liquid culture with both KH 1060 and 9-cis-RA at $10^{-8}$ M for 60 h, thoroughly washed, and plated in soft agar, more than 99% of clonal growth was inhibited (Fig. 2). These results suggest that the combination of both analogues could irreversibly inhibit the clonal growth of HL-60 cells.

Fig. 4. Morphological changes before (A) and after (B) treatment with a combination of KH 1060 and 9-cis-RA ($10^{-8}$ M; 60 h).

Fig. 3. Induction of differentiation of HL-60 cells, as measured by their ability to reduce NBT and to stain with NSE after culture with either KH 1060, 9-cis-RA, or both compounds ($10^{-8}$ M; 60 h). Results represent means of three or more experiments; bars, SD.
accumulation of mature granulocyte-like differentiation. Fully mature granulocytes or macrophages were not observed (Fig. 4).

Analysis of Cell Surface Markers. Two-color flow analysis with mAbs CD11b (β-integrin subunit, expressed by both polymorphonuclear cells and monocytes) and CD14 (late monocyte cell surface marker) showed that less than 3% of HL-60 cells expressed either CD11b or CD14 (Table 1). The KH 1060 (10^{-7} M) induced HL-60 to express either CD14 (20%) or both CD14/CD11b (67%). In contrast, 9-cis-RA (10^{-7} M) induced expression of CD11b (20%). The combination of both ligands (10^{-7} M) increased expression of CD11b (30%) but decreased CD14-expressing cells as compared to those cultured with KH 1060 alone. Interestingly, although the combination induced approximately equivalent numbers of CD11b^+/CD14^- cells (59%) as KH 1060 alone (67%; Table 1), the mean fluorescence intensity of HL-60 cells for CD14 was about 2-fold lower after culture with the combination of both analogues as compared to KH 1060 alone (P < 0.01; Fig. 5).

Effect of KH 1060 and 9-cis-RA on Phagocytic Activity of HL-60 Cells. The HL-60 cells developed the ability to phagocytose C. albicans after exposure to either 9-cis-RA, KH 1060, or the combination of both analogues (10^{-8} M; 60 h; Fig. 6). Wild-type HL-60 control cells were unable to engulf yeast, whereas 24 and 3% of the cells ingested one or more Candida after they were cultured with either KH 1060 or 9-cis-RA, respectively (10^{-8} M; 60 h). The combination of both analogues (10^{-8} M; 60 h) synergistically (CI < 1) increased to 93% the percentage of HL-60 cells capable of phagocytosis (Fig. 6).

Cell Cycle Analysis. Analysis of the cell cycle of HL-60 cells after exposure to KH 1060, 9-cis-RA, or a combination of both analogues (10^{-8} M; 60 h) is shown in Figure 7. Forty-seven % of the untreated cells were in G0-G1 compared to 63, 64, and 70% when cultured with KH 1060, 9-cis-RA, or both, respectively. The percentage of HL-60 cells in S phase decreased from 40% (untreated control) to 32, 25, and 20% for those cells cultured in the presence of KH 1060, 9-cis-RA, or the combination of both compounds, respectively. The combination of both analogues caused a statistically significant increase in the number of cells in G0-G1 and a decrease in these in the S phase of the cell cycle (P < 0.05).

Induction of Apoptosis. The strong antiproliferative effects of the combination of KH 1060 and 9-cis-RA on HL-60 cells in vitro may be caused by induction of apoptosis. As shown by analysis of DNA fragmentation by 3' labeling of DNA strand breaks (Fig. 8), apoptosis was induced in 14 and 3% of HL-60 cells after 60 h of culture with...
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Increased Ratio of Expression of bax to bcl-2 after Exposure of HL-60 Cells to the Combination of KH 1060 and 9-cis-RA. Because of the apoptosis-inducing activity of KH 1060 and 9-cis-RA, we studied their effects on the expression of apoptosis-related genes. Expression of bcl-2 protein, as analyzed by immunohistochemistry, was nearly 100% in wild-type HL-60 cells, which became nearly undetectable (9%) when they were cultured with the combination of KH 1060 and 9-cis-RA (Figs. 4B and 8).

DISCUSSION

Cancer can result from dysregulation of proliferation, differentiation, and/or apoptosis. In analogy with aggressive chemotherapy, the combination of new, potent, antiproliferative, differentiation- and apoptosis-inducing drugs working through different receptors and signaling pathways may be useful to eliminate the malignant clone before resistance can develop.

KH 1060 belongs to the 20-epi family of vitamin D3 analogues that differ markedly from 1,25(OH)2D3 in their conformational distribution (31). The side-chain of the 20-epi vitamin D3 analogues is directed to the left, whereas it is directed to the right in the "normal" isomers. This can induce significant change in the conformation of the receptor upon ligand-binding and thereby produce differences in the biological selectivities of these compounds. The 20-epi analogues can efficiently enhance dimerization of VDR with RXR as compared to 1,25(OH)2D3 (8, 32). The competition between the RAR and VDR for association with RXR may provide an additionally important control step for these ligands (9). We examined KH 1060 and 9-cis-RA separately and in combination for their abilities to inhibit clonal proliferation, induce cellular differentiation, and trigger apoptosis of HL-60 cells. KH 1060 is a very potent inhibitor of proliferation of HL-60 cells, decreasing their clonal growth by 50 and 95% at 10^{-10} and 10^{-9} M, respectively. The combination of KH 1060 at 10^{-11} M with 9-cis-RA (10^{-8} M) caused a synergistic decrease of clonal proliferation of HL-60 cells. Importantly, the arrest of clonal growth produced by pulse-exposure to either compound (10^{-8} M; 60 h) was in part reversible, whereas the pulse-exposure to the combination of these analogues (10^{-8} M; 60 h) resulted in nearly completely irreversible arrest of clonal proliferation.

