Research Article

MART-10, a New Generation of Vitamin D Analog, Is More Potent than 1α,25-Dihydroxyvitamin D₃ in Inhibiting Cell Proliferation and Inducing Apoptosis in ER+ MCF-7 Breast Cancer Cells

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1. Introduction

Breast cancer ranks first globally among the most commonly diagnosed and cancer-related deaths in women [1]. Over 1.38 million new breast cancer cases and 458,400 breast cancer-related deaths have been reported worldwide in 2008. Estrogen receptor (ER), which is present in nearly 70% of all breast cancer patients, plays a crucial role in the progression of breast cancer [2]. Thus, ER antagonists, tamoxifen and raloxifene, have been widely used to treat breast cancer and have contributed to a better prognosis for ER positive (ER+) breast cancer. However, only a 50% reduction in tumor relapse has been achieved by ER antagonist therapy [3]. Furthermore, the antagonists have serious side effects on bone [4], which highlights the necessity of seeking alternative treatments for ER+ breast cancer.

Vitamin D is well known as a modulator of calcium and bone metabolism. For the past three decades, abundant
evidence has been accumulated to indicate that the active form of vitamin D, 1α,25-dihydroxyvitamin D₃, 1α,25(OH)₂D₃, or calcitriol, possesses many actions not associated with calcium and bone metabolism [5]. They include antiproliferation, antiangiogenesis, proapoptosis, prodifferentiation, and immune regulation in a cell- and tissue-specific manner [5–9].

1α,25(OH)₂D₃ exerts its effects through binding to vitamin D receptor (VDR). The receptor is expressed in most human cancer cell lines and its growth can be inhibited by 1α,25(OH)₂D₃ [10–14]. However, the clinical application of 1α,25(OH)₂D₃ is hindered by its lethal hypercalcemic side-effect after its systemic administration at a concentration sufficient to inhibit tumor cell growth [15]. To overcome this drawback, thousands of vitamin D analogs have been synthesized aiming to minimize its calcemic side effect while maintaining or even potentiating the antitumor activities [16, 17].

For breast cancer, 1α,25(OH)₂D₃ and its analogs, including EB1089, ILX 23-7533, and 22-oxa-1α,25(OH)₂D₃, have been shown to be effective in suppressing breast cancer cell growth in vitro and in vivo either alone or in combination with other drugs [18]. However, no significant benefit on survival has been observed in clinical trials [19, 20].

MART-10 (19-nor-2α-(3-hydroxypropyl)-1α,25-dihydroxyvitamin D₃) [21] has been shown to be more active in VDR transactivation [22]. Most importantly, MART-10 is far more potent in inhibiting liver and prostate cancer cell proliferation [11, 22, 23] and prostate cancer cell invasion [24], and it did not raise serum calcium in vivo in an animal model [24]. These findings suggest that MART-10 could be a good candidate for breast cancer treatment. We, therefore, study the antiproliferative and proapoptotic effects of MART-10 in ER+ MCF-7 breast cancer cells and the potential mechanisms involved.

2. Materials and Methods

2.1. Vitamin D Compounds. 1α,25(OH)₂D₃ was purchased from Sigma (St. Louis, MO, USA). MART-10 was synthesized as previously described [21].

2.2. Cell Culture. Human breast cancer cell lines, MCF-7 and MDA-MB-231, were obtained from Bioresource Collection and Research Center (BCRC, Taiwan). Both MCF-7 and MDA-MB-231 cells were grown in DMEM (Sigma) supplemented with 5% fetal bovine serum (FBS). Culture medium was changed 3 times per week.

2.3. Cell Proliferation Assay by Cell Number Counting. Cell counting was conducted using a hemocytometer as previously described [11]. Cells were treated every two days and counted on day 7.

2.4. Western Blot for Protein Expression. The procedures for protein extraction, blocking, and detection were described previously [11]. The primary antibodies used in this study were monoclonal antibodies against VDR (D-6, Santa Cruz Biotechnology, Santa Cruz, CA, USA), p21 (2946, Cell Signal, Beverly, MA, USA), p27 (3698, Cell Signal), cytochrome C (clone 7H8.2C12, BD Biosciences Pharmingen), Bax (554104, BD), and Bcl-2 (50-729, Millipore, Bedford, MA, USA). The secondary antibodies (1 : 5000) were anti-rabbit (111-035-003, Jackson Immunoresearch, West Grove, PA, USA) or anti-mouse secondary antibodies (Zymed 81-6520). The blots were detected using ECL reagents (WBKLS0500, Millipore, Billerica, MA, USA). Membranes were detected by VersaDoc Imaging System (Bio-Rad, Hercules, CA, USA) for analysis.

2.5. Cell Cycle Analysis by Flow Cytometry. Flow cytometry for cell cycle analysis was performed using a FACScalibur (BD Biosciences, San Jose, CA, USA) as described previously [11, 25]. Briefly, after exposure for two days to indicated concentrations of 1α,25(OH)₂D₃, the cells were collected and fixed in ice-cold 75% ethanol at 20°C overnight. The fixed cells were stained in propidium iodide (PI) buffer containing 100 mM sodium citrate, 0.1% Triton X-100, 0.2 mg/mL RNase, and 50 μg/mL PI at 4°C for 1 h. Flow cytometry and cell cycle analysis were then performed using a FACScalibur.

2.6. Apoptosis Analysis by Flow Cytometry. MCF-7 cell apoptosis was analyzed using a flow cytometer with Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) staining kit (Strong Biotech Corporation, Taiwan) to distinguish early apoptotic from necrotic cells as previously described [11, 26]. Briefly, three days after the indicated concentrations of MART-10 or 1α,25(OH)₂D₃ treatment, MCF-7 cell apoptosis was analyzed using a flow cytometer with Annexin V-FITC (fluorescein isothiocyanate) and propidium iodide (PI) staining. Apoptosis Detection Kit (Strong Biotech Corporation, Taiwan) was applied in the present study. Briefly, cells from each sample were suspended in a mixture of 2 μL Annexin V-FITC, 2 μL propidium iodide (PI), and 100 μL Annexin-V-FITC binding buffer and then incubated at room temperature for 15 min. According to the cell density, 0.4–0.8 mL binding buffer was added. The samples were analyzed using a flow cytometer FACS Calibur (BD Biosciences). The cell population was separated into three groups, that is, live cells with a low level of fluorescence, apoptotic cells in the earlier period with green fluorescence, and PI positive).

2.7. Apoptosis Analysis by TUNEL Assay. TUNEL assay was used to measure DNA fragmentation [27]. Briefly, cells were plated on autoclaved glass coverslips in six-well culture plates and treated with MART-10 or 1α,25(OH)₂D₃ as indicated in the figure legends. Cellular DNA was stained with apoptosis detection kits (Millipore Billerica, MA, USA), and the assay was performed according to the recommendations from the manufacturer (Millipore Billerica).

2.8. Statistical Analysis. The data from each group were compared by the student t-test. P-value < 0.05 was considered as
3. Results

3.1. VDR Expression in MCF-7 Cells. Since the genomic actions of 1α,25(OH)₂D₃ are mediated through VDR, we first analyzed the expression of VDR in MCF-7 cells. The expression in MDA-MB-231 cells served as a negative control [28]. As demonstrated in Figure 1(a), VDR was highly expressed in MCF-7 cells (lanes 1, 3, and 5), whereas very little or no expression (lanes 2, 4, and 6) was found in MDA-MB-231 cells as previously reported [28].

3.2. Antiproliferative Effect of MART-10 and 1α,25(OH)₂D₃ on MCF-7 Cells. To compare the antiproliferative activity of
MART-10 and 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} in MCF-7 cells, the cells were treated with either MART-10 or 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3}, and the cell numbers were counted on 7th day as previously described [11]. As shown in Figure 1(b), either 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} or MART-10 caused a dose-dependent inhibition of cell growth. However, MART-10 caused a 50 ± 9% inhibition at 10\textsuperscript{-10} M, whereas, no inhibition was observed with 10\textsuperscript{-10} M of 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3}. Only when 10\textsuperscript{-7} M 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} was used, a 58 ± 6% inhibition was obtained. Thus, it is concluded that MART-10 is about 500- to 1000-fold as potent as 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3}.

Figure 1(c) shows a time course inhibition of MCF-7 cell growth by 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} and MART-10 at 10\textsuperscript{-7} M. 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} inhibited MCF-7 cell growth by 14 ± 5, 46 ± 6 and 61 ± 3% on the 3rd, 5th, and 7th day, whereas a 20 ± 3, 60 ± 3, or 84 ± 4% growth inhibition by MART-10 was observed at the same time points. A greater inhibition by MART-10 was observed at each time point.

Figure 1(d) demonstrates that MDA-MB-231 cells were not as responsive as MCF-7 cells to 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} and MART-10 treatments. Only a 13 ± 6% and a 16 ± 5% inhibition were observed in the presence of 10\textsuperscript{-6} M 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} and 10\textsuperscript{-7} M MART-10, respectively. The results are in agreement with the VDR expression data obtained by western blot analysis showing much less expression of VDR in MDA-MB-231 cells than in MCF-7 cells (Figure 1(a)).

3.3. Induction of Cell Cycle Arrest at G\textsubscript{0}/G\textsubscript{1} Phase and the Cyclin Dependent Kinase (CDK) Inhibitors, p21 and p27, by MART-10 and 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} in MCF-7 Cells. Since MART-10 and 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} showed a significant inhibition in the growth of MCF-7 cells, we next conducted cell cycle analysis by flow cytometry to further understand the mechanisms mediating the inhibition. When MCF-7 cells were treated with 10\textsuperscript{-8}, 10\textsuperscript{-7}, and 10\textsuperscript{-6} M 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} for two days, the fraction of cells arrested at G\textsubscript{0}/G\textsubscript{1} phase increased by 5.81%, 13.34%, and 13.78%, respectively, whereas we observed an increase in cell arrest at G\textsubscript{0}/G\textsubscript{1} by 10.45%, 15.36%, and 19.93% in the presence of 10\textsuperscript{-6} M 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} and 10\textsuperscript{-7} M MART-10, respectively. The results are in agreement with the VDR expression data obtained by western blot analysis showing much less expression of VDR in MDA-MB-231 cells than in MCF-7 cells (Figure 1(a)).

Since p21 and p27 have been implicated in the G\textsubscript{0}/G\textsubscript{1} progression at G\textsubscript{0}/G\textsubscript{1}, MART-10 is much more potent than 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} in this respect.

4. Discussion

The focus of this study was to investigate the antiproliferative and proapoptotic activities of MART-10 in the ER+ MCF-7 breast cancer cells which express high level of VDR (Figure 1(a)). MART-10 is a new generation of 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} analogs with a skeleton of “2\alpha-(3-hydroxy)propyl group” and “19-nor” integrated into one molecule. Therefore, MART-10 possesses the combined characteristics of the noncalcemic nature of the 19-nor vitamin D compounds [30] as exemplified by the FDA-approved drug Zemplar or 19-nor-1\alpha, 25(OH)\textsubscript{2}D\textsubscript{2} for the treatment of the secondary hyperparathyroidism, and the enhanced VDR binding property of 2\alpha-(3-hydroxy)propyl compound [31, 32]. Similar to Zemplar, MART-10 did not raise serum calcium in an in vivo animal model [23] and was more potent than 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} in inducing VDR transactivation [22].

The effects of vitamin D are mainly mediated through the VDR-dependent genomic actions. Our results confirm the high level of VDR expression in MCF-7 cells and accordingly highly sensitive growth inhibitory responses to 1\alpha,25(OH)\textsubscript{2}D\textsubscript{3} and MART-10 in a dose- and time-dependent manner (Figures 1(b) and 1(c)).
Figure 2: Flow cytometry analysis of cell cycle distribution for MCF-7 cells treated by 1α,25(OH)₂D₃ and MART-10. Effects of 1α,25(OH)₂D₃ and MART-10 on the relative distribution of MCF-7 cells at G₁/G₀, S and G₂/M phase. MCF-7 cells were treated with 1α,25(OH)₂D₃ from 10⁻⁸ M to 10⁻⁶ and MART-10 from 10⁻⁹ M to 10⁻⁷ M for two days before cell cycle analysis was performed with a flow cytometer. A representative DNA histogram for control, 1α,25(OH)₂D₃-, or MART-10-treated MCF-7 cells was shown. The total DNA content of cells (x-axis) was obtained by staining with propidium iodide. Cells were analyzed by flow cytometry. The percentage of cells in each cell cycle phase was determined with the program ModFit. The first large peak represents population of cells (y-axis) in G₀/G₁ phase, the second small peak shows population of cells in G₂/M phase, and the gray area between both peaks represents cells in S phase.
Figure 3: Western blot analysis for the expressions of p21 and p27 after treating MCF-7 cells with 1α25(OH)2D3 and MART-10. (a) A western blot (30 μg protein was loaded for each individual lane) depicting a typical dose-dependent upregulation of p21 protein expression in response to the treatment with 1α25(OH)2D3 or MART-10 for 2 days (upper panel). Actin was used as the loading control. The lower panel shows the average ratio of the dose-dependent p21 expression relative to actin expression from three independent experiments. Each value is a mean ± SD of three independent determinations. (b) A western blot (30 μg protein was loaded for each individual lane) depicting a typical dose-dependent up-regulation of p27 protein expression in response to the treatment with 1α25(OH)2D3 or MART-10 for 2 days (upper panel). Actin was used as the loading control. The lower panel depicts the average radio of the dose-dependent p27 expression relative to actin expression from three independent experiments. Each value is a mean ± SD of three independent determinations. *P < 0.05, **P < 0.001 versus control.

Table 1: The distribution of different phases of MCF-7 cell cycle under the influence of 1α25(OH)2D3 or MART-10.

| Condition | G1  | S   | G2/M |
|-----------|-----|-----|------|
| Control   | 50.36% | 33.51% | 16.13% |
| 1,25D+ 10−8 M | 56.17% | 30.06% | 13.77% |
| 1,25D+ 10−7 M | 63.70% | 23.85% | 12.36% |
| 1,25D+ 10−6 M | 64.14% | 21.96% | 13.90% |
| M-10 10−9 M | 60.81% | 23.65% | 15.54% |
| M-10 10−8 M | 65.72% | 21.32% | 12.96% |
| M-10 10−7 M | 70.29% | 12.98% | 16.73% |

*1,25D: 1α25(OH)2D3.
*M-10: MART-10.

expression of VDR in MDA-MB-231 cells (Figure 1(a)) is in agreement with the low antiproliferative activity caused by 1α25(OH)2D3 and MART-10 (Figure 1(d)) in these VDR-null cells. Thus, the results clearly suggest that VDR plays a crucial role in the response of MCF-7 breast cancer cells to 1α25(OH)2D3. Along this line, Lopes et al. recently reported that VDR expression was high in benign breast lesions and diminished gradually in invasive breast cancer as the tumor progressed [33]. VDR expression has also been shown to be inversely related to breast cancer incidence [34]. Collectively, the findings suggest that dysregulation of VDR expression may contribute to the incidence and progression of breast cancer.

In addition, our data, showing a greater cell growth inhibition induced by MART-10 than by 1α25(OH)2D3 on day 5 and day 7 (Figure 1(c)), suggest that the effective dose of MART-10 may be higher than that of 1α25(OH)2D3, possibly because MART-10 is more bioavailable than 1α25(OH)2D3 due to the nature that MART-10 is more resistant to CYP24A1 degradation [22, 23].

Our results show that although both 1α25(OH)2D3 and MART-10 are active in inhibiting the proliferation (Figures 1(b) and 1(c)), inducing the cell cycle arrest at G0/G1 phase (Figure 2 and Table 1) and promoting the apoptosis of MCF-7 cells (Figure 4), MART-10 is far more potent than 1α25(OH)2D3. The greater antiproliferative activity with MART-10 over 1α25(OH)2D3 may be explained at least in part by its greater stimulatory effects on the expression of two tumor suppressor genes, p21 and p27, which act as CDK inhibitors to inhibit the progression of cells into the S phase of the cell cycle (Figure 3). This finding is consistent with
Figure 4: Effects of 1α25(OH)₂D₃ and MART-10 on MCF-7 cell apoptosis analyzed by flow cytometry with Annexin V-FITC, PI staining, and TUNEL assay. (A) Annexin V-FITC in conjunction with PI staining was used to distinguish early apoptotic (Annexin V-FITC positive, PI negative; bottom right quadrant of each panel) from late apoptotic or necrotic cells (Annexin V-FITC positive, PI positive; top right quadrant of each panel). Fluorescence intensity for Annexin V-FITC is plotted on the x-axis, and PI is plotted on the y-axis. (B) The apoptotic effects induced by MCF-7 cells were analyzed by TUNEL assay to measure the extent of DNA fragmentation visualized by fluorescence microscopy: (a) control; (b) cells treated with 10⁻⁷ M MART-10; (c) cells treated with 10⁻⁷ M 1α25(OH)₂D₃. The cells showing positive DNA fragmentation were circled; (d) relative apoptotic index. Each value represents the average of three determinations. *P < 0.05, **P < 0.001 versus control.
several previous reports that showed that p21 and p27 were the genes targeted by 1α,25(OH)₂D₃ and, therefore, leading to the arrest of cell growth [11, 35, 36].

As demonstrated in Figure 4 and Table 2, MART-10 is also more active than 1α,25(OH)₂D₃ in inducing apoptosis. Bax, a proapoptotic protein, works toward the initiation of apoptosis through promoting the release of cytochrome C from mitochondria into cytosol. Whereas, Bcl-2, an antiapoptotic protein, functions as a protector to stabilize the mitochondrial membrane from releasing cytochrome C [37].

Studying MCF-7 breast cancer cells, James et al. [38] and Simboki-Campbell et al. [39] reported that 1α,25(OH)₂D₃ induced apoptosis by downregulating Bcl-2 protein expression, increased TRPM-2 (clusterin) mRNA expression, and increased DNA fragmentation after 1α,25(OH)₂D₃ treatment. In our studies with MCF-7 cells, both 1α,25(OH)₂D₃ and MART-10 increased the ratio of Bax/Bcl-2 and the subsequent release of cytochrome C (Figures 5(a) and 5(b)). However, MART-10 is more potent than 1α,25(OH)₂D₃.

The release of cytochrome C from mitochondria to cytoplasm is a trigger of apoptosis pathway, leading to the activation of intrinsic initiator caspase 9, which in turn activates executioner caspase 3 and caspase 7 [40]. To investigate whether caspases were involved in the vitamin D-induced apoptosis in MCF-7 cells, we performed western blotting to detect the expression of the active form of caspases 3, 7, 8, and 9 in the presence of 10⁻⁷M of 1α,25(OH)₂D₃ or MART-10 for 5 days. We found that none of them was detected either with or without 1α,25(OH)₂D₃ or MART-10 treatment (unpublished data). Our results are in agreement with the previously published observations by Narvaez and Welsh [41] and Jänicek et al. [42]. Collectively, we conclude that MART-10 and 1α,25(OH)₂D₃-mediated apoptosis in MCF-7 cells may be cytochrome C-related but caspases-independent, and MART-10 is more potent than 1α,25(OH)₂D₃ in inducing apoptosis in MCF-7 cells.
5. Conclusion

For premenopausal women with ER+ breast cancer, the choice for antihormone treatment is tamoxifen or raloxifene which binds to ER, whereas aromatase inhibitors are the major therapeutic antihormone agents for the postmenopausal women with ER+ breast cancer. The drawback of tamoxifen or raloxifene and aromatase inhibitors is that they globally attenuate estrogen receptor transactivation or estrogen synthesis. It may be undesirable for some tissues where estrogen is essential to maintain normal functions, such as bone which needs estrogen to stimulate bone formation. On the contrary, 1α,25(OH)2D3 can selectively down-regulate aromatase and ER-α expression in breast cancer cells [43, 44]. Along this line, we have performed preliminary studies indicating that MART-10 is far more potent than 1α,25(OH)2D3 in inhibiting ER-α expression in MCF-7 cells (unpublished observation). In conclusion, we show that MART-10 is much more potent than 1α,25(OH)2D3 in inhibiting cell growth through arresting cell cycle progression at G1 phase and inducing apoptosis. In addition, the more bioavailable character of MART-10 as compared to 1α,25(OH)2D3 in MCF-7 cells and its noncalcemic nature in an animal model suggest that MART-10 has potential as a superior chemotherapeutic agent to replace or to be in combination with traditional antihormone therapy for the treatment of breast cancer, such as the ER+ breast cancer patients, to decrease the tumor recurrence and eliminate the side effect on bone caused by the antihormone treatments.

Abbreviations

ER: Estrogen receptor
1α,25(OH)2D3: 1α,25-Dihydroxyvitamin D
MART-10: 19-nor-2α-(3-Hydroxypropyl)-1α,25(OH)2D3
VDR: Vitamin D receptor
VDRE: Vitamin D response element
RXR: Retinoid X receptor
PI: Propidium iodide
E2F-1: E2F transcription factor 1
FBS: Fetal bovine serum
FITC: Fluorescein isothiocyanate.

Conflict of Interests

The authors declare that they have no conflict of interests.

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