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POTENCY OF NOVEL 1,25 D3 ANALOGS ON INDUCING DIFFERENTIATION IN A HUMAN MYELOID LEUKEMIA CELL MODEL

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Objective:

The hormonally active form of Vitamin D, dihydroxyvitamin D_3 ($1,25D_3$), is known as an important factor for maintaining calcium homeostasis. This is accomplished through the regulation of genes in the intestine, kidney, and bone. $1,25D_3$ has also been identified as an important regulator of basic cellular processes such as proliferation and differentiation. The potency of $1,25D_3$ to induce differentiation and inhibit proliferation of different types of cancer cells has led to increasing research efforts leading to the design and investigation of $1,25D_3$ analogs that exploit these characteristics. Additionaly, analogs have been studied because the clinical use of 1,25 dihydroxyvitamin D_3 as a differentiation agent for treatment of myeloid leukemia is impeded by the development of toxic hypercalcemia and resistance to differentiation. In some studies, analogs reduced calcemic activities yet increased anti-proliferative and differentiation inducing properties, which raises the expectations for these analogs to be useful in the treatment of human neoplastic diseases. In this study we compared the abilities of ten novel analogs, BXL-0044, BXL-0045, BXL-0046, BXL-0053, BXL-0054, BXL-0055, RO-3884, RO-2198, PRI-2201, and PRI-2191 to induce markers (CD14, CD11b, and MSE) of differentiation, and molecular events associated in human promyeloblastic leukemia cells (HL60-G).

Methods:

Cell Culture: HL60-G cells were grown in RPMI 1640 medium supplemented with 10% BCS in a 37 ºC environment.

Proliferation and Viability: Total cells and non-viable cells were counted using Neubauer hemocytometer at a 1:1 dilution with Trypan blue (0.2%). Counts were repeated in duplicate.

Differentiation: 10^6 cells were harvested, washed and stained with MY4-RD1 (anti-CD14) and M01-FITC (anti-CD11b) antibodies from Beckman Coulter. Two parameter analysis was performed using a Beckman Coulter EPICs XL flow cytometer.

Cell smears were prepared with aliquots of 10^6 cells for cytochemical assay. Slides from each sample were stained for MSE, a monocyte specific esterase. Counts were repeated in triplicate.

RT-PCR: 10^6 cells were harvested and washed twice with cold 1 x PBS. Total RNA extracted with Qiagen RNeasy mini kit and treated with Rnase-free DNase following manufacturer's protocol. GeneAmp PCR core kit and AmpliTaq gold polymerase enzyme was used. Primers were as follows: (1) CYP24 upstream (5'CTCATGCTAAATACCCAGGTG 3') and downstream (5'AGGCGTATTATCCGCTGGCAAA 3'), (2) C/EPB β upstream primer (5-GTTCTTGACGTTCTTCGGCCG-3) and C/EPB β downstream primer (5-TGGACAAGCACAGCGACGAGT-3), (3) β-actin upstream (5'

TGACGGGGTCACCCACACTGTGCCCAGCTA 3') and downstream (5'CTAGAAGCATTTGCCGGTGGACGATGGAGGG 3').

250 ng total RNA was reverse transcribed following manufacturer's protocol, using MuLV RT and oligo d(T) as primers. PCR reaction in Eppendorf thermal cycler with settings, 95°C for 12', 40 and 35 cycles of [95°C: 45s, T_m: 45s, 72°C: 45s], and 72°C: 7'. Bands visualized with EtBr on 1.5% agarose gel, scanned by Typhoon image scanner. Optical density calculated with ImageQuant software.

Summary:

We found that the potencies of the analogs to induce differentiation paralleled their ability to increase expression of CAAT/ Enhancer Binding Protein (C/EBP β) and 1,25D_3 24-hydroxylase (CYP24). Analysis of CYP24 mRNA and C/EBP β, by RT-PCR demonstrated a significant increase in this gene transcript when HL60 cells were treated with the most potent analogs. The data suggest that novel BXL analogs 53,55 and Roche analog Ro-2198 are more potent than 1,25D_3 at inducing markers of differentiation, while BXL-0054 was found to be equally as potent as 1,25D_3 **(See Figure 1-3)**. A comparison of structure showed that analogs that are more potent than 1,25 D_3 contain a gemini configuration at C20, 26, 27-hexafluoro substitutions, 23-double bond, and 23-triple bond are less susceptible to 24 hydroxylation (inactivation). Our data suggest that enhanced potency of the novel analogs is at least partly due to increased metabolic stability of the analogs, resulting in more elevated levels of transcription.

