The selective retinoid X receptor agonist bexarotene (LGD1069, Targretin) prevents and overcomes multidrug resistance in advanced breast carcinoma

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Abstract
Acquired drug resistance represents a major challenge in the therapeutic management of breast cancer patients. We reported previously that the retinoid X receptor–selective agonist bexarotene (LGD1069, Targretin) was efficacious in treating animal models of tamoxifen-resistant breast cancer. The goal of this study was to evaluate the effect of bexarotene on development of acquired drug resistance and its role in overcoming acquired drug resistance in advanced breast cancer. Paclitaxel, doxorubicin, and cisplatin were chosen as model compounds to determine the effect of bexarotene on the development of acquired drug resistance. Human breast cancer cells MDA-MB-231 were repeatedly treated in culture with a given therapeutic agent with or without bexarotene for 3 months. Thereafter, cells were isolated and characterized for their drug sensitivity. Compared with parental cells, cells treated with a single therapeutic agent became resistant to the therapeutic agent, whereas cells treated with the bexarotene combination remained chemosensitive. Cells with acquired drug resistance, when treated with the combination, showed increased sensitivity to the cytotoxic agent. Furthermore, cells treated with the combination regimen had reduced invasiveness and angiogenic potential than their resistant counterparts. These in vitro findings were further confirmed in an in vivo MDA-MB-231 xenograft model. Our results suggest a role for bexarotene in combination with chemotherapeutic agents in prevention and overcoming acquired drug resistance in advanced breast carcinoma.

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Introduction
Breast cancer is the most common cancer among women and is the second leading cause of cancer deaths in women (1). The American Cancer Society estimates that ~215,990 women in the United States will be found to have breast cancer and 40,110 women will die from the disease in 2004. Although chemotherapy provides the major therapeutic modality for treatment of advanced breast cancer, the 5-year survival for disseminated breast carcinoma is <20% due to relapse with drug-resistant disease. Several mechanisms of drug resistance have been characterized in breast cancer cell lines. The best understood mechanism is P-glycoprotein (Pgp)–mediated drug resistance (2). Pgp has been shown to contribute to resistance to natural product–based chemotherapeutic agents, including taxanes, anthracyclines, Vinca alkaloids, podophyllotoxins, and camptothecins (3). The relationship among Pgp expression, Pgp function, and drug resistance in clinical samples of breast carcinoma was best illustrated by Mechetner et al. (4). These investigators showed that the level of Pgp expression in freshly resected breast tumors was strongly correlated with the degree of clinical resistance to paclitaxel and doxorubicin. Their findings also revealed that both intrinsic and acquired expression of Pgp in breast cancer was likely to contribute in part to therapeutic failure and relapse. Other resistance mechanisms in breast cancer include alterations of glutathione metabolism, altered topoisomerase, increased thymidylate synthase levels, decreased reduced folate carrier, and mutations in the tumor suppressor p53 (5–9). Thus, developing effective treatment strategies to enhance cytotoxic effect of chemotherapeutic agents while reduce the risk of developing drug resistance is highly desired.

Bexarotene (LGD1069, Targretin), a selective retinoid X receptor ligand (10), has been shown to be an efficacious chemopreventive and chemotherapeutic agent in preclinical rodent models of breast cancer (11–14). Furthermore, in the rat N-nitroso-N-methylurea–induced mammary carcinoma model, tumors that were resistant to tamoxifen responded to both bexarotene and bexarotene/tamoxifen combination (15). Mechanistically, tumor regression by bexarotene in the rat N-nitroso-N-methylurea–induced mammary tumors involved differentiation induction along the adipocyte lineage leading to terminal cell division followed by cell death (16). More recently, we have shown that bexarotene when combined with paclitaxel produced a synergistic growth inhibition (combination index < 1) in a rat N-nitroso-N-methylurea–induced mammary tumor–derived cell line in vitro. In the rat N-nitroso-N-methylurea–induced tumor model in vivo, the bexarotene/paclitaxel combination regimen produced a statistically significant decrease, when compared with single agents alone, in total tumor burden and an
increase in overall objective response without further intensifying the major side effects of paclitaxel (17). Phase I/II clinical trials indicated that bexarotene is safe and well tolerated (18).

The encouraging results of bexarotene alone or in combination in preclinical models of early-stage mammary carcinoma led us to examine the efficacy of bexarotene in more advanced preclinical models of breast cancer. To evaluate the role of bexarotene in treatment of breast cancer further, we studied the efficacy of bexarotene/cytotoxic combinations and determined the influence of bexarotene on the development and treatment of multidrug resistance in advanced human breast cancer. Bexarotene represents a good candidate for chemotherapy-based combinations due to its nonoverlapping side effect profile with most chemotherapeutic agents and its unique mechanism of action. MDA-MB-231 cells was chosen as a model cell line because of its sensitivity to several cytotoxic agents used in the treatment of breast cancer (19–21). In addition, MDA-MB-231 cells do not express the estrogen or progesterone receptors, are HER-2/neu positive, and have mutant p53 (22). Thus, MDA-MB-231 cells represent a model of advanced breast cancer in which to examine the role of bexarotene in combination with cytotoxic agents for breast cancer therapy. Paclitaxel (a microtubule-stabilizing agent, Pgp substrate), doxorubicin (a topoisomerase II inhibitor, Pgp substrate), and cisplatin (an alkylating agent, non-Pgp substrate) were used to determine the effect of bexarotene on the development of acquired drug resistance.

Materials and Methods

Chemicals and Reagents

RPMI 1640, fetal bovine serum, glutamine, and gentamicin were obtained from Cambrex Bioscience (Walkersville, MD). Paclitaxel in sterile solution was obtained from Bristol Myers Squibb (Princeton, NJ). Bexarotene was synthesized at Ligand Pharmaceuticals, Inc. (San Diego, CA). Paclitaxel, vincristine, doxorubicin, cisplatin, methotrexate, and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma Chemicals (St. Louis, MO).

Cell Line

The human breast cancer cell line MDA-MB-231 and human umbilical vein endothelial cells (HUVEC) were obtained from the American Type Culture Collection (Manassas, VA) and Cambrex Bioscience, respectively. MDA-MB-231 cells were routinely cultured in RPMI 1640 supplemented with 10% fetal bovine serum and 2 mmol/L glutamine in 95% air-5% CO2. HUVECs were maintained in RPMI 1640, fetal bovine serum, glutamine, and gentamicin (1:100). HUVECs were obtained from the American Type Culture Collection (Manassas, VA) and Cambrex Bioscience, respectively. HUVECs were maintained in EGM-2 supplemented with basic fibroblast growth factor, heparin, epidermal growth factor, hydrocortisone, vascular endothelial growth factor, ascorbic acid, and 5% fetal bovine serum.

In vitro Drug Sensitivity Assay

To determine the drug effect after a single exposure, MDA-MB-231 cells were seeded in 96-well tissue culture plates. Stock solutions of each cytotoxic compound or bexarotene were dissolved in DMSO, and the final concentration of the solvent was <0.1%. MDA-MB-231 cells were exposed to the cytotoxic agent for 3 days and bexarotene for 6 days. Drug-induced growth inhibition was measured by MTT assay as described previously (17). To determine the effect of bexarotene/cytotoxic combination after multiple exposures, MDA-MB-231 cells were seeded at 2 × 10^4 in T-225 flasks overnight. The various combinations and treatment schemes are illustrated in Fig. 1. Briefly, the cells were exposed to the combination regimens on a 10-day cycle. Typically, exposures used a 3-day treatment cycle with the cytotoxic agent (or combination) and then were washed, counted, and replated followed by a 7-day exposure either to bexarotene or to control medium. At the end of each treatment cycle, the cells were trypanized, when possible counted, replated, and exposed to the same treatment regimen again. This procedure was repeated 10 times. For the cytotoxic agent alone regimen (Fig. 1, scheme 1), the cells were exposed to 30 nmol/L paclitaxel, 50 nmol/L doxorubicin, or 5 μmol/L cisplatin alone for 3 days followed by 7 days in control medium. For the sequential combination (Fig. 1, scheme 2), the cells were treated with a cytotoxic agent for 3 days followed by 1 μmol/L bexarotene for 7 days. For the simultaneous drug combination (Fig. 1, scheme 3), the cells were exposed to a cytotoxic agent and 1 μmol/L bexarotene for 3 days followed by a 7-day drug holiday. For the combination of intermittent cytotoxic agent with continuous bexarotene (Fig. 1, scheme 4), the cells were exposed to a cytotoxic agent and 1 μmol/L bexarotene for 3 days followed by 1 μmol/L bexarotene for 7 days. Control cells were treated similarly with fresh medium containing 0.1% solvent (not shown in Fig. 1). Cell growth in the presence of 1 μmol/L bexarotene either given continuously or on a 7-day on, 3-day off cycle was also evaluated as an additional control. Drug-induced growth inhibition was measured by trypan blue exclusion. The sensitivity of resistant variants to various cytotoxic agents was evaluated on 96-well tissue culture plates for 3 days. Drug effect was measured by MTT assay as described previously.

Measurement of Pgp Activity

The degree of Pgp efflux activity was quantified by measuring the accumulation of a fluorescent Pgp substrate calcein in the cytosol according to the manufacturer’s instruction (Molecular Probes, Eugene, OR). The intracellular accumulation of free calcein was measured with a fluorescence microplate reader (BioTek Instruments, Winnoski, VT) at excitation/emission wavelength of 485/530 nm.

RNA Preparation and Quantitative Real-time PCR

Total RNA was isolated using RNeasy Mini kit (Qiagen, Valencia, CA) according to the manufacturer’s instructions. RNA samples were eluted in RNase-free water and stored at −80°C. Total RNA (1 μg) was reverse transcribed into cDNA in a 50 μL reaction volume containing 1X reverse transcription buffer, 5.5 mmol/L MgCl2, 2 mmol/L deoxynucleotide triphosphates, 25 μmol/L random hexamer, 0.4 unit RNase inhibitor, and 1.25 unit MuLV reverse
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Figure 1. Treatment scheme for the bexarotene/cytotoxic combination. (1) Cytotoxic agent alone regimen: the cells were exposed to a single cytotoxic agent for 3 d followed by 7 d in control medium. (2) Sequential combination: the cells were treated with a single cytotoxic agent for 3 d followed by 1 μmol/L bexarotene for 7 d. (3) Simultaneous combination: the cells were exposed to a single cytotoxic agent in the presence of 1 μmol/L bexarotene for 3 d followed by a 7-d drug holiday. (4) Intermittent cytotoxic agent + continuous bexarotene: the cells were exposed to a single cytotoxic agent and 1 μmol/L bexarotene for 3 d followed by 1 μmol/L bexarotene for 7 d. Not shown: control cells were treated similarly with fresh medium containing 0.1% solvent or cell growth in the presence of 1 μmol/L bexarotene either given continuously or on a 7-d on, 3-d off cycle.

Transcriptase (Applied Biosystems, Foster City, CA). Human brain and liver cDNA were used as standards (Ambion, Austin, TX). The resulting cDNA was diluted in RNase-free water, aliquoted, and stored at −80°C. Real-time PCR was done using a dual-fluorescent nonextendable probe containing a 5′ 6-carboxyfluorescein reporter dye and a 3′ 6-carboxytetramethylrhodamine (TAMRA). cDNAs (50 ng each) were used for real-time PCR in a final volume of 50 μL containing 1× Taqman buffer (Applied Biosystems), 300 nmol/L of each forward and reverse primer, and 100 nmol/L probe. Reactions were carried out in an ABI PRISM 7700 sequence detection system (Applied Biosystems) for 40 cycles of 15 seconds at 95°C and 60°C. The level of expression of the target gene was normalized to the expression level of the housekeeping gene 36B4.

Primers and Probes for Real-time PCR

The primers and probes used in this study are as follows: mdr1 (Genbank accession no. M14758) forward 5′-AGGAAAGCCAATGCTATGACTTTA-3′, reverse 5′-CAACTTGGCCCCCTCTCTC-3′, and probe 6-carboxyfluorescein-ATGAAACTGCCTCATAAATTIGACACCTGG-TAMRA (23); human 36B4 (Genbank accession no. M17885) forward 5′-GCAAGACCCATGTTCCTT-3′, reverse 5′-TGTTTTCCAGGTCCTCCTG-3′, and probe 6-carboxyfluorescein-AGGGCTGTGGGTGCTGGTAM-3′; human abc11 (Genbank accession no. NM_003742) forward 5′-GACATGTCITGGAAGACCTT-3′, reverse 5′-GACGTTGGCCGATG-3′, and probe 6-carboxyfluorescein-AGCCCTAAAACTATGCTGTATCCTGGTC-GTAMRA; abc1 (Genbank accession no. NM_004996) forward 5′-TCATGTTGCCCCGTAATG-3′, reverse 5′-GATGTTGCTCTCCTATGGT-3′, and probe 6-carboxyfluorescein-ACCTGATACGTCTTGGTCTTCATCGCCA-3′; abcc1 (Genbank accession no. NM_000392) forward 5′-GTTCGATATACCAAATCCAGGCT-3′, reverse 5′-CCAGAAATTAGGAAGAACCAG-3′, and probe 6-carboxyfluorescein-TCTGTACACACCATTGTCTGGTC-3′; bcrp (Genbank accession no. AF098951) forward 5′-AGATGGGGITTCCAAGGACAT-3′, reverse 5′-GCCGACCCAGCACC-3′, and probe 6-carboxyfluorescein-CATCTCTCCACCTGTC-CAAGCCTA-TAMRA (24); bcrp (Genbank accession no. AF098951) forward 5′-AGATGGGGTTCCAAGGACAT-3′, reverse 5′-GCCGACCCAGCACC-3′, and probe 6-carboxyfluorescein-TGCAGGTGTTATAAGCCTCTT-3′; abcc3 (Genbank accession no. NM_003786) forward 5′-M17885) forward 5′-ACGCTCGCAAG-3′, reverse 5′-ACCACCTGCAATGCCTATGCTTTAA-3′, and probe 6-carboxyfluorescein-ACCTGATACGTCTTGGTCTTCATCGCCA-3′; abcc3 (Genbank accession no. NM_003786) forward 5′-M17885) forward 5′-ACGCTCGCAAG-3′, reverse 5′-ACCACCTGCAATGCCTATGCTTTAA-3′, and probe 6-carboxyfluorescein-ACCTGATACGTCTTGGTCTTCATCGCCA-3′; abcc3 (Genbank accession no. NM_003786) forward 5′-M17885) forward 5′-ACGCTCGCAAG-3′, reverse 5′-ACCACCTGCAATGCCTATGCTTTAA-3′, and probe 6-carboxyfluorescein-ACCTGATACGTCTTGGTCTTCATCGCCA-3′.

Cell Invasion and Angiogenesis Assays

The invasion assay was carried out in a Matrigel-coated 24-well Transwell unit (BD Biosciences, Bedford, MA). Briefly, MDA-MB-231 parental and drug-resistant cells were grown in culture medium until 80% confluence. Cells were trypsinized, resuspended in serum-free medium, and seeded at 1×10^5 in triplicate wells. The lower chambers were filled with culture medium containing 5% fetal bovine serum as a chemoattractant. The fraction of cells invading into and through the Matrigel matrix after 18 to 20 hours was quantified by MTT assay. To determine the ability of MDA-MB-231 parental and drug-resistant cells to modulate angiogenesis, 3×10^5 parental cells or drug-resistant variants were cultured in 10 mL
serum-free medium. The conditioned medium was collected 48 hours later. HUVECs (5 × 10^5) were seeded into the upper chamber of the Matrigel-coated Transwells. The conditioned medium from MDA-MB-231 parental cells and drug-resistant variants was used as chemoattractant and added to the lower chambers. The fraction of cells invading into and through the Matrigel matrix after 24 hours was quantified by MTT assay. Results of invasiveness and angiogenic potential were normalized with parental cells and expressed as an invasion index.

Fluctuation Analysis

MDA-MB-231 cells were seeded at 1,000 per flask in 75 cm^2 tissue culture flasks. The flasks were divided into two groups, with one group of cells grown in culture medium and the other group in 1 μmol/L bexarotene. Cells were allowed to grow to 80% confluence (average of 4.2 × 10^6 cells per flask from cells grown in the culture medium and 4.5 × 10^6 cells per flask from cells grown in the bexarotene-containing medium). The total cell population from each flask from both treatment groups was seeded onto separate 96-well plates overnight. For cultures that contained >3.5 × 10^6 cells per flask, only 3.5 × 10^6 cells were seeded on the plate to avoid high cell density per well. Cells grown in the culture medium during the expansion period were treated with 30 nmol/L paclitaxel for 7 days, whereas cells grown in 1 μmol/L bexarotene during the expansion period were treated with a combination of 30 nmol/L paclitaxel and 1 μmol/L bexarotene. Drug-containing medium was changed every other day for 7 days and then replaced by drug-free medium. Surviving colonies were counted 3 weeks later. In a control experiment, the bulk population of MDA-MB-231 cells (4 × 10^7 cells at 2 × 10^6 cells per plate) without expansion of the population before drug treatment was treated directly with paclitaxel to determine the number of drug-resistant variants in the total population. Mutation rate was calculated according to the method of Catcheside (26).

In vivo Animal Studies

For the human xenograft tumor model, MDA-MB-231 cells in log phase were harvested and resuspended in 1:1 (v/v) mixture of culture medium and Matrigel (BD Biosciences, San Diego, CA). Tumor cells were implanted s.c. into right and left axial regions of 6-week-old female athymic nude mice (Harlan, Madison, WI) with 25 gauge needle containing 5 × 10^6 cells/100 μL. Thereafter, fresh tumors harvested from donor mice were minced into 1 to 3 mm^3 in size. Approximately 25 to 30 fragments in 1:1 culture medium and Matrigel were injected s.c. into recipient mice with a 16 gauge needle. This process was repeated twice before initiation of the study. Animals were randomized 2 days after tumor injection. Treatment began when tumors were palpable (4–5 days after tumor injection). Each group consisted of 6 to 10 animals bearing two tumors per animal. Bexarotene was prepared in an aqueous solution containing 10% (v/v) polyethylene glycol (M_r 400)/Tween 80 (99.5:0.5) and 90% of 1% (w/v) carboxymethylcellulose (Sigma Chemicals) and dosed orally once daily at 100 mg/kg. Doxorubicin and cisplatin were dissolved in sterile saline. Paclitaxel was prepared freshly each time from the concentrated stock solution (6 mg/mL) with sterile saline. The cytotoxic agents were given i.p. once weekly. Doses used for cytotoxic agents and bexarotene were at the maximum tolerated dose, the dose that caused <10% weight loss during the study. Animals receiving no drugs were given vehicle for bexarotene orally daily and saline i.p. weekly. Animals receiving bexarotene only were given saline i.p. once weekly; animals receiving cytotoxic agent only were given vehicle for bexarotene orally daily. The treatment continued for 6 weeks. Tumor growth was measured with an electronic caliper (Mitutoyo, Inc., Utsunomiya, Japan) twice weekly. Tumor volumes were calculated using the formula: t/2ab^2, where a is the longest and b is the shortest axis of the tumor. Animal weights were recorded once weekly. The animals used in this study were housed in a U.S. Department of Agriculture–registered facility in accordance with NIH guidelines for the care and use of laboratory animals.

Data Analysis

Dose-response curves for growth inhibition were generated and plotted as a percentage of untreated control. The drug concentration needed to produce 50% growth inhibition, IC_50, was determined by nonlinear least squares regression (JMP, Cary, NC). Differences in mean values between groups were analyzed by unpaired Student’s t test with two-tailed comparison. Multiple comparisons used two-way ANOVA followed by Tukey-Kramer test. Differences of P < 0.05 are considered significant. Software for statistical analysis was by SigmaStat (SPSS, Inc., Chicago, IL).

Results

Effect of Bexarotene/Cytotoxic Combination on the Development of Acquired Drug Resistance In vitro

When used as a single agent, paclitaxel produced a sigmoidal concentration-dependent growth inhibition in MDA-MB-231 cells with an IC_50 of 3.17 ± 0.2 nmol/L (mean ± SD, n = 3), whereas bexarotene showed limited growth inhibitory activity up to 10 μmol/L. Bexarotene did not interfere or enhance paclitaxel activity in various combinations with paclitaxel after single exposure (data not shown). To determine the effect of the bexarotene/paclitaxel combination after multiple exposures, cells were subjected to repeated treatment for 10 cycles (see Fig. 1). After four treatment cycles, paclitaxel activity was not enhanced nor was it inhibited by the combination with bexarotene when compared with cells treated with paclitaxel alone (Fig. 2A). Cells treated with continuous bexarotene grew similarly as vehicle-treated cells (Fig. 2A). Repeated treatment with intermittent paclitaxel alone resulted in the development of paclitaxel resistance within 80 days (Fig. 2B; Fig. 1, scheme 1). Treatment with intermittent paclitaxel and continuous bexarotene (Fig. 1, scheme 4) resulted in complete growth inhibition, whereas the sequential combination (Fig. 1, scheme 2) and the
simultaneous combination (Fig. 1, scheme 3) delayed the development of paclitaxel resistance for 1 and 2 months, respectively, compared with cells treated with paclitaxel alone (Fig. 2B). Repeated exposure to bexarotene for 10 cycles did not alter cell growth (data not shown).

Because the combination of intermittent paclitaxel and continuous bexarotene prevented the development of paclitaxel resistance, we sought to determine whether this treatment regimen could be applied to other cytotoxic agents to prevent or delay acquired drug resistance. Two additional cytotoxic compounds, doxorubicin (a Pgp substrate) and cisplatin (a non-Pgp substrate), were used to evaluate the efficacy of the bexarotene/cytotoxic combination. Similar to paclitaxel, bexarotene did not interfere with the activity of either doxorubicin or cisplatin after single exposure (data not shown). Repeated exposures to the single cytotoxic agent alone resulted in development of acquired drug resistance, whereas the combination treatment delayed the growth of the cells (Fig. 2C and D) and remained chemosensitive (see below).

Characteristics of Resistant Variants
The surviving cells after prolonged treatment with a single cytotoxic agent or with bexarotene were isolated and evaluated for their drug sensitivity. Cells isolated from paclitaxel or doxorubicin alone were cross-resistant to other Pgp substrates but responded to the non-Pgp substrate cisplatin (Table 1A and B). Cells isolated from the cisplatin-treated culture were resistant to cisplatin alone (Table 1C). On the other hand, the surviving cells isolated from the intermittent cytotoxic agent and continuous bexarotene (Fig. 1, scheme 4) remained chemosensitive toward all the compounds tested (Table 1).

It was apparent that the resistant cells isolated from the various bexarotene/paclitaxel combinations showed a differential multidrug-resistant (MDR) phenotype (Table 1A). The resistant cells from the sequential combination (Fig. 1, scheme 2) showed a 20-fold resistance to tubulin agents and a 3-fold resistance to doxorubicin, whereas the resistant cells from the simultaneous combination (Fig. 1, scheme 3) showed a 6-fold resistance to paclitaxel but were sensitive to other tubulin agents and doxorubicin. To further examine the mechanism of the MDR phenotype in paclitaxel-resistant cells, the efflux activity of Pgp and mdr1 gene expression was determined. As seen in Fig. 3A, the extent of paclitaxel resistance (Table 1A) was correlated with the efflux activity of Pgp, being highest in paclitaxel-resistant cells followed by cells derived from the sequential combination and lowest in cells derived from simultaneous combination. Addition of

Figure 2. Effect of bexarotene/cytotoxic combination on growth of MDA-MB-231 after multiple exposures. A, comparison of total cell numbers in vehicle control, bexarotene alone, paclitaxel alone, and various bexarotene/paclitaxel combinations for the first four cycles. Effect of bexarotene on the development of acquired drug resistance in MDA-MB-231 cells: (B) paclitaxel, (C) doxorubicin, and (D) cisplatin. MDA-MB-231 cells were subjected to various treatments as described in Fig. 1. At the end of each treatment cycle (10 d), cells were harvested by trypsinization. Viable cells were determined by trypan blue exclusion and reexposed to the same treatment. The procedure was repeated 10 times. Because only 5% of the cells for the vehicle control and bexarotene-treated groups were reseeded for the following cycle, and assuming the growth rate of these cells represents the growth rate of the total population, projected cell numbers were shown for these two groups in A.
bexarotene had no effect on the efflux activity in the paclitaxel-resistant cells, whereas the Pgp inhibitor verapamil significantly decreased the efflux activity of Pgp in these cells (data not shown). To elucidate the mechanism of increased Pgp efflux activity in the resistant cells, real-time PCR was used to analyze mRNA levels for genes known to be involved in drug resistance: \textit{mdr1} (Pgp), \textit{abcb11} (MDR/TAP), \textit{abcc1} (MRP1), \textit{abcc2} (MRP2), \textit{abcc3} (MRP3), \textit{bcrp} (breast cancer–resistant protein), and \textit{mvp} (lung-resistant protein). Our data showed that \textit{mdr1} mRNA was barely detected in MDA-MB-231 parental cells or cells treated with bexarotene but was increased 150-fold in cells treated with paclitaxel alone, 50-fold in cells treated with sequential combination, and 20-fold in cells treated with simultaneous combination (Fig. 3B). The expression of other resistant genes was not altered in the paclitaxel-resistant variants (data not shown). The level of \textit{mdr1} mRNA expression was consistent with the degree of efflux activity of Pgp and MDR phenotype (compare Fig. 3 and Table 1A). Taken together, these results showed that increased \textit{mdr1} mRNA expression was responsible for the MDR phenotype in paclitaxel-resistant cells. Our data further showed that bexarotene can influence the MDR phenotype in paclitaxel-resistant cells through modulation of \textit{mdr1} mRNA expression.

Effect of Bexarotene/Cytotoxic Combinations on Overcoming Acquired Drug Resistance

To determine whether acquired drug resistance can be overcome by the combination of intermittent cytotoxic agent and continuous bexarotene, resistant cells derived from a single cytotoxic agent were repeatedly treated intermittently with the same cytotoxic agent and continuous bexarotene (similar to Fig. 1, scheme 4). Treatment with the cytotoxic agent alone or bexarotene alone had no

**Table 1. Comparison of drug sensitivity between MDA-MB-231 parental cells and acquired drug-resistant cells**

<table>
<thead>
<tr>
<th>Cells</th>
<th>Drugs</th>
<th>Paclitaxel RF</th>
<th>Vincristine RF</th>
<th>Doxorubicin RF</th>
<th>Cisplatin RF</th>
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<td></td>
<td></td>
<td></td>
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<td>1</td>
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<td><strong>B. Doxorubicin</strong></td>
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<td>Intermittent doxorubicin + continuous bexarotene</td>
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**NOTE:** Mean ± SD, \( n = 3 \) from three separate experiments. MDA-MB-231 parent cells and cells recovered from the cytotoxic agent treatment alone or from the combination were tested for the sensitivity to paclitaxel, vincristine, doxorubicin, and cisplatin at various concentrations for 3 days. Drug effects were measured by MTT assay. IC\(_{50}\), the concentration needed to inhibit 50% of cell growth for each drug, was determined. Results were normalized with the parental cells and expressed as the resistant factor (RF).

Figure 3. Analysis of efflux activity of Pgp and \textit{mdr1} expression in MDA-MB-231 parental cells and paclitaxel-resistant variants. A, Pgp activity was quantified by the fluorescence intensity of free calcine inside the cells. B, \textit{mdr1} expression was analyzed by real-time PCR. The expression of a housekeeping gene was used to normalized gene expression level. Columns, mean (\( n = 3 \)); bars, SD. BEX, bexarotene; PAL, paclitaxel. *, \( P < 0.05 \), statistically different from parental cells.
effect on the growth of drug-resistant cells. However, the drug-resistant cells were resensitized to the cytotoxic agent after multiple exposures to the bexarotene/cytotoxic combination (Fig. 4A–C). In general, the reversal of drug resistance became apparent after repeated treatment for three cycles. The remaining viable fraction of cells continued to decrease with increasing treatment cycles, reaching <1% of untreated control after 8 to 10 treatment cycles.

**Effect of Bexarotene on Invasion and Angiogenic Ability**

Several lines of evidence have suggested that tumor cells selected for drug resistance are more invasive than their parental cells (27, 28). To determine whether bexarotene can influence invasiveness and angiogenesis, cells derived from single cytotoxic agents (drug-resistant) and from the combination (drug-sensitive) were subjected to Matrigel invasion and angiogenesis assays. As seen in Fig. 5, drug-resistant cells showed a 2-fold increase in invasiveness and angiogenic potential compared with the parental cells. In contrast, cells treated with the combination regimen showed a 50% reduction in invasiveness and angiogenic ability compared with the parental cells. The extent of invasiveness and angiogenesis of these cells was further correlated with the degree of drug resistance (see Table 1). Resistant cells that were treated with bexarotene alone or in combination also showed a decreased invasiveness and angiogenic ability compared with cells treated with single cytotoxic agents (data not shown).

**Effect of Bexarotene on Mutation Rate**

The ability of bexarotene to prevent and overcome multidrug resistance and reduce invasion and angiogenesis suggested that bexarotene may maintain and/or increase genomic integrity of the cells to prevent the cancer cell from modifying its genome resulting in the acquisition of drug resistance. To test this possibility, the effect of bexarotene on the frequency of developing drug resistance was evaluated using Luria-Delbrück fluctuation analysis (29). As seen in Table 2, no surviving clones were found in the bulk population of 2 × 10^7 cells treated with paclitaxel in a single step (control group), indicating that preexisting paclitaxel-resistant variants in the MDA-MB-231 cell population was minimal. In the fluctuation groups, the average number of colonies per plate in paclitaxel-selected cells was 2.4, whereas the average number of colonies per plate in the bexarotene-treated cultures decreased 6-fold. Because the variance in the number of surviving colonies per plate exceeded the mean in the fluctuation groups, the above findings suggested that paclitaxel resistance in these clones arose spontaneously rather than being induced by environmental selection. The mutation rate in the cells expanded in control medium was calculated to be 7.3 × 10^-8 per cell generation, whereas those treated with bexarotene during the expansion period had a 7-fold reduction in the mutation rate (P < 0.05, t test). Similar results were obtained by MSS maximum likelihood method (ref. 30; data not shown). The above findings suggest that the interference with developing paclitaxel resistance by the combination was due to a reduction in the spontaneous mutation rate by bexarotene.

**Effect of Bexarotene/Cytotoxic Combination In vivo**

As seen in Fig. 6, MDA-MB-231 tumors grew continuously throughout the course of study in both control and drug-treated animals. When compared with vehicle control, bexarotene given alone at 100 mg/kg daily had no effect on tumor growth. Treatment with doxorubicin (5 mg/kg)

![Figure 4. Effect of bexarotene/cytotoxic combination on reversal of acquired drug resistance: (A) paclitaxel, (B) doxorubicin, and (C) cisplatin. Paclitaxel-, doxorubicin-, or cisplatin-resistant MDA-MB-231 cells were repeatedly treated with vehicle, cytotoxic agent alone, bexarotene alone, or intermittent drug with continuous bexarotene. Parental cells were used as reference control and treated with a given cytotoxic drug for 3 d. Viable cells were determined by trypan blue exclusion at the end of each treatment cycle. Points, mean (n = 3); bars, SD.](mct.aacrjournals.org)
decreased tumor volume by ~40% (P < 0.05 versus control; Fig. 6B), whereas no significant difference in tumor volumes were seen in animals treated with paclitaxel (20 mg/kg) or cisplatin (5 mg/kg) relative to the vehicle control (Fig. 6A and C). On the other hand, the combination of bexarotene/cytotoxic agent reduced tumor volume by ~40% relative to cytotoxic agents alone (P < 0.05 in all cases) and by 60% relative to vehicle control (P < 0.05 in all cases). The benefit of adding bexarotene to the cytotoxic regimen became apparent beyond 3 weeks as the tumor volume in mice receiving the combination therapy began to diverge from the tumor volume in mice treated with the cytotoxic agent alone. In a separate study, animals were treated with paclitaxel for 2 weeks followed by the bexarotene/paclitaxel combination. Our data showed that treatment with paclitaxel followed by the combination resulted in a 40% decrease in tumor growth compared with paclitaxel alone (Fig. 6D). Taken together, the above findings indicated that the combination regimen produced significant decrease in tumor volume than single agents in MDA-MB-231 xenograft model. Our data further showed that treatment with a single cytotoxic agent followed by the combination resulted in a much greater delay in tumor growth compared with the single cytotoxic agent alone.

**Discussion**

The present study shows that the bexarotene/cytotoxic combination can delay or prevent the development of acquired drug resistance in MDA-MB-231 cells. The extent of acquired paclitaxel resistance correlated with the MDR phenotype and efflux activity of Pgp and mdr1 gene expression. Cells with acquired drug resistance (paclitaxel, doxorubicin, and cisplatin), when treated with a bexarotene/cytotoxic combination, were resensitized to the cytotoxic agent. Interestingly, cells treated with the combination regimen showed reduced invasiveness and angiogenic ability than their resistant counterparts. The *in vivo* MDA-MB-231 xenograft model showed that the bexarotene/cytotoxic combination produced a statistically significant greater antitumor efficacy than single agents. Our findings suggest that the bexarotene/cytotoxic combination may be an efficacious treatment for advanced breast cancer by interfering with tumor cell progression to a more malignant drug-resistant, invasive phenotype.

Genetic instability of cancer cells is thought to be one of the major factors giving rise to drug-resistant mutant or variant subpopulations (31, 32). Our data show that long-term exposure to the bexarotene/cytotoxic combination can...
delay and overcome acquired drug resistance and decrease invasive and angiogenic capacity. We hypothesize that bexarotene may increase and/or maintain the genomic integrity of cells to prevent the cancer cell from modifying its genome resulting in the development of acquired drug resistance. This hypