Myeloma Cell Growth Arrest, Apoptosis, and Interleukin-6 Receptor Modulation Induced by EB1089, a Vitamin D3 Derivative, Alone or in Association With Dexamethasone

By Denis Puthier, Régis Bataille, Sophie Barillli, Marie-Paule Mellerin, Jean-Luc Harousseau, Anne Ponzio, Nelly Robillard, John Wijdenes, and Martine Amiot

We have previously shown that malignant plasma cells expressed the specific receptor for 1,25-dihydroxyvitamin D3 and that this derivative could significantly inhibit the proliferation of such malignant cells. More recently, new vitamin D3 derivatives have been generated with extraordinarily potent inhibitory effects on leukemic cell growth in vitro. These new data prompted us to (re)investigate the capacity of such new vitamin D3 derivatives to inhibit myeloma cell growth in comparison with that of dexamethasone, a potent antitumor agent in multiple myelomas. In the current study, we show that EB1089, a new vitamin D3 derivative, (1) induces G1 growth arrest of human myeloma cells, which is only partially reversed by interleukin-6 (IL-6); (2) induces apoptosis in synergy with dexamethasone, IL-6, leukemia-inhibitory factor, and Oncostatin M, with an agonistic anti-gp130 monoclonal antibody being unable to prevent this apoptosis; (3) downregulates both the gp80 (ie, the α chain of the IL-6 receptor [IL-6Rα] expression on malignant plasma cells and the production of soluble IL-6Rα, and finally (4) inhibits the deleterious upregulation of gp80 expression induced by dexamethasone while limiting the dexamethasone-induced upregulation of gp130 expression. Considering that these in vitro effects of EB1089 have been observed at doses obtainable in vivo (without hypercalcemic effects), our present data strongly suggest that EB1089 could have a true interest in the treatment of multiple myeloma, especially in association with dexamethasone.

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MATERIALS AND METHODS

Reagents. Dexam was obtained from Merck Sharp USA (Paris, France). 1,25(OH)2D3 and the derivative EB1089 were obtained from Leo Pharmaceuticals (Ballerup, Denmark). Anti-gp80 MoAb (MT18) and anti-gp130 MoAb (AM64) were provided by Dr Taga (Institute for Molecular and Cellular Biology, Osaka University, Osaka, Japan). The agonistic anti-gp130 MoAb (BS12) was kindly provided by Dr J. Wijdenes (Diaclone Research, Besançon, France). Human recombinant IL-6 and Oncostatin M (OSM) were obtained from Sandoz (East Hanover, NJ) and Dako (Glostrup, Denmark), respectively. Leukemia-inhibitory factor (LIF) was kindly provided by Dr A. Godard (U211, Nantes, France).

HMCL and culture conditions. The HMCL LP1 and NCI H929 were purchased from DSM (Braunschweig, Germany), and RPMI 8226 was from American Type Culture Collection (Rockville, MD). They were maintained in RPMI-1640 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 μg/mL streptomycin, 100 μU/mL penicillin, 5 μg/mL gentamicin, and 10 ng/mL interleukin-6 (IL-6). RPMI-1640 supplemented with 10% fetal calf serum, 2 mmol/L glutamine, 100 μg/mL streptomycin, 100 μU/mL penicillin, 5 μg/mL gentamicin, and 10 ng/mL interleukin-6 (IL-6).

Freshly expanded, pure, and slowly growing, 6-month-old myeloma cells were obtained from Sandoz (East Hanover, NJ) and Dako (Glostrup, Denmark), respectively. Leukemia-inhibitory factor (LIF) was kindly provided by Dr A. Godard (U211, Nantes, France).

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after Ficoll-Hypaque separation from the peripheral blood of a patient with MM during a terminal leukemic phase.

Proliferation assays. Proliferation assays were performed in 96-well round-bottom microtiter plates at a cell density of 10⁵ cells/mL. As previously described, 12 cell cultures were incubated for 3 days at 37°C in a 5% CO₂ humidified atmosphere. Then, 1 mCi of [³H]-thymidine was added during the last 18 hours. [³H]-thymidine incorporation was quantified by liquid scintillation spectroscopy.