Separately, KH 1060 promoted monocytic differentiation as measured by NSE and CD14 expression, whereas 9-cis-RA (10^{-8} M) was a weak inducer of granulocytic differentiation, as measured by morphology, NBT reduction, and CD11b expression. The combination of KH 1060 and 9-cis-RA stimulated HL-60 cells to express markers of monocytic differentiation (CD14 expression; NSE-positive cells), but morphologically many of the cells were proceeding down the granulocytic pathway. In addition, in the presence of both compounds, the percentage of NBT-positive cells (myeloid differentiation) was three times higher than the percentage of NSE-positive cells (monocytic differentiation; P < 0.05). Furthermore, when cultured with both compounds, the percentage of HL-60 cells that expressed CD14+/CD11b− and their mean fluorescent intensity of expression of CD14 was 20-fold (P < 0.005) and 2-fold (P < 0.05), respectively, less than their expression when HL-60 cells were exposed to KH 1060 alone (Table 1; Fig. 4). Taken together, these results suggest that at least a population of HL-60 cells proceeded down the granulocytic pathway of differentiation after their exposure to the combination of KH 1060 and 9-cis-RA.

Other studies have provided a mixed picture of myeloid differentiation induced by a retinoid and a vitamin D$_3$ compound. In one study, all-trans-RA and 1,25(OH)$_2$D$_3$ cooperated in promoting differentiation of HL-60 cells along the monocytic pathway (33, 34), whereas no additive or synergistic effects were observed with KH 1060 and 9-cis-RA in inducing HL-60 toward monocyte-like cells (NSE-positive cells), and the granulocytic pathway of differentiation was not studied (34). In a second investigation, the combination of very low concentrations of 1,25(OH)$_2$D$_3$ (10^{-12}–10^{-10} M) with a high concentration of either all-trans-RA or 9-cis-RA resulted in differentiation of HL-60 cells toward neutrophils (35). Studies with another...
The apoptosis-suppressor and -promoter proteins (bcl-2 and bax, respectively) of the granulocytic pathway (36). Normal human committed myeloid stem cells (CFU-GM) when cultured with both 1,25(OH)2D3 and all-trans-RA appeared to proceed down the granulocytic pathway (36). Future experiments need to examine a variety of features of differentiation to determine the effect of the combination of KH 1060 and 9-cis-RA on fresh myeloid leukemia cells from a variety of patients with acute myeloid leukemia.

To gain insight into the remarkable antileukemic effect of the combination of KH 1060 and 9-cis-RA, apoptosis and expression of the apoptosis-suppressor and -promoter proteins (bcl-2 and bax, respectively) were examined in HL-60 cells. Apoptosis is an active process that contributes to the shaping of organs during embryogenesis; to the maintenance, growth, or involution of tissues; and to the elimination of damaged cells. Dysregulation of this pivotal process can contribute to cancer. The bcl-2 protein, which is overexpressed in many types of human tumors and suppresses apoptosis, is induced by a wide variety of stimuli, including chemotherapy drugs and γ-irradiation (37–39). The product of the bax gene promotes rather than blocks cell death (40). It has about 20% homology to the bcl-2 protein, can form heterodimers with bcl-2, and abrogate the latter’s ability to suppress apoptosis. Thus, the bax:bcl-2 ratio helps to determine cell survival following an apoptotic stimulus (40). Our experiments regarding apoptosis in HL-60 cells make several points: (a) apoptosis can be induced in the absence of p53, as the latter is completely deleted in HL-60 cells; this confirms prior observations (41). The rapid induction of other genes, such as gadd153, c-fos, and c-jun, could conceivably have a role in induction of apoptosis in HL-60 cells (42); (b) KH 1060 decreased bcl-2 in 50% of HL-60 cells but induced no apoptosis. These data are in agreement with data from other groups that found down-regulation of bcl-2 by other vitamin D3 compounds with protection from apoptosis (43). Perhaps more is required to induce apoptosis than merely decreasing intracellular levels of bcl-2; (c) 9-cis-RA induced apoptosis in HL-60 cells, and the addition of KH 1060 synergistically increased the number of apoptotic HL-60 cells, which was associated with a prominent increase in the bax:bcl-2 ratio. Why the combination of KH 1060 and 9-cis-RA is synergistically able to induce apoptosis is unclear. Perhaps, this combination can cause the activation of RAR/RAR, RXR/RXR, and VDR/VDR homodimers and VDR/RXR, VDR/RAR, and RAR/RXR heterodimers, which can functionally induce and/or suppress expression of a wide range of growth-related genes (10, 11). Further studies are required to identify the key target genes of these ligands that mediate apoptosis and then to dissect how they mediate their activities.

In conclusion, our data demonstrate that the combination of KH 1060 and 9-cis-RA synergistically inhibited clonal growth and induced differentiation and apoptosis in HL-60 cells associated with a markedly decreased expression of bcl-2 and increased bax:bcl-2 ratio. The combination of these seco-steroids may provide alternative approaches to therapy of acute myelogenous leukemia as well as other malignancies.

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34. Brown, G., Bunce, C. M., Rowlands, D. C., and Williams, G. R. All-trans retinoic acid and 1α,25-dihydroxyvitamin D3 co-operate to promote differentiation of the human promyeloid leukemia cell line HL60 to monocytes. Leukemia (Baltimore), 8: 806–815, 1994.


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