5,6-trans-16-ene-Vitamin D₃: A New Class of Potent Inhibitors of Proliferation of Prostate, Breast, and Myeloid Leukemic Cells

Jun-ichi Hisatake, Tetsuya Kubota, Yasuko Hisatake, Milan Uskokovic, Shigeru Tomoyasu, and H. Phillip Koeffler

Division of Hematology/Oncology, Cedars-Sinai Research Institute, UCLA School of Medicine, Los Angeles, California 90048 [J. H., T. K., Y. H., H. P. K.]; Hoffmann LaRoche, Inc., Nutley, New Jersey 07110 [M. U.]; and Department of Hematology, Showa University School of Medicine, Tokyo, Japan [S. T.]

ABSTRACT

The 1,25-dihydroxyvitamin D₃ [1,25(OH)₂D₃] is the physiologically active form of vitamin D₃ that inhibits proliferation and induces differentiation of a variety of malignant cells. We evaluated a newly synthesized vitamin D₃ analogue [1,25(OH)₂-16-ene-5,6-trans-D₃ (Ro 25-4020)] that has a novel 5,6-trans motif. Dose-response studies showed that 1,25(OH)₂-16-ene-5,6-trans-D₃ had 10-100-fold greater antiproliferative activities than 1,25(OH)₂D₃ when measuring clonal growth of breast (MCF-7) and prostate (LNCaP) cancer cell lines as well as a myeloid leukemia cell line (HL-60). Because the chief toxicity of vitamin D₃ is hypercalcemia, we examined the calcemic activity of 1,25(OH)₂-16-ene-5,6-trans-D₃ in mice. Remarkably, 1,25(OH)₂-16-ene-5,6-trans-D₃ was at least 40-fold less calcemic as compared with 1,25(OH)₂D₃ and 1,25(OH)₂-16-ene-16-D₃ (Ro 24-2637). To explore the mechanism by which the 1,25(OH)₂-16-ene-5,6-trans-D₃ analogue mediated its antiproliferative activity, several studies were performed. Pulse-exposure studies showed that a 4-day pulse exposure to 1,25(OH)₂-16-ene-5,6-trans-D₃ (10⁻⁷ M) in liquid culture was adequate to achieve a 40% inhibition of MCF-7 clonal growth in the absence of the analogue, suggesting that the growth inhibition mediated by 1,25(OH)₂-16-ene-5,6-trans-D₃ was at least in part irreversible. Cell cycle studies showed that 1,25(OH)₂-16-ene-5,6-trans-D₃ increased the proportion of MCF-7 cells in the Go-G₁ phase and decreased those in the S phase. Furthermore, 1,25(OH)₂-16-ene-5,6-trans-D₃ induced an elevated expression of the cyclin-dependent kinase inhibitors, p21WAF and p27KIP1. In addition, 1,25(OH)₂-16-ene-5,6-trans-D₃ almost completely inhibited telomerase activity, as measured by telomeric repeat amplification protocol assay and human telomerase reverse transcriptase mRNA. For each of the growth-related parameters that were examined, the vitamin D₃ analogue was more active than 1,25(OH)₂D₃. In contrast, 1,25(OH)₂-16-D₃ was more calcemic than 1,25(OH)₂-16-ene-5,6-trans-D₃. In summary, 1,25(OH)₂-16-ene-5,6-trans-D₃, having a novel 5,6-trans motif, strongly inhibited clonal proliferation and reduced telomerase activity with low calcemic activity, suggesting further testing in in vivo cancer models. This analogue may gain a therapeutic niche for selected malignancies.

INTRODUCTION

In the United States, breast and prostate cancers are two of the most prevalent, nonskin malignancies. Improvement in hormonal and cytotoxic therapies has not led to either a major lengthening of remissions or increase in cures in advanced breast cancer. In prostate cancer, blockade of androgen stimulation often leads to a remission; however, a subsequent relapse almost invariably occurs within a few years, resulting in a poorly differentiated, androgen-independent cancer. The present chemotherapeutic of cancer uses agents that are toxic to normal cells. On the other hand, induction of cellular differentiation may be useful for several forms of neoplasia, like the successful use of all-trans-retinoic acid in the treatment of acute promyelocytic leukemia.