BM stromal cell culture. BM mononuclear cells were isolated by ficoll-hypaque density sedimentation. The cells were cultured in RPMI-1640 supplemented with 10% fetal calf serum. After 2 or 3 weeks, a confluent adherent cell monolayer was obtained. Then, after 2 passages using trypsin/EDTA solution, they were used for cell cycle analysis. LP1 cells (1 x 10⁶) were fixed in 70% cold ethanol for 30 minutes at 4°C, washed twice in phosphate-buffered saline, and stained with 40 μg/mL propidium iodide for 10 minutes at room temperature. Flow cytometry analysis was performed on a FACSCalibur using CELLQuest program (Becton Dickinson). Data were gated on the Fl2-Area versus Fl2-Width cytogram to exclude doublets and aggregates, and a minimum of 1.5 x 10⁴ gated cells were collected per sample. Analysis of the cell cycle was performed using the Modfit LT for Mac V1.01 program (Verity Software House, Inc). Apoptotic cells were detected as a subdiploid peak as described.

Statistical methods. For statistical analyses, we used the Wilcoxon rank sum test.

RESULTS

Inhibition of myeloma cell growth by 1,25(OH)₂D₃: Comparison with the new vitamin D₃ derivative EB1089.

![Graphs showing proliferation and growth inhibition](image-url)
INHIBITION OF MYELOMA CELL GROWTH BY A VITD~ DERIVATIVE 4661

Fig 2. Comparative effect of Dex and EB1089 on growth inhibition of the myeloma cell lines. Dex was used at a concentration of 10^{-7} mol/L, and EB1089 was used at a concentration of 10^{-8} mol/L. DNA synthesis was measured by [3H]-TdR incorporation after a 3-day culture.

EB1089 is a new vitamin D derivative with potent inhibitory effects on leukemic cell growth in vitro. Thus, it was tested on the three HMCLs and the fresh IL-6-dependent myeloma cells SBN1 and compared with 1,25-(OH)_{2}D_{3}. As previously emphasized by ourselves on RPMI 8226 and as outlined in Fig 1, 1,25-(OH)_{2}D_{3} inhibits not only the growth of the three HMCLs but also that of the IL-6-dependent SBN1 cells in a dose-dependent way. However, whereas a progressive inhibition is observed with RPMI 8226 with significant effects at doses as low as 10^{-12} mol/L, no effect is observed with LP1, NCI H929, and SBN1 cells until a dose of 10^{-10} mol/L is used, with significant effects beginning at 10^{-9} mol/L. Of note, EB1089 gives similar response curves but turns out to be more efficient than 1,25-(OH)_{2}D_{3} on the three HMCLs and SBN1 cells at least at 10^{-8} and 10^{-9} mol/L (Fig 1). This is especially true for LP1 and SBN1 cells (54% and 51% inhibition, respectively, with EB1089 vs 7% and 3%, respectively, with vitamin D_3 at 10^{-9} mol/L). Of major interest and as previously emphasized, the inhibition of SBN1 is observed in the presence of rIL-6 because the growth of these cells (as opposed to that of the other HMCLs) is totally dependent on IL-6. The concentration of 10^{-8} mol/L was chosen for the next experiments performed with EB1089 because a maximum of inhibitory effects is observed at this concentration (71% of inhibition for LP1, 58% for NCI H929, 42% for RPMI 8226, and 73% for SBN1 cells).

