1,25-Dihydroxyvitamin D3 and All-trans-Retinoic Acid Sensitize Breast Cancer Cells to Chemotherapy-induced Cell Death

Qin Wang, Wen Yang, Myrna S. Uytengco, Sylvia Christakos, and Robert Wieder

Division of Oncology/Hematology, Department of Medicine [Q. W., M. S. U., R. W.], and Department of Biochemistry and Molecular Biology [W. Y., S. C.], University of Medicine and Dentistry of New Jersey, New Jersey Medical School, Newark, New Jersey 07103

ABSTRACT

We investigated the capacity of 1,25-dihydroxyvitamin D3 [1,25(OH)2D3] and all-trans-retinoic acid (ATRA) to sensitize three breast cancer cell lines to the cell killing effects of paclitaxel (Taxol) and Adriamycin, two chemotherapeutic agents commonly used in the treatment of breast cancer. In tissue culture colony assays, 1,25(OH)2D3 and ATRA were synergistic in inhibiting the clonogenicity of MCF-7 and T-47D cells that expressed estrogen receptor; vitamin D receptor; retinoic acid receptors (RARs) α, β, and γ; and retinoid X receptors α, β, and γ but were not additive in MDA-MB-231 cells that lacked expression of estrogen receptor, RARα, and RARβ. The hormones used individually or in combination induced up to 40–50% cell death by a trypan blue exclusion assay in a dose-dependent manner up to concentrations of 10−7 M in MCF-7 and T-47D cells, more modestly in MDA-MB-231 cells, and not at all in MCF-10 and MCF-12 nontransformed mammary epithelial cells. Pretreating the cancer cell lines with 1,25(OH)2D3 and ATRA individually or in combination for 3 days prior to a 1-h incubation with paclitaxel or Adriamycin decreased the ED50 for inhibition of colony formation or for cell death by trypan blue by up to 2 logs for paclitaxel and up to 1 log for Adriamycin in all three cell lines but had no effect on chemotherapy-induced MCF-12 cell death. The effects of the hormones were synergistic with those of the chemotherapeutic agents in all of the breast cancer cell lines, generally at the higher concentrations. Cell death took place by apoptosis. To determine one potential reason for the greater potentiation of the effects of paclitaxel than those of Adriamycin, we determined the effects of preincubation of MCF-7 cells with 1,25(OH)2D3 and ATRA on the phosphorylation of Bcl-2. Pretreatment of MCF-7 cells with either 1,25(OH)2D3 or ATRA increased the phosphorylation of Bcl-2 by variable concentrations of paclitaxel. These data suggest that pretreatment of breast cancer with 1,25(OH)2D3 or ATRA lowers the threshold for cell killing by chemotherapy agents and may provide a novel treatment option for this disease.

INTRODUCTION

Although providing short-term benefits, chemotherapy does not produce long-term disease-free survival in patients with unresectable breast cancer (1). Treatment strategies for solid tumors must incorporate chemotherapy administered in doses high enough to affect a substantial reduction in the number of tumor cells and frequent enough to prevent a significant regrowth between treatment cycles (dose density) (2). Many patients with recurrent or metastatic breast cancer, however, may be precluded from receiving dose dense therapy because of substantial prior therapy. Novel strategies must be devised to administer therapy that is effectively dose dense without the side effects of high-dose chemotherapy. One such strategy is the potentiation of chemotherapy with agents that are well tolerated yet capable of lowering the threshold for chemotherapy-induced cell death in the cancer cells. Two candidate agents are ATRA and 1,25(OH)2D3. Both compounds belong to classes of agents reported to inhibit proliferation and induce cell death in breast cancer cells (3–8).

Vitamin D3 and retinoic acid derivatives exert their effects by binding to related members of the nuclear receptor family that, together with their ligands, modulate transcription through cognate response elements in the promoters of their target genes (9). Both 1,25(OH)2D3 and ATRA inhibit cell cycle progression in the G1 phase through up-regulating transcription of the CDK inhibitor p21WAF1/CIP1 through vitamin D response elements (10) and retinoic acid response elements (11) in the waf1 promoter. The 1,25(OH)2D3- and ATRA-induced increases in p21WAF1/CIP1 are p53 independent (6, 10). 1,25(OH)2D3 also up-regulates p27KIP1 (12) and inactivates CDK 6 (13). 9-cis-Retinoic acid and ATRA also inhibit CDK 4 and CDK 2 activity, expression of cyclins D1 and D3, and proliferation in MCF-7 and T-47D breast cancer cells, without affecting the proliferation of MCF-10 nontransformed mammary epithelial cells (14). Both 1,25(OH)2D3 and ATRA-induced effects result in dephosphorylation of Rb (15, 16). Retinoid treatment also reduces E2F1 promoter activity, E2F1 mRNA and protein levels (16), and inhibits AP1 activity in MCF-7 cells but not in MDA-MB-231 cells that lack ER, RARα, and RARβ and express mutant p53 (5). Retinoids can, however, promote cell death in a p53-independent manner and increase the expression of cell death factor Bax and decrease Bcl-2 levels (6), and analogues of 1,25(OH)2D3 can down-regulate Bcl-2 in cells expressing mutant p53 (3). In addition, ATRA derivatives can induce apoptosis in a RARγ-specific manner, abrogating the need for RARα and RARβ expression for that effect (17).

Combinations of 1,25(OH)2D3 and retinoic acid family members can work cooperatively to inhibit proliferation (18, 19) and promote apoptosis (20) in MCF-7 cells and squamous cell carcinoma cells (21). The combined effects of retinoids and vitamin D3 derivatives are attributable to heterodimeric interactions between members of the nuclear receptor family. VDRs and RARs both heterodimerize with RXR, greatly enhancing their transcriptional activity (22–24). In addition, VDRs and RARs form heterodimers that provide the signaling crossroads between the two classes of compounds (25).

In this study, we attempted to modulate the susceptibility of breast cancer cells to chemotherapy agents by preincubating them with 1,25(OH)2D3 and ATRA, either individually or in combination, to render them more susceptible to the proapoptotic effects of two chemotherapeutic agents. Our data demonstrate a substantial increase in the sensitivity of three breast cancer cell lines to the effects of paclitaxel and Adriamycin while sparing nontransformed mammary epithelial cells.

MATERIALS AND METHODS

Cell Lines and Tissue Culture. MCF-7, T-47D, MDA-MB-231, MCF-10, and MCF-12 cells were purchased from the American Type Culture Collection.

1 The abbreviations used are: ATRA, all-trans retinoic acid; 1,25(OH)2D3, 1,25-dihydroxyvitamin D3; CDK, cyclin-dependent kinase; ER, estrogen receptor; RAR, retinoic acid receptor; RXR, retinoid X receptor; VDR, vitamin D receptor; TUNEL, terminal deoxynucleotidyl transferase-mediated nick end labeling.

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7 To whom requests for reprints should be addressed, at University of Medicine and Dentistry of New Jersey-New Jersey Medical School, Division of Medical Oncology/Hematology, MSB I-594, 185 South Orange Avenue, Newark, NJ 07103. Phone: (973) 972-4871; Fax: (973) 972-2384.

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(Rockville, MD). MCF-7, T-47D, and MDA-MB-231 cells were cultured in DMEM (Life Technologies, Inc., Gaithersburg, MD) with 15 mg/l phenol red, 2 mM glutamine, 10% heat-inactivated FCS, 50 units/ml penicillin and 50 μg/ml streptomycin (Gemini Bioproducts, Calabasas, CA; standard medium) or standard medium in which 10% FCS was replaced by 2% charcoal-stripped FCS (Life Technologies; stripped medium). MCF-10 and MCF-12 cells were cultured in MCF-10 culture medium, described elsewhere (26), or MCF-10 culture medium in which the 5% horse serum was replaced by 2% horse serum (Life Technologies; stripped MCF-10 medium). 1,25(OH)2D3, the analogues 1,25(OH)2-16-ene-23-yne-D3 and 1,25(OH)2-16-ene-23-yne-26,27-hexafluoro-D3, and ATRA were kind gifts of Milan Uskokovic (Hoffmann-La Roche, Nutley, NJ). These compounds were dissolved in ethanol, and the stock solutions were stored in the dark at −20°C.

**Northern Blots.** Polyadenylated RNA was prepared from guanidinium thiocyanate-extracted total cellular RNA (27) using a poly-dT column (Life Technologies) as described (28), electrophoresed in 1% agarose gels, and transferred to nylon filters by capillary transfer techniques. The membranes were hybridized with a 32P-labeled cDNA fragments for ER (a kind gift of B. Katzenellenbogen, University of Illinois, Urbana, IL) for VDR, RARα, and RARβ (kind gifts of J. W. Pike, Baylor College of Medicine, Houston, TX) and RXRα (kind gift of D. Mangelsdorf, University of Texas Southwestern Medical Center, Dallas TX). A cdna probe for β-actin was used as a loading control. Sequences were identified by exposure to Kodak XAR-5 film at −80°C. Band intensity was quantitated using a Shimadzu C5900U densitometer dual flying spot scanner (Shimadzu Scientific Instruments, Inc., Princeton, NJ) and normalized against the actin loading control.

**Western Immunoblots.** For receptor determinations, cells cultured in standard medium were harvested when 60–70% confluent, and lysates were prepared using NP40 lysis buffer, containing 50 mM Tris-HCl (pH 7.4), 150 mM NaCl, 0.5% NP40, 1 mM NaVO4, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 1 μg/ml leupeptin (Sigma Chemical Co., St. Louis, MO), and 0.01 unit/ml aprotinin (26), and analyzed as before (29). Western blots were stained with antibodies to ER, VDR, RARα, RARβ, RXRα, RXRβ, and RXRγ (Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and to α-tubulin (Sigma) used as a loading control.

To determine the effects on Bcl-2, cells were cultured to ~70% confluence in standard medium, switched to stripped medium with and without ATRA or 1,25(OH)2D3 for 3 days, and then incubated with paclitaxel in standard medium for 24 h. Cells were scraped from the plates in NP40 lysis buffer and analyzed by Western immunoblot using a mouse monoclonal antibody to human Bcl-2 (Dako Corp., Carpenteria, CA) or a monoclonal antibody to α-tubulin (Sigma) as a loading control, as described (29). All blots were carried out at least twice.

**Colony Assays.** To determine the colony-inhibitory effects of 1,25(OH)2D3 and ATRA, 2000 cells were incubated in triplicate 6-cm tissue culture plates and allowed to adhere for 24 h in standard medium. The medium was replaced by stripped medium or stripped medium supplemented with...
CHEMOPOTENTIATION BY 1,25(OH)₂ D₃ AND ATRA

Table 1. Concentrations of 1,25(OH)₂ D₃ and ATRA causing a 50% inhibitory effect (ED₅₀ M) in colony formation

<table>
<thead>
<tr>
<th></th>
<th>MCF-7</th>
<th>T-47D</th>
<th>MDA-MB-231</th>
</tr>
</thead>
<tbody>
<tr>
<td>1,25(OH)₂ D₃</td>
<td>5.9 × 10⁻⁸</td>
<td>7.4 × 10⁻⁸</td>
<td>1.2 × 10⁻⁷</td>
</tr>
<tr>
<td>ATRA</td>
<td>6.8 × 10⁻⁸</td>
<td>1.3 × 10⁻⁷</td>
<td>7.3 × 10⁻⁸</td>
</tr>
<tr>
<td>1,25(OH)₂ D₃ + ATRA</td>
<td>7.4 × 10⁻⁸</td>
<td>9.9 × 10⁻⁸</td>
<td>4.4 × 10⁻⁸</td>
</tr>
</tbody>
</table>

* Synergistic by Loewe’s Additivity Model (see text).

Results

Characterization of Cell Lines. The five cell lines used in our studies were characterized with respect to their ER, VDR, RAR, and RXR status by Western blot (Fig. 1) and Northern blot (not shown) analysis. ER, RARα, and RARβ were only expressed in MCF-7 and T-47D cells. VDR, RARγ, RXRα, RXRβ, and RXRγ were expressed in all of the cells. The results from Northern blots were in general agreement with the Western blot data.

Effects of 1,25(OH)₂ D₃ and ATRA on Clonogenic Potential and Cell Death. To demonstrate the effects of 1,25(OH)₂ D₃ and ATRA, either individually or in combination, on the survival of breast cancer cells, we used a clonogenic assay (Ref. 30; Fig. 2A). Both 1,25(OH)₂ D₃ and ATRA inhibited colony formation in a dose-dependent manner in all three breast cancer cell lines. The doses causing a 50% inhibitory effect (ED₅₀) are displayed in Table 1. Colony inhibition by the two compounds used in combination was synergistic in MCF-7 and T-47D cells, with Loewe’s Additivity Model combination indices of 0.024 and 0.021, respectively (31, 32). However, the effect of combining 1,25(OH)₂ D₃ and ATRA in MDA-MB-231 cells yielded a combination index of 0.96, indicating additive effects within experimental error by the two compounds. Nontransformed mammary epithelial cells did not form colonies in tissue culture and, thus, could not be assayed using this technique.

To determine whether noncalcemic analogues of 1,25(OH)₂ D₃ are also effective in their ability to inhibit clonogenic potential in breast cancer cells, we incubated MCF-7 cells with variable doses of the 1,25(OH)₂ D₃ analogues 1,25(OH)₂16-ene-23-yne-D₃ and 1,25(OH)₂16-ene-23-yne-18,20-ene-D₃, and 1,25(OH)₂16-ene-23-yne-18,20-ene-19,21-diene-D₃.

Fig. 2A. Measurement of DNA Fragmentation. Cells were incubated on sterile microscope slide coverslips overnight in standard medium. Medium was removed 24 h later and replaced with either stripped medium or stripped medium containing 1,25(OH)₂ D₃ or ATRA at 10⁻⁸ M alone or in combination for 72 h. Cells were then incubated with Adriamycin or paclitaxel at the concentrations indicated for 1 h in standard medium, washed, and incubated in standard medium. One, 2, or 3 days later, the cells were permeabilized, and the DNA was ³²P end labeled with FITC-dUTP using a Boehringer Mannheim (Indianapolis, IN) Fluorescein In Situ Cell Death Detection kit to measure apoptosis. Cells were photographed at ×400 with the Olympus BX40 fluorescence microscope and the PM20 photographic system.

Fig. 3. Percentage of cell death determined by trypan blue uptake in MCF-7, T-47D, and MDA-MB-231 breast cancer cells and MCF-10 and MCF-12 nontransformed mammary epithelial cells incubated with 1,25(OH)₂ D₃ and ATRA at concentrations shown for 3 days. Bars, SD.
26,27-hexafluoro-D₃. The effects of these compounds were tested for activity in breast cancer cells because any potential clinical application of 1,25(OH)₂D₃ will require the use of a noncalcemic analogue. Fig. 2B demonstrates that these analogues of 1,25(OH)₂D₃ were even more effective than the parent compound in inhibiting colony formation. The ED₅₀ for 1,25(OH)₂D₃, 1,25(OH)₂-16-ene-23-yne-D₃, and 1,25(OH)₂-16-ene-23-yne-26,27-hexafluoro-D₃ were 5.6 \times 10^{-2}, 4.7 \times 10^{-2}, and 6.3 \times 10^{-2}, respectively.

To complement the clonogenicity studies, we investigated the effects of 1,25(OH)₂D₃ and ATRA on their capacity to induce cell death in breast cancer cells and in nontransformed mammary epithelial cell lines. Fig. 3 demonstrates that 1,25(OH)₂D₃ and ATRA, either individually or in combination, induced cells to die in a dose-dependent manner after a 3-day incubation. The ED₅₀ for 1,25(OH)₂D₃ and ATRA were 1.0 \times 10^{-8} (extrapolated) and 5.27 \times 10^{-8}, respectively, for MCF-7 cells but did not reach ED₅₀ for any of the other cells assayed at doses up to 1 \times 10^{-7} M. The maximum cell death achieved was 39% in T-47D cells by either 1,25(OH)₂D₃ or ATRA.

Table 2. Effects of preincubation with 1,25(OH)₂D₃ and ATRA on the ED₅₀ of paclitaxel and Adriamycin in colony formation assays

<table>
<thead>
<tr>
<th></th>
<th>Paclitaxel</th>
<th>Adriamycin</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>ED₅₀ (M)</td>
<td>Fold decrease</td>
</tr>
<tr>
<td>MCF-7 Control</td>
<td>4.8 \times 10^{-7}</td>
<td>9.8 \times 10^{-8}</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>6.3 \times 10^{-9}</td>
<td>6.9 \times 10^{-9}</td>
</tr>
<tr>
<td>ATRA</td>
<td>7.0 \times 10^{-9}</td>
<td>68.6</td>
</tr>
<tr>
<td>1,25(OH)₂D₃ + ATRA</td>
<td>6.5 \times 10^{-9}</td>
<td>73.8</td>
</tr>
<tr>
<td>T-47D Control</td>
<td>6.0 \times 10^{-7}</td>
<td>8.4 \times 10^{-8}</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>2.8 \times 10^{-8}</td>
<td>21.4</td>
</tr>
<tr>
<td>ATRA</td>
<td>1.0 \times 10^{-8}</td>
<td>60.0</td>
</tr>
<tr>
<td>1,25(OH)₂D₃ + ATRA</td>
<td>5.2 \times 10^{-9}</td>
<td>115.4</td>
</tr>
<tr>
<td>MDA-MB-231 Control</td>
<td>6.2 \times 10^{-7}</td>
<td>9.0 \times 10^{-8}</td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>9.6 \times 10^{-9}</td>
<td>64.6</td>
</tr>
<tr>
<td>ATRA</td>
<td>2.2 \times 10^{-8}</td>
<td>28.2</td>
</tr>
<tr>
<td>1,25(OH)₂D₃ + ATRA</td>
<td>1.8 \times 10^{-8}</td>
<td>34.4</td>
</tr>
</tbody>
</table>

a 1,25(OH)₂D₃ and ATRA concentrations were 10⁻⁹ M each when used individually or in combination.
and 24 and 29% for 1,25(OH)₂D₃ and ATRA, respectively, in MDA-MB-231 cells. Combining the two agents caused a 55% cell kill at 10⁻⁷ M in T-47D cells that was statistically greater than killing by either compound alone (P<0.05 and P<0.02 for 1,25(OH)₂D₃ and ATRA, respectively). However, combining the two hormones did not increase the percentage of cell kill over that achieved by either compound alone in MCF-7 and MDA-MB-231 cells, suggesting a less than additive effect on inducing cell death in these cell lines. These data, demonstrating different effects on colony inhibition and cell killing by these agents, suggest that different mechanisms are responsible for these two effects and that cell killing is only a subset of the effects that result in colony inhibition.

The effects on the nontransformed mammary epithelial cell lines MCF-10 and MCF-12 were tested. The background rate of cell death after 3 days in 2% horse serum was 23.5±3.2% and 30.3±0.8% for MCF-10 and MCF-12, respectively, and did not exceed 31% with 3-day incubations with either 1,25(OH)₂D₃, ATRA, or both in either cell line. These differences were not statistically significant. These experiments demonstrate that, unlike the effects in cancer cells, 1,25(OH)₂D₃ and ATRA do not induce a significant amount of cell death in nontransformed mammary epithelial cell lines MCF-10 and MCF-12.

### Table 3

Effects of preincubation with 1,25(OH)₂D₃ and ATRA on the ED₅₀ of paclitaxel and Adriamycin in trypan blue cell death assays

<table>
<thead>
<tr>
<th></th>
<th>Paclitaxel</th>
<th>Adriamycin</th>
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<tbody>
<tr>
<td></td>
<td>ED₅₀ (μM)</td>
<td>Fold decrease</td>
</tr>
<tr>
<td>MCF-7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.8 × 10⁻⁸</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>9.5 × 10⁻⁷</td>
<td>16.7</td>
</tr>
<tr>
<td>ATRA</td>
<td>8.1 × 10⁻⁸</td>
<td>195.8</td>
</tr>
<tr>
<td>1,25(OH)₂D₃ + ATRA</td>
<td>3.2 × 10⁻⁷</td>
<td>49.1</td>
</tr>
<tr>
<td>T-47D</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>8.5 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>6.5 × 10⁻⁷</td>
<td>13.0</td>
</tr>
<tr>
<td>ATRA</td>
<td>6.0 × 10⁻⁷</td>
<td>14.2</td>
</tr>
<tr>
<td>1,25(OH)₂D₃ + ATRA</td>
<td>5.6 × 10⁻⁷</td>
<td>15.3</td>
</tr>
<tr>
<td>MDA-MB-231</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>7.1 × 10⁻⁶</td>
<td></td>
</tr>
<tr>
<td>1,25(OH)₂D₃</td>
<td>8.4 × 10⁻⁸</td>
<td>84.5</td>
</tr>
<tr>
<td>ATRA</td>
<td>8.8 × 10⁻⁸</td>
<td>80.1</td>
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<tr>
<td>1,25(OH)₂D₃ + ATRA</td>
<td>4.6 × 10⁻⁸</td>
<td>13.3</td>
</tr>
</tbody>
</table>

*1,25(OH)₂D₃ and ATRA concentrations were 10⁻⁸ M each when used individually or in combination.

*Extrapolated.
Fig. 6. A, apoptosis as determined by dUTP-FITC 3'-OH end labeling analysis in MCF-7 cells incubated on coverslips with $10^{-9}$ M 1,25(OH)$_2$D$_3$ and ATRA in combination for 3 days prior to 1-h incubation with medium; 0.01, 0.1, or 1.0 μM paclitaxel (Taxol); or 0.01, 0.05, or 0.1 μM Adriamycin. The cells were photographed after a subsequent 3-day incubation in DMEM 10% FCS at ×400 using an Olympus BX40 fluorescence microscope and PM20 photographic system. B, graphic representation of TUNEL-positive percentages obtained from manual counts of three to five random (∼×400) fields of all of the cancer cell lines and the MCF-10 nontransformed cells. **, statistically significant differences of the pretreatment points of MDA-MB-231 over those of nonpretreated cells.
combination, prior to treatment with paclitaxel and Adriamycin, caused a greater decrease in colony formation than treatment with paclitaxel or Adriamycin alone. Comparisons of fractional products of the measured effects on colony inhibition with the effects predicted from the Bliss Interdependence Model indicated synergy in MCF-7 cells with either paclitaxel or Adriamycin at all concentrations tested (33). The interactions between 1,25(OH)2D3 and ATRA and the chemotherapeutic agents in T-47D and MDA-MB-231 cells were mixed between synergistic and additive and varied with the concentrations of the drugs. The synergistic effects are presented in graphic form in the lower graphs of Fig. 4, where the data are graphed as a percentage of untreated controls. Statistically significant divergence of the values at each concentration are indicated by asterisks and denote synergy. The ED50s for colony inhibition by paclitaxel and Adriamycin are presented in Table 2. The table includes the calculated fold decreases in the ED50s by pretreatment of the cancer cells with 1,25(OH)2D3 and ATRA. The potentiation of the effects of chemotherapy was observed with both drugs, but the effects were consistently greater with paclitaxel than with Adriamycin in all three cell lines.

To determine whether these additive and synergistic effects of 1,25(OH)2D3 and ATRA on colony inhibition by chemotherapeutic agents are attributable, at least in part, to a permissive effect on cell death, we determined the effects of these compounds on the cell killing effects of paclitaxel and Adriamycin, using trypan blue uptake, as before. Fig. 5 demonstrates that preincubation with 10^{-8} M 1,25(OH)2D3 or ATRA alone or in combination for 3 days sensitized breast cancer cells but not MCF-12 nontransformed mammary epithelial cells to the killing effects of paclitaxel and Adriamycin. All three cell lines were subject to synergistic killing by the combined effects of both chemotherapeutic agents and 1,25(OH)2D3 and ATRA, as determined by the Bliss Interdependence Model (33). The combined treatments were synergistic at the higher concentrations of the drugs, generally, as denoted by the asterisks in the graphs. The ED50s for cell killing for paclitaxel and Adriamycin are presented in Table 3. The table also lists the fold decreases in the ED50s for the two drugs when the cells were preincubated with 1,25(OH)2D3 and ATRA. The decreases in ED50s for paclitaxel-treated cells were all >10-fold and in the range of 100-fold under some conditions. The decreases in ED50 for Adriamycin treatment were generally lower than with paclitaxel but were still in the 10-fold range under most conditions. These data confirm that pretreatment of all of the breast cancer cells tested with 1,25(OH)2D3, ATRA or the combination were synergistic with both chemotherapeutic agents in their capacity to induce cell death. No interactions between the two treatment modalities were observed in MCF-12 nontransformed mammary epithelial cells.

To determine whether the mode of cell death was apoptotic, we carried out confirmatory TUNEL assays on the cell lines treated with variable doses of paclitaxel and Adriamycin for 1 h with and without pretreatment with a combination of 1,25(OH)2D3 and ATRA at 1 \times 10^{-8} M for 3 days. Fig. 6A demonstrates that increased doses of paclitaxel induced 3’OH end labeling with FITC-dUTP in a dose-dependent manner. Pretreatment of cells with 1,25(OH)2D3 and ATRA caused a minimal increase in the amount of baseline labeling, but combining preincubation with paclitaxel or Adriamycin treatment caused substantial increases in the percentage of TUNEL-positive cells. Although treatment of cells with paclitaxel or Adriamycin at 1.0 or 0.1 \mu M, respectively, resulted in ~40% TUNEL-positive cells, pretreated cells were 100% TUNEL positive at 0.1 and 0.05 \mu M paclitaxel and Adriamycin, respectively, and demonstrated ~50% positive TUNEL staining at 0.01 \mu M paclitaxel or Adriamycin, or a 2-log increase in sensitivity to paclitaxel and a 1-log increase in sensitivity to Adriamycin. Fig. 6B demonstrates in graphic form the TUNEL-positive percentages obtained from manual counts of 3–5 random fields of all of the cancer cell lines and the MCF-10 nontransformed cells. The increases in the percentage of MDA-MB-231 cells attributable to preincubation prior to exposure to chemotherapy were statistically significant over those of nonpretreated cells but represented substantial underestimates of the true percentages of cell death. This was because of the loss of adherence of dying cells that were preferentially lost with the multiple washing steps required for the staining procedure. These data confirm the >10-fold potentiation of the effects of chemotherapy by pretreatment with 1,25(OH)2D3 and ATRA and demonstrate that the cell death observed was attributable to apoptosis.

Modulation of Bcl-2 Phosphorylation by Paclitaxel by Pretreatment with 1,25(OH)2D3 and ATRA. To understand at least one mechanism that may play a role in the greater potentiation of the effects of paclitaxel than those of Adriamycin by 1,25(OH)2D3 and ATRA, we investigated the effects of these hormones on the phosphorylation of Bcl-2 by paclitaxel. Paclitaxel stabilizes microtubules and induces cell death of cells arrested in the G2 phase of the cell cycle (34). However, paclitaxel also initiates signaling through Raf-1 and the c-Jun NH2-terminal kinase pathway that results in phosphorylation and inactivation of Bcl-2 (35, 36). Retinoids and vitamin D3 analogues activate the mitogen-activated pathway that includes Raf-1 and may play a role by potentiating this pathway (37, 38). We assayed the effects of 1,25(OH)2D3 and ATRA on the capacity of paclitaxel to phosphorylate Bcl-2. Fig. 7 demonstrates that preincubation of MCF-7 cells with 1 \times 10^{-8} M 1,25(OH)2D3 or ATRA caused an increase in the phosphorylation of Bcl-2 at 0.01, 0.1, and 1.0 \mu M paclitaxel with ATRA pretreatment and 0.1 and 1.0 \mu M paclitaxel with 1,25(OH)2D3 pretreatment. These data suggest that at least part of the observed effect may be secondary to a potentiation of the ability of paclitaxel to phosphorylate Bcl-2.
DISCUSSION

Data presented in this report demonstrate that treatment of breast cancer cells with 1,25(OH)2D3 or ATRA, either individually or in combination, sensitize cells to the effects of two chemotherapy agents with different mechanisms of action used in the treatment of breast cancer. At concentrations of 10−8 m, 1,25(OH)2D3 and ATRA can decrease the ED50 for cell death of paclitaxel by up to 2 logs and of Adriamycin by up to 1 log in three breast cancer cell lines. The mode of cell death occurs by apoptosis. The effects of 1,25(OH)2D3 or ATRA when combined with the chemotherapeutic agents paclitaxel or Adriamycin were synergistic at the higher concentrations tested.

Pretreatment of these cancer cells with 10−9 m 1,25(OH)2D3 and ATRA also potentiated the effects of these chemotherapy agents on decreasing colony formation in tissue culture. The effects of the hormones and the chemotherapy drugs were synergistic in all of the cells, in at least some of the concentration ranges of chemotherapy drugs tested, and were additive under other conditions. The effects were once again greater with paclitaxel than with Adriamycin. The ED50 of the chemotherapy drugs for inhibition of colony formation and the concentration of hormones required to affect sensitization were ~10-fold lower that the values needed to affect cell death. This is probably attributable to the factors that affect colony formation. Clonogenic potential is affected by modulating the capacity of the cells to proliferate as well as to survive, and both the hormones and the chemotherapy agents affect both proliferation and cell death.

The effects of retinoids and vitamin D3 on the proliferation of MCF-7 and T-47D, two ER-positive cell lines, are greater than on cells to proliferate as well as to survive, and both the hormones and the chemotherapy drugs were synergistic in all of the cells, in at least some of the concentration ranges of chemotherapy drugs tested, and were additive under other conditions. The effects of retinoids and vitamin D3 on the proliferation of MCF-7 and T-47D, two ER-positive cell lines, are greater than on cells to proliferate as well as to survive, and both the hormones and the chemotherapy agents affect both proliferation and cell death.

As alluded to above, the effects observed with paclitaxel were greater than those obtained with Adriamycin. We investigated one of the most important mechanisms that may cause a sensitizing effect by ATRA in MCF-7 cells treated with paclitaxel, that of the effects on Bcl-2. In addition to acting in its well-defined role as a chemotherapy agent through stabilization of microtubules, paclitaxel induces the phosphorylation and thereby the inactivation of Bcl-2 (42). In this report, we present data that demonstrate, for the first time, that both 1,25(OH)2D3 and ATRA potentiate the ability of paclitaxel to phosphorylate Bcl-2. The mechanism of Bcl-2 phosphorylation by paclitaxel has been shown to be mediated through Raf-1 by direct phosphorylation by cytoplasmic Raf-1 (35). Both 1,25(OH)2D3 and ATRA have been shown to activate the mitogen-activated protein kinase pathway (37, 38), but the mechanism of potentiation of paclitaxel-induced Bcl-2 phosphorylation by these compounds remains undefined and the subject of further investigation. What roles other mechanisms play in potentiation and whether the accen-
tuation of the ability of paclitaxel to mediate phosphorylation of Bcl-2 is physiologically significant remain to be determined (43).

The sensitization of breast cancer cells to the killing effects of chemotherapy agents and the relative insensitivity of nontransformed mammary epithelial cells may provide a useful therapeutic modality in the treatment of breast cancer.

REFERENCES

20. James, S. Y., Mackay, A. G., and Colston, K. W. Vitamin D derivatives in combi-


1,25-Dihydroxyvitamin D$_3$ and All -trans-Retinoic Acid Sensitize Breast Cancer Cells to Chemotherapy-induced Cell Death

Qin Wang, Wen Yang, Myrna S. Uytingco, et al.

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