Conclusion:

Gemini analogs containing a 23-triple bond and a 26,27-hexafluoro substitutions have enhanced the potency of the analog in comparisn to 1,25$(OH)_2$ D_3. These types of analogs also exhibit enhanced trancriptional activity *in vitro* and induce greater or equal cellular differentiation then 1,25$(OH)_2$ D_3.

Introduction of a 23-double bond and a 26,27-hexafluoro substitutions has proven to be the most effective at enhancing the potency of these analogs in both cellular differentiation and transcriptional expression.

Based on the modifications introduced to block 24 hydroxylation in combination with an increased ligand binding from the 26,27-hexafluoro substitutions, it can be concluded that ligand stability is most likely the main contributor to the observed increase in potency.

The analogs tested in this study have the potential to be antineoplastic agents if they are found to have little to no hypercalcemic affect. For this reason, further studies testing the calcemic affects of these analogs will provide more insight into the usefulness of each compound.

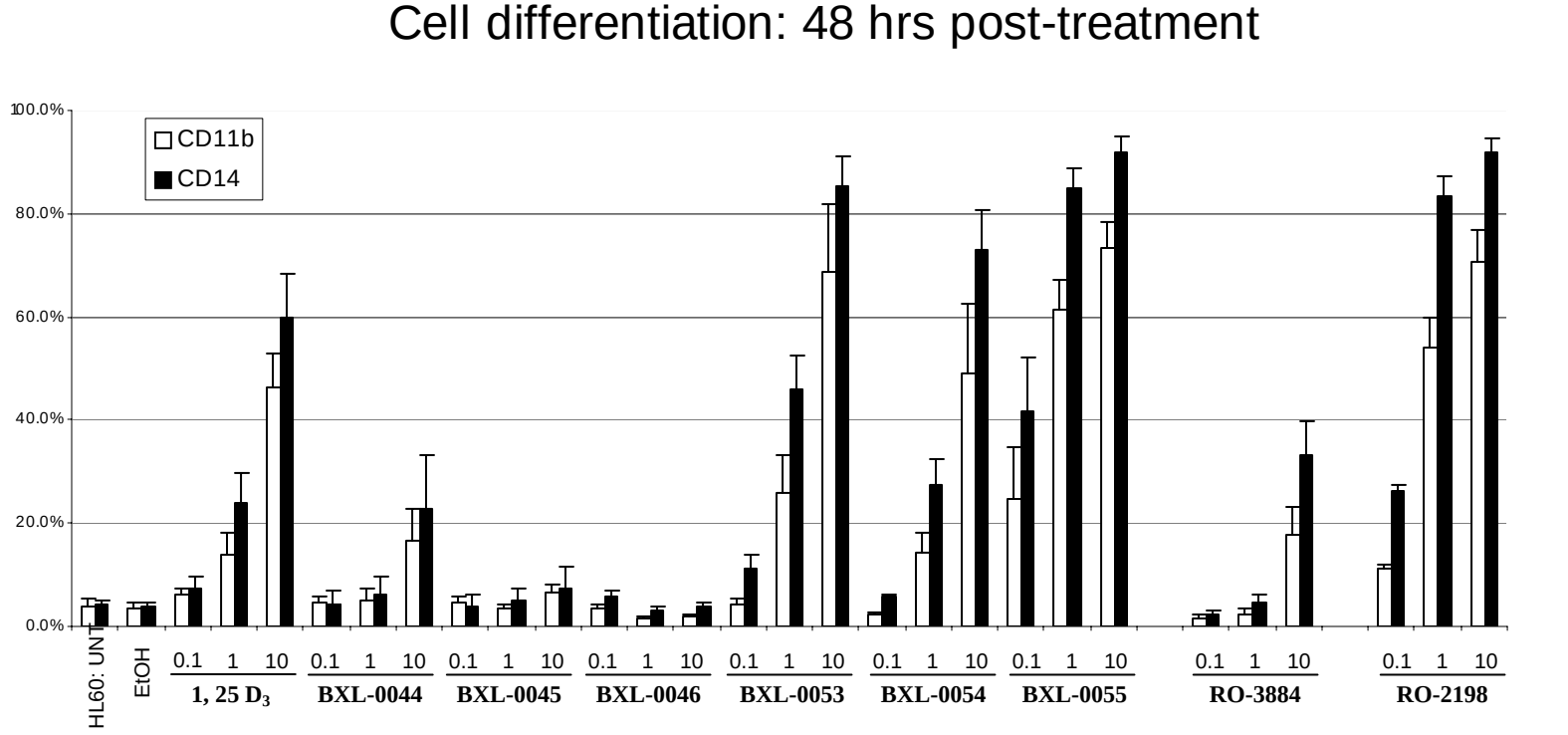

Figure1: Analysis of markers of monocytic differentiation by FACS. HL60-G cells expressed equal amounts of CD11b and CD14 when left untreated or treated with EtOH. When treated with 1,25 D3 a gradual increase in the expression of CD11b and CD14 was observed with increasing concentrations (0.1nM, 1nM, and 10nM). BXL 53, 55 and RO-2198 are more potent at inducing expression of CD11b/CD14, than 1,25 D3 at 0.1nM, 1nM, and 10nM concentrations.

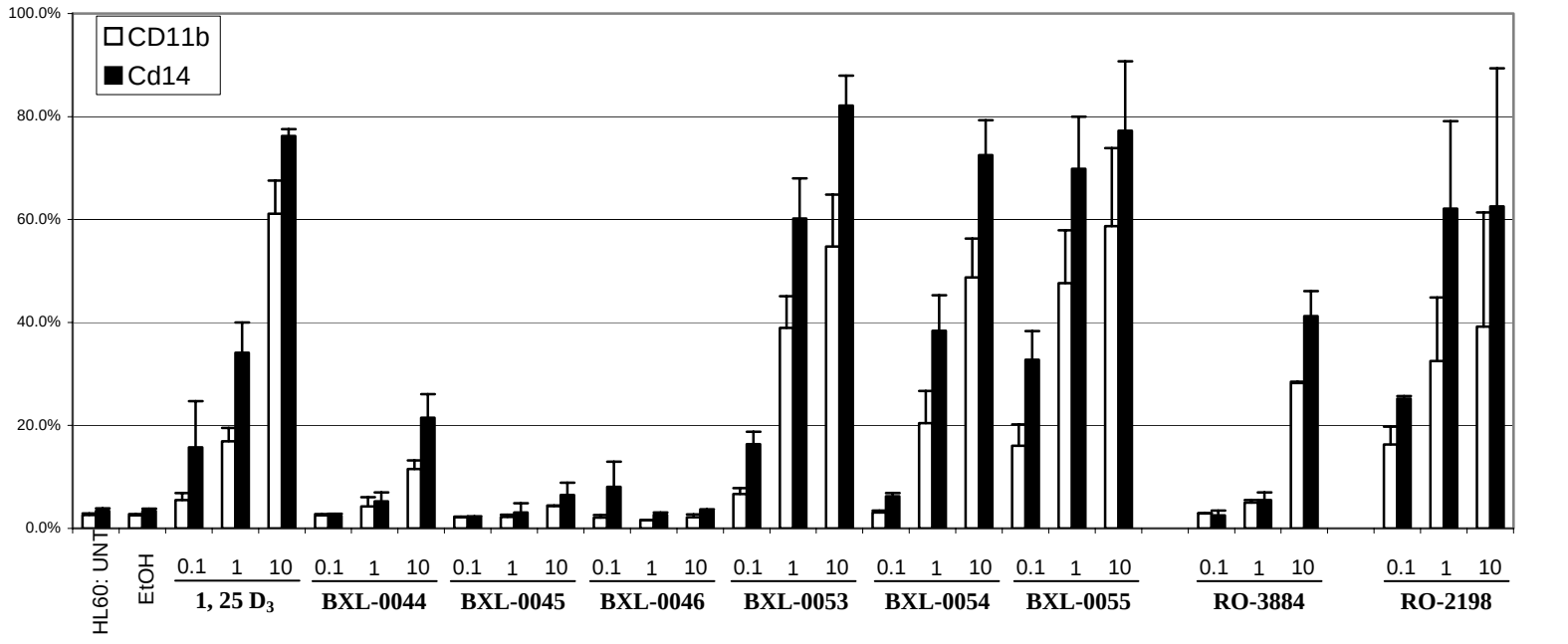

Figure 2: Analysis of markers of monocytic differentiation by FACS. Untreated and EtOH treated HL60-G cells, expressed similar levels of CD11b and CD14. When treated with 1,25 D3 a gradual increase in the expression of CD11b and CD14 was observed with increasing concentrations. BXL 53 and 55 were more potent and inducing differentiation of CD11b/CD14 than 1,25 D3 at all concentrations, while BXL 54 and RO-2198 were equally as potent as 1,25 D3.

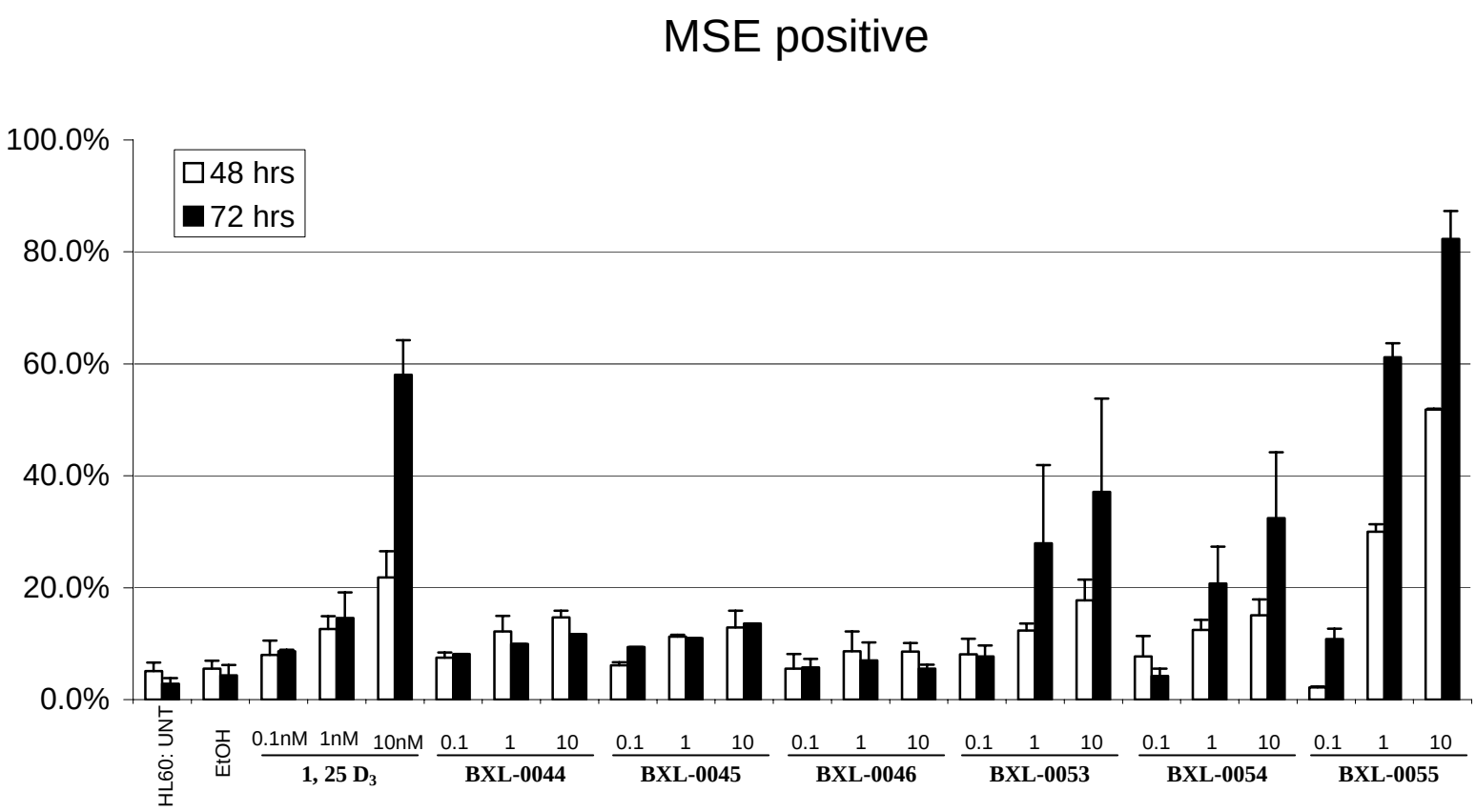

Figure 3: MSE Analysis of Cell Differentiation. The untreated and EtOH samples of HL60-G cells showed an equal percentage of cellular differentiation, which indicates no influence of vehicle. Beginning at 48 hours, an increase in MSE positivity was observed, with 10nM 1,25 D3, indicating an effective induction of MSE differentiation by parent compound. When compared to 1,25 D3, BXL 55 was the most potent in inducing differentiation of MSE starting at 48 hours. Analog BXL 53 and 54 induced an elevation of differentiation equal to 1,25 D3, indicating equal potency to 1,25 D3 on cellular differentiation.