1,25(OH)₂D₃ is a member of the seco-steroid hormone family, which controls calcium homeostasis. The effects of 1,25(OH)₂D₃ are mediated mainly via interaction with a specific nuclear vitamin D₃ receptor, which heterodimerizes with the retinoic acid receptor (retinoid X receptor; Ref. 1). 1,25(OH)₂D₃ can inhibit the growth and induce differentiation of a variety of types of malignant cells, including breast (2–6), prostate (7–10), blood (11–14), colon (15, 16), skin (17), and brain (18). However, the calcemic side effects of 1,25(OH)₂D₃ have prevented its application as a therapeutic agent (19). Synthesis of analogues of 1,25(OH)₂D₃ with potent antiproliferative and differentiation activity against cancer cells with decreased risk of inducing hypercalcemia have been reported (20–25).

In the present study, we analyzed 1,25(OH)₂D₃ analogues that have a novel 5,6-trans motif. The results indicated that 1,25(OH)₂-16-ene-5,6-trans-D₃ (Ro 25-4020) was more potent than 1,25(OH)₂D₃ in its ability to inhibit the clonal cell growth of breast (MCF-7) and prostate (LNCaP) cancer cells and myeloid leukemia cells (HL-60) in vitro. Further studies showed that 1,25(OH)₂-16-ene-5,6-trans-D₃ arrested MCF-7 cells in G₀-G₁, which was associated with the rapid and prominent accumulation of the p21WAF and p27KIP1 CDKIs. In addition, we showed that 1,25(OH)₂-16-ene-5,6-trans-D₃ markedly inhibited telomerase activity as measured by TRAP assay and hTERT mRNA expression in HL-60 cells.

MATERIALS AND METHODS

Cell Lines. The breast cancer (MCF-7), prostate cancer (LNCaP), and myeloid leukemia (HL-60) cell lines were obtained from American Type Culture Collection (Rockville, MD). MCF-7 cells were maintained in DMEM with 10% FCS. LNCaP and HL-60 were cultured in RPMI 1640 with 10% FCS. All three cell lines were maintained in a 37°C incubator containing 5% CO₂.

Vitamin D₃ Compounds. In this study, 10 vitamin D₃ analogues were used: [1,25(OH)₂-16-ene-5,6-trans-D₃], [1,25(OH)₂-16-ene-5,6-trans-D₃]; [1,25(OH)₂-16-ene-D₃]; [1,25(OH)₂-16,23Z-diene-5,6-trans-D₃]; [1,25(OH)₂-16-ene-23-yne-26,27-F₆-5,6-trans-D₃]; [1,25(OH)₂-16,23E-diene-26,27-F₆-epi-5,6-trans-D₃]; [1,25R(OH)₂-16,23E-diene-26-F₆-5,6-trans-D₃]; [1,25S(R)-OH-16,23-eine-26,27-F₆-5,6-trans-D₃]; and [25(OH)-16-ene-23-yne-5,6-trans-D₃]. All analogues were synthesized by Hoffmann-La Roche, Inc. The analogues studied in greatest detail are shown in the Fig. 1. The vitamin D₃ compounds were dissolved in absolute ethanol at 10⁻⁷ M as stock solution, which were stored at −20°C and protected from light. For in vitro use, analogues were diluted in DMEM or RPMI 1640. For in vivo use, analogues were diluted with PBS. An aliquot was used only once.

Soft Agar Colony Assay. Cells were cultured in a two-layer soft agar system for either 14 days (MCF-7 and LNCaP) or 10 days (HL-60) as described previously (26). MCF-7 and LNCaP cells were trypsinized. Washed single-cell suspensions of cells were enumerated and plated into 24-well, flat-bottomed plates with a total of 1 × 10³ cells/well in a volume of 400 µl/well. The feeder layer was prepared with agar that had been equilibrated at

Received 1/6/99; accepted 6/14/99.

The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked advertisement in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

1 This work was supported by United States Defense and NIH grants and by the Lymphoma Foundation, Parker Hughes Trust, CAP Cure, Aaron Eshcan Trust, and the C. and H. Koeffler Fund.

2 To whom requests for reprints should be addressed, at Division of Hematology/Oncology, Cedars-Sinai Medical Center, UCLA School of Medicine, B-213, 8700 Beverly Boulevard, Los Angeles, CA 90048. Phone: (310) 855-4609; Fax: (310) 659-9741.