Inhibition of myeloma cell growth by EB1089: Comparison with Dex. As previously emphasized\(^6\) and as recently reassessed by ourselves,\(^16\) Dex is a potent inhibitor of myeloma cell growth in vitro. In our experience, a \(\simeq 20\%\) inhibition of growth is observed in six of six HMCLs independent of exogenous IL-6 to grow (the current study and Juge-Morineau et al\(^15\)). For treatment in vivo, Dex is used at a concentration of 10^{-7} mol/L;\(^13\) this concentration has also been chosen for the following experiments. As shown in Fig 2, Dex significantly inhibits the growth of LP1 and NCI H929 (99% and 58% inhibition, respectively) but has a less significant effect on RPMI 8226. Again, EB1089 at 10^{-8} mol/L has inhibitory effects on the three cell lines. Clearly, EB1089 alone is less inhibitory than Dex on LP1 (71% vs 99%). However, when compared with Dex, it is as efficient on NCI H929 and more efficient on RPMI 8226. Of note, the effect of the combination of EB1089 and Dex is always superior to the effect of each one alone, and, on RPMI 8226, does EB1089 have additive effects to those of Dex. With respect to the SBN1 cells, which are totally dependent on IL-6 to grow, it is impossible to inhibit their growth with Dex in the presence of IL-6. On the other hand, and as indicated in the previous section (Fig 1), a significant inhibition of these cells is observed with EB1089, despite the presence of IL-6. Finally, the combination of EB1089 and Dex has no greater effect than that of EB1089 alone (data not shown).

Table 1. Effects of Dex and EB1089 on the Cell Cycle Distribution of LP1 Cells

<table>
<thead>
<tr>
<th>Drug Exposure*</th>
<th>G1 (%)</th>
<th>S (%)</th>
<th>G2 (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>43.3 ± 2.2</td>
<td>40.6 ± 3.5</td>
<td>15.3 ± 2.3</td>
</tr>
<tr>
<td>Dex</td>
<td>90.2 ± 1.8</td>
<td>6.0 ± 1.3</td>
<td>3.7 ± 0.8</td>
</tr>
<tr>
<td>EB1089</td>
<td>73.91 ± 5.7</td>
<td>15.3 ± 4.2</td>
<td>11.47 ± 2.0</td>
</tr>
<tr>
<td>Dex + EB1089</td>
<td>94.0 ± 2.1</td>
<td>3.5 ± 1.3</td>
<td>2.7 ± 0.7</td>
</tr>
</tbody>
</table>

* Two-day drug exposure. Dex and EB1089 were used at 10^{-7} mol/L and 10^{-8} mol/L, respectively.
† The percentage of cells in different phases of the cycle (among nonapoptotic cell population) after treatment with the different drugs is presented.

Data are expressed as the mean ± SD of five experiments.
EB1089 induces G1 growth arrest by itself but induces apoptosis in synergy with Dex. When the cell cycle was analyzed, EB1089 turned out to have potent cytostatic effects without inducing apoptosis in our experimental conditions at 3 days. Indeed, in five experiments, a significant reduction of the percentages of myeloma cells in S phase was observed (40.6% ± 3.5% vs 15.3% ± 4.2%; P = .01), with a significant increase of the percentages of myeloma cells in the G1 phase (43.3% ± 2.2% vs 73.9% ± 5.7%; P = .01; see Table 1). Of note, these effects are similar to although less marked than those of Dex as shown in the Table 1.

Flow cytometry analysis of the DNA content indicates that EB1089 or Dex used alone cannot trigger apoptosis of myeloma cells in our current experimental conditions within 3 days. On the contrary, treatment of LP1 cells with a combination of the two drugs leads to a strong induction of apoptosis that is shown after 2 days by the appearance of a hypodiploid DNA peak that identified the apoptotic cell population (29%), as shown in the typical experiment represented in Fig 3.

IL-6, LIF, OSM, and an agonistic anti-gp130 MoAb cannot prevent the apoptotic effects of EB1089 with Dex. We12 and others13 have recently shown that IL-6, even at low doses (10 pg/mL), and some other cytokines belonging to the gp130 family such as LIF and OSM could be the major factors responsible for the resistance of myeloma cells to Dex. Because EB1089 has cytostatic effects on myeloma cells too, we have examined the capacity of IL-6, LIF, and OSM to reverse these effects in comparison with those of Dex. As shown in Fig 4A, on LP1 cells, IL-6 only partially reverses the inhibitory effects of EB1089, whereas it reverses totally those of Dex. On the other hand, LIF and OSM totally reverse the inhibitory effects of both EB1089 and Dex. However, because EB1089 and Dex have potent synergistic effects to induce apoptosis of myeloma cells, we have examined the capacity of IL-6, LIF, and OSM to prevent these effects. As shown in Fig 4A, IL-6, LIF, and OSM have only minimal effects to prevent those of EB1089 in combination with Dex. Of note, IL-6 at doses as high as 20 ng/mL (which are generally encountered in the myeloma intermediate milieu) are unable to prevent the effects of both EB1089 and Dex (data not shown). BS12 has been described as an agonistic anti-gp130 MoAb with IL-6-like effects on HMCLs.14 In the current study, such agonistic effects have been reproduced on LP1 (Fig 4B), with the BS12 MoAb being able to reverse the inhibitory effect of Dex better than IL-6. Of major interest, BS12 is unable to prevent the effects induced by the combination of EB1089 and Dex, although it partially reverses those of EB1089 alone (Fig 4B).

EB1089 downregulates the expression of the gp80 on myeloma cells and reduces the production of soluble IL-6Ra. Considering that IL-6 and BS12 only partially reverse the inhibitory effects of EB1089, we have investigated the effect of EB1089 on the expression of both gp80 and gp130 in
INHIBITION OF MYELOMA CELL GROWTH BY A VIT D \textsubscript{3} DERIVATIVE

**Fig 4.** (A) Effect of IL-6, LIF, and OSM on the inhibitory effects induced by Dex, EB1089, and Dex plus EB1089. Cells \(10^6/\text{well}\) were cultured for 72 hours in the different conditions as indicated. Dex and EB1089 were used at the concentration of \(10^{-7}\) and \(10^{-8}\) mol/L, respectively. IL-6 was added at 125 pg/mL and LIF and OSM were added at 10 ng/mL. (B) Effects of BS12, an agonist anti-gp130 MoAb, on the inhibitory effects induced by Dex, EB1089, and Dex plus EB1089. Dex and EB1089 were used at the concentrations of \(10^{-7}\) and \(10^{-8}\) mol/L, respectively. BS12 was added at 0.1 \(\mu\text{g/mL}\).

**DISCUSSION**

The generation of new vitamin D\(_3\) derivatives such as EB1089 with potent antileukemic effects in vitro\(^5\) at least prompted us to investigate them on human myeloma cells, considering the presence of specific receptors for vitamin D\(_3\) on such tumor cells.\(^8\) In the current study, we have shown that 1,25(OH)\(_2\)D\(_3\) and EB1089, a new vitamin D\(_3\) derivative, inhibit the growth of all myeloma cells tested, even those growing in the presence of IL-6, a major survival and growth factor for myeloma cells.\(^1,2\) EB1089 is clearly more potent than 1,25(OH)\(_2\)D\(_3\) and is significantly efficient at \(10^{-9}\) and \(10^{-8}\) mol/L on the three HMCLs (even at \(10^{-12}\) mol/L on RPMI 8226) and on fresh proliferative myeloma cells. The efficacy of EB1089 was compared with that of Dex, first, because Dex is well known to exert an inhibitory effect on the proliferation of myeloma cells in vitro\(^10,12,14,17-19\) and, second, because Dex is a steroid of reference in the treatment of MM.\(^11\) In the current study, on three HMCLs independent of exogenous IL-6 administration, EB1089 was clearly less efficient than Dex on LP1, the HMCL most sensitive to Dex, but was as efficient on NCI H929 and was more efficient on RPMI 8226. On the other hand, Dex lacked any inhibitory effect on SBN1 cells, which are myeloma cells totally dependent on IL-6 for growth, whereas EB1089 had significant inhibitory effects. The lack of correlation between the effects of EB1089 and those of Dex suggests that both drugs have different mechanisms of action. This is highly likely because it has been shown on a human breast cancer cell line that the antiproliferative effects of EB1089 might be related to promoter selectivity and were not mediated by inhibition of
the AP-1 pathway, as opposed to those of Dex.\textsuperscript{20} The density of vitamin D\textsubscript{3} receptors and their upregulation by Dex\textsuperscript{22} could also be critical to the explanation of some of the differences of sensitivity we observed between the HMCLs. These critical points deserve further investigation.

Our cell cycle analysis has shown that EB1089, similar to Dex, was able to induce G1 growth arrest by itself, as recently published for 1,25(OH)\textsubscript{2}D\textsubscript{3}.\textsuperscript{22} Of note, whereas the Dex-induced G1 growth arrest was reversed by low doses of IL-6, IL-6 only partially reversed the G1 growth-arrest induced by EB1089. We must emphasize that, in our experimental conditions, within 3 days, neither EB1089 or Dex alone was able to induce apoptosis. When we tried to understand why IL-6 only partially reversed the G1 growth arrest induced by EB1089 while it easily reversed the growth arrest induced by Dex, we discovered that EB1089 downregulated both gp80 expression and the production of the soluble IL-6Ra, which is a potent agonist of IL-6. These data are remi-

\begin{table}
\centering
\begin{tabular}{|c|c|c|c|c|}
\hline
Drug & LP1 & Patient 1 & \hline
\hline
Control & gp80 & 2.15 ± 0.15 & 2.58 ± 0.26 & 2.49 ± 0.02 & 1.48 ± 0.07 \\
 & gp130 & 2.49 ± 0.02 & 1.48 ± 0.07 & & \\
Dex & gp80 & 3.75 ± 0.27 & 6.96 ± 0.29 & 4.25 ± 0.04 & 2.1 ± 0.12 \\
 & gp130 & 4.25 ± 0.04 & 2.1 ± 0.12 & & \\
EB1089 & gp80 & 1.38 ± 0.01 & 2.39 ± 0.19 & 1.96 ± 0.04 & 1.21 ± 0.12 \\
 & gp130 & 1.96 ± 0.04 & 1.21 ± 0.12 & & \\
Dex & gp80 & & & & \\
 & gp130 & & & & \\
EB1089 & 1.55 ± 0.24 & 5.37 ± 0.14 & 3.47 ± 0.13 & 1.77 ± 0.15 \\
 & gp130 & 3.47 ± 0.13 & 1.77 ± 0.15 & & \\
\hline
\end{tabular}
\caption{Effects of Dex and EB1089 on the IL-6Ra (gp80) and \( \beta \) (gp130) Expression Analyzed by Flow Cytometry}
\end{table}

Fig 5. (A) Regulation of gp80 on LP1 after treatment with EB1089 or Dex or EB1089 plus Dex for 24 hours: (\ldots), staining with an isotype control antibody; (\ldots), staining with MT18 MoAb without treatment; and (\ldots), staining with MT18 MoAb after the indicated treatment. (B) Regulation of gp130 on LP1 after treatment with EB1089 or Dex or EB1089 plus Dex for 24 hours: (\ldots), staining with an isotype control antibody; (\ldots), staining with AM64 MoAb without treatment; and (\ldots), staining with AM64 MoAb after the indicated treatment.

Fig 6. Northern blot analysis of gp80 expression on LP1 cells. Lane A, EB1089 treatment; lane B, Dex treatment; lane C, control.
niscient of those showing the downregulation of epidermal growth factor receptor levels by 1,25(OH)2D3 in human breast cancer cells. The regulation of some critical growth factor receptors could be a key physiological role of this hormone. In parallel, we also found that Dex could strongly upregulate both gp80 (and the production of soluble IL-6Ra) and gp130 expression, as previously observed on epithelial cells, hepatocytes, and osteoblasts and as recently shown on myeloma cells by Chen et al. However, we found that EB1089 could inhibit the upregulation of gp80 expression by Dex while significantly limiting that of gp130. Whereas Dex significantly inhibited the IL-6 production by stromal cells, EB1089 had a weak but significant stimulatory effect. However this effect is always annihilated in the presence of Dex. The fact that EB1089 could activate the IL-6 gene in stromal cells contrary to Dex is reminiscent of the IL-6 gene induction by 1,25(OH)2D3 in M1 cells. However, no activation was observed in the HMCLs. Whereas neither EB1089 or Dex alone was able to induce apoptosis, we discovered that EB1089 could induce apoptosis in about one third of myeloma cells in synergy with Dex. A striking observation is that IL-6 (at doses as high as 20 ng/mL), LIF, OSM, and even an agonistic anti-gp130 MoAb were unable to prevent it.

From a therapeutic point of view, all these observations are of major importance. Higher doses of EB1089 than those we have used in vitro can be achieved in vivo without any hypercalcemic effects. Although an excessive osteoclastic resorption is generally observed in patients with MM, the efficacy of EB1089 at 10^-9 mol/L or even at 10^-12 mol/L suggests that this drug could be used in vivo, especially if agents able to limit calcium uptake (glucocorticoids) and bone resorption (bisphosphonates) are associated. Although Dex has a clear-cut antitumoral effect in MM, some patients do not respond to this treatment, and finally all become resistant. There is more and more evidence that Dex inhibits the IL-6 gene transcription, at least in stromal cells and myeloma cells, by interacting directly with the autocrine/paracrine IL-6 network in MM. As a consequence, we and others have shown that low doses of IL-6 could counteract the antiproliferative effects of Dex on myeloma cells in vitro. Thus, in vivo, the overproduction of IL-6 observed in the microenvironment of patients with MM (ie, several nanograms per milliliter) could be a major cause for the resistance of myeloma cells to the inhibitory effects of Dex. Furthermore, its newly described effects on the IL-6Raβ expression (ie, upregulation) and production (ie, increase) of soluble IL-6Ra could lead to the selection of myeloma clones sensitive to low amounts of IL-6 to survive and to grow. In this context, the effects of EB1089, if observed in vivo could be of major therapeutic interest as a maintenance treatment. Because EB1089 (1) has potent cytostatic effects on human myeloma cells by itself even in presence of IL-6, (2) acts in synergy with Dex to induce unescapable apoptosis, and (3) inhibits both gp80 expression and production of the soluble IL-6Ra while counteracting the deleterious effects of Dex (ie, upregulation of both gp80 and gp130 expression), its association with Dex could be very helpful in the treatment of MM. Finally, it has been recently shown that the sensitivity of human myeloma cells to the cytotoxicity of immune cells inversely correlates with the degree of gp80 expression. In this context, EB1089 could facilitate the cytotoxicity of immune cells against myeloma cells.

Table 3. Effects of Dex and EB1089 on the Production of the Soluble IL-6Ra Determined by ELISA

<table>
<thead>
<tr>
<th>48-h Drug Exposure</th>
<th>Soluble IL-6Ra (ng/mL)</th>
<th>Exp 1</th>
<th>Exp 2</th>
<th>Exp 3</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4.6</td>
<td>5.6</td>
<td></td>
<td>11.5</td>
</tr>
<tr>
<td>Dex</td>
<td>5.3</td>
<td>7.3</td>
<td></td>
<td>16.9</td>
</tr>
<tr>
<td>EB1089</td>
<td>1.5</td>
<td>1.6</td>
<td></td>
<td>6.2</td>
</tr>
<tr>
<td>Dex + EB1089</td>
<td>1.6</td>
<td>1.9</td>
<td></td>
<td>6.3</td>
</tr>
</tbody>
</table>

Fig 7. IL-6 production by stromal cells derived from two myeloma patients. Stromal cells were cultured for 48 hours with EB1089, Dex, or a combination of Dex plus EB1089; then, the supernatant was collected for IL-6 determination by ELISA.

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