3 The abbreviations used are: 1,25(OH)₂D₃, 1,25-dihydroxyvitamin D₃; Ro 25-4020, 1,25(OH)₂-16-ene-5,6-trans-D₃; CDKI, cyclin-dependent kinase inhibitor; TRAP, telomeric repeat amplification protocol; hTERT, human telomerase reverse transcriptase; RT-PCR, reverse transcription-PCR; GAPDH, glyceraldehyde-3-phosphate dehydrogenase.
**RESULTS**

**Effect of Vitamin D3 Analogues on Clonogenic Growth of Prostate, Breast, and Myeloid Leukemic Cells.** The LNCaP, MCF-7, and HL-60 cells were cloned in soft agar in the presence of various concentrations of vitamin D3 analogues at 10⁻¹¹ to 10⁻⁷ M. Dose-response curves were drawn, and the effective dose that inhibited 50% colony formation (ED₅₀) was determined. Initially, 10 analogues were examined (see "Materials and Methods"). The most effective was 1,25(OH)₂-16-ene-5,6-trans-D₃ (Ro 25-4020; data not shown). Thus, additional experiments focused on this analogue. For comparison, 1,25(OH)₂-5,6-trans-D₃, 1,25(OH)₂-16-ene-D₃, and 1,25(OH)₂-D₃ were also studied in the additional experiments (Fig. 1). Each of the four vitamin D₃ compounds was effective in inhibition of clonal proliferation of the three cell lines in a dose-dependent manner (Fig. 2). The 1,25(OH)₂-16-ene-D₃ was the most potent. The ED₅₀ of 1,25(OH)₂-16-ene-D₃ was 5.0 x 10⁻¹¹ M, 2.4 x 10⁻¹¹ M, and 1.9 x 10⁻¹² M for LNCaP, MCF-7, and HL-60 cells, respectively (Table 1). The ED₅₀ of 1,25(OH)₂-16-ene-5,6-trans-D₃ was 1.4 x 10⁻⁹ M for LNCaP cells, 4.3 x 10⁻⁹ M for MCF-7 cells, and 3.0 x 10⁻¹¹ M for HL-60 cells, which were about 10–100-fold more potent than 1,25(OH)₂-D₃. The potency of 1,25(OH)₂-5,6-trans-D₃ was nearly equivalent to 1,25(OH)₂-D₃.

**Serum Calcium Levels in Vivo.** Because hypercalcemia is a major toxicity of vitamin D₃ compounds, we compared their calcemic effects (Fig. 3). The mice that received 0.1 μg of either 1,25(OH)₂-D₃ or 1,25(OH)₂-16-ene-5,6-trans-D₃ (three times per week) were hypercalcemic, with mean serum calcium levels of approximately 11.6 ± 0.1 and 13.8 ± 0.5 mg/dl (normal, 8.5–10.5 mg/dl) at week 3, respectively. In contrast, mice that received 1,25(OH)₂-16-ene-5,6-trans-D₃ (0.1–2.0 μg/mouse) had almost the same calcium level (7.9–10.4 mg/dl) as the control mice (8.4–9.7 mg/dl) at week 3. When 6.0 μg/mouse 1,25(OH)₂-16-ene-5,6-trans-D₃ were administered, serum calcium levels increased to a mean 12.9 ± 0.7 and 11.4 ± 0.3 mg/dl at weeks 3 and 5, respectively. When 4.0 μg/mouse were administered, serum calcium levels increased to a mean 11.7 ± 0.8 mg/dl at week 3; but at weeks 4 and 5, levels returned to the normal range.

**Pulse-Exposure Experiments.** To investigate whether the inhibition of clonogenic proliferation by 1,25(OH)₂-16-ene-5,6-trans-D₃ was reversible, we performed pulse-exposure experiments. The MCF-7 cells were exposed to either 1,25(OH)₂-16-ene-5,6-trans-D₃...
or 1,25(OH)D₃ for various durations, washed thoroughly, and plated in soft agar; clonal growth was determined on day 14 of culture (Fig. 4). Approximately 40 and 30% of the clonogenic cells were inhibited by 4 days of exposure to 1,25(OH)₂-16-ene-5,6-trans-D₃ and 1,25(OH)₂ D₃, respectively, suggesting that these vitamin D₃ compounds were capable of mediating a partial, irreversible inhibition of growth of MCF-7 cells.

**Analysis of the Cell Cycle and Expression of p21waf1 and p27kip1.** To understand better the mechanism by which the vitamin D₃ analogue prevented cell growth, its effect on the cell cycle of the MCF-7 cells was determined. A significant accumulation (P < 0.05) of the number of cells in the G₀-G₁ phase of the cell cycle occurred with a concomitant decrease in the proportion of cells in S phase after culture with either 1,25(OH)₂-16-ene-5,6-trans-D₃ or 1,25(OH)₂D₃ (10⁻⁷ M; 4 days; Fig. 5).

The CDKIs known as p21waf1 and p27kip1 are able to inhibit the activity of cyclin kinase and thus slow the progression of the cells through the cell cycle. The control MCF-7 cells constitutively had a moderate level of expression of p21waf1 and p27kip1, as determined by Western blot analysis (Fig. 6A). Exposure for 1 day to 1,25(OH)₂-16-ene-5,6-trans-D₃ (10⁻⁷ M) increased levels of p21waf1 and p27kip1 by about 3.2–3.5-fold, whereas culture with 1,25(OH)₂ D₃ (10⁻⁷ M; 1 day) increased expression of p21waf1 and p27kip1 about 1.6–1.8-fold. By 3 days, 1,25(OH)₂-16-ene-5,6-trans-D₃ (10⁻⁷ M) increased expression of p21waf1 and p27kip1 by 2.8- and 3.4-fold, respectively; and 1,25(OH)₂ D₃ increased expression of p21waf1 and p27kip1 by 4.8- and 3.3-fold, respectively. The 1,25(OH)₂-16-ene-5,6-trans-D₃ and 1,25(OH)₂ D₃ also up-regulated expression of p27kip1 in HL-60 cells in a dose-dependent manner (Fig. 6B).

**Telomerase Activity.** Maintenance of telomeres is important for cellular well-being, and progressive telomere shortening limits the replicative capacity of cells (29–33). Telomerase activity is inhibited by diverse agents including 1,25(OH)₂ D₃ during terminal differentiation of leukemia cell lines (34–36). Therefore, we evaluated the effect 1,25(OH)₂-16-ene-5,6-trans-D₃ and 1,25(OH)₂ D₃ (10⁻⁷ M; 4 days) on telomerase activity using the TRAP assay. Telomerase activity markedly decreased in HL-60 cells cultured with either 10⁻⁹ M 1,25(OH)₂-16-ene-5,6-trans-D₃ or 10⁻⁷ M 1,25(OH)₂ D₃ (Fig. 7).

Recent investigations have revealed that hTERT is the catalytic human telomerase subunit, and it plays an important role in the activation of telomerase (37–40). The effects of vitamin D₃ analogues on hTERT expression in HL-60 cells were evaluated using RT-PCR.

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**Table 1** Inhibition of clonal proliferation of tumor cells by vitamin D₃ analogues

<table>
<thead>
<tr>
<th>Analogue</th>
<th>HL-60 ED₅₀ (M)</th>
<th>MCF-7 ED₅₀ (M)</th>
<th>LNCaP ED₅₀ (M)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)₂D₃</td>
<td>4.0 × 10⁻⁹</td>
<td>7.3 × 10⁻⁸</td>
<td>2.3 × 10⁻⁸</td>
</tr>
<tr>
<td>1,25(OH)₂-5,6-trans-D₃ (Ro 22-3790)</td>
<td>3.5 × 10⁻⁹</td>
<td>1.3 × 10⁻⁷</td>
<td>4.3 × 10⁻⁷</td>
</tr>
<tr>
<td>1,25(OH)₂-16-ene-5,6-trans-D₃  (Ro 25-4020)</td>
<td>3.0 × 10⁻¹¹</td>
<td>4.3 × 10⁻⁹</td>
<td>1.4 × 10⁻⁹</td>
</tr>
<tr>
<td>1,25(OH)₂-16-ene-D₃            (Ro 24-2637)</td>
<td>1.9 × 10⁻¹²</td>
<td>2.4 × 10⁻¹¹</td>
<td>5.0 × 10⁻¹¹</td>
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**Fig. 2.** Dose-response effects of vitamin D₃ compounds on clonal proliferation of HL-60, LNCaP, and MCF-7 cells. Results are expressed as a mean percentage of control plates containing no vitamin D₃ analogues. Each point represents a mean of three independent experiments with triplicate dishes. Bars, SD.
Fig. 3. The effect of vitamin D₃ analogues on serum calcium in mice: □, control; ⊙, 1,25(OH)₂D₃ (0.1 μg); ◇, 1,25(OH)₂-5,6-trans-D₃ (0.1 μg); ■, 1,25(OH)₂-16-ene-D₃ (0.1 μg); ▲, 1,25(OH)₂-16-ene-5,6-trans-D₃ (6.0 μg); ◆, (4.0 μg); ▼, (2.0 μg); ▼, (1.0 μg); ▼, (0.5 μg); ◇, (0.1 μg). Each data point represents the mean; bars, SD. If the SD was <0.2 mg/dl, it does not appear on the graph. The compounds were delivered i.p. on Monday, Wednesday, and Friday.

DISCUSSION

Previously, we showed that the vitamin D₃ analogue having the C-16-ene motif [1,25(OH)₂-16-ene-D₃] was more potent than 1,25(OH)₂ D₃ in inhibiting the proliferation of HL-60 cells, but the calcemic activity of this analogue was nearly identical to 1,25(OH)₂ D₃ (22). The desaturation of the side chain with the addition of a C-23-triple bond [1,25(OH)₂-16-ene-23-yne-D₃] also was potent in its ability to induce cell differentiation and inhibit leukemic clonal proliferation with low calcemic activity (13, 14, 22). This latter analogue, however, did not have a broad range of activity against breast and prostate cancer cells (27, 41). The addition of six fluorines to the end of the side chain (C-26,27-F₆) markedly enhanced the range and potency of the analogues (24–27, 41, 42). In particular, 1,25(OH)₂-16-ene-23-yne-19-nor,26,27-F₆D₃ is one of the most potent vitamin D₃ analogues in its ability to inhibit clonal proliferation of breast and prostate cancer cells as well as leukemic cells. But a major toxicity with these fluorine-substituted analogues was their potent induction of hypercalcemia in experimental animals (23, 24).

In this study, we evaluated in detail a compound from the newly synthesized family of analogues having a C-5,6-trans motif. The 1,25(OH)₂-16-ene-5,6-trans-D₃ strongly inhibited clonal proliferation of each of the cell lines (ED₅₀: HL-60, 3.0 × 10⁻¹¹ M; MCF-7, 4.3 × 10⁻⁹ M; LNCaP, 1.4 × 10⁻⁹ M). It was approximately 10–
100-fold more active than 1,25(OH)D₃. In contrast, the 1,25(OH)₂16-ene-5,6-trans-D₃ inhibited cell growth almost to the same degree as 1,25(OH)D₃. The 1,25(OH)₂16-ene-D₃, which has a 16-ene motif, was almost 1000-fold more potent than 1,25(OH)D₃ in HL-60 cells as described previously (22). It was ~1000-fold more potent than 1,25(OH)D₃ against breast (MCF-7) and prostate (LNCaP) cancer cell lines. These findings show the importance of the 16-ene motif for antiproliferative activity toward cancer cells.

The dose-limiting toxicity of vitamin D₃ compounds is hypercalcemia. The calcium studies in mice highlighted the importance of the 5,6-trans motif. The 1,25(OH)₂16-ene-5,6-trans-D₃ had very weak calcemic effects, causing no hypercalcemia at week 5 of administration of 4.0 µg, which was given i.p. three times per week. When the dose of the analogue was increased to 6.0 µg, serum calcium levels became elevated at approximately 11 mg/dl on week 5. In contrast, 1,25(OH)D₃ and 1,25(OH)₂16-ene-D₃ at a 40-fold lower dose (0.1 µg/mouse) induced hypercalcemia. Thus, the addition of the 5,6-trans motif to the 16-ene-containing analogue plays an important role in reducing the calcemic activity of the vitamin D₃ analogues.

Although the addition of the 5,6-trans motif decreased the calcemic potential of the 16-ene-analogue, it also decreased its antiproliferative potency against the target cancer cell lines. To compare the ability of the compounds to inhibit clonal growth and to cause hypercalcemia, we calculated the relative therapeutic potency of the compounds (Table 2). When the results were standardized for their potential to cause hypercalcemia, 1,25(OH)₂16-ene-5,6-trans-D₃ showed a 2.5- and 1.4-fold greater therapeutic index than 1,25(OH)₂16-ene-D₃ for growth inhibition of leukemic cells (HL-60) and prostate cancer cells (LNCaP), respectively. For the breast cancer cells (MCF-7), however, 1,25(OH)₂16-ene-5,6-trans-D₃ had a 4.5-fold lower therapeutic potency than 1,25(OH)₂16-ene-D₃.

We explored the mechanism by which the vitamin D₃ analogues decreased the clonal growth of cancer cells. The vitamin D₃ compounds, including 1,25(OH)D₃, increased the number of MCF-7 cells in G₁ and decreased those in S phase. Recently, studies reported that the CDKIs, p21waf1 and p27kip1, mediated the G₁ arrest induced by 1,25(OH)D₃ in HL-60 cells (43, 44). We have shown previously that vitamin D₃ compounds induced increased expression of p27kip1 and p21waf1 in several cancer cell lines (26, 27). In this study, 1,25(OH)₂-
16-ene-5,6-trans-D_3 markedly enhanced expression of p27kip1 and p21waf1 in MCF-7 cells and 25(OH)D_3 expression in HL-60 cells. These results were consistent with the hypothesis that the p27kip1 and p21waf1 mediated at least in part the antiproliferative effects of the vitamin D_3 compounds by producing a G_1-S phase block of the cell lines.

Furthermore, pulse-exposure of MCF-7 breast cancer cells to 1,25(OH)_2-16-ene-5,6-trans-D_3 (10^-7 M; 4 days), followed by extensive washing, plating in soft agar, and enumerating colony formation at 14 days resulted in a 40% inhibition of colony formation. These results showed that 1,25(OH)_2-16-ene-5,6-trans-D_3 inhibited growth of these cancer cells by a mechanism other than one that was merely cytostatic, and only a relatively brief exposure (4 days) was required to cause this growth suppression. This suggests that brief pulse exposures in vivo might suffice for a cancer-suppressive effect.

Telomere length correlates closely with cellular senescence. Cellular senescence appears to be impaired in telomerase-deficient mice (45). Studies have observed that several reagents reduced the telomerase activity during the induction of terminal differentiation of leukemia cell lines (34–36). In this study, the TRAP assay showed that 1,25(OH)_2-16-ene-5,6-trans-D_3 (10^-8 M; 4 days) almost completely inhibited telomerase activity in HL-60 cells; and within the limits of the TRAP assay, 1,25(OH)_2-16-ene-5,6-trans-D_3 was more potent than 1,25(OH)_2D_3. H_TERT is probably the human telomerase catalytic subunit (37–40), the expression of which significantly correlates with telomerase activity in malignant cell lines and cancer tissue (46, 47). hTERT expression in tumor cells was down-regulated by several inducers of differentiation [retinoic acid (39) or phorbol diester (48)]. We have found that vitamin D_3 compounds caused the down-regulation of hTERT mRNA expression, and this correlated with down-regulation of telomerase activity. These observations suggest that vitamin D_3 compounds inhibit telomerase activity by reducing hTERT mRNA expression. We do not know if this marked decrease in telomerase activity is the cause or the result of CDKI-induced inhibition of growth and terminal differentiation of HL-60 cells.

Taken together, the new vitamin D_3 compound 1,25(OH)_2-16-ene-5,6-trans-D_3 strongly inhibited cell proliferation, caused a G_1-G_0 block in the cell cycle associated with an elevation of expression of several CDKIs, and markedly decreased telomerase activity; and yet, it had extremely low calcemic activity. This analogue should be studied in vivo with several cancer models as a prelude to possible future clinical trials.

ACKNOWLEDGMENTS

We thank Chloe Koeffler and Seth Robinson for enthusiastic help. We also thank Patricia Lin (Flow Cytometry Core Facility, Cedars-Sinai Medical Center, Los Angeles, CA) for generous technical assistance and Kim Burgin for excellent secretarial help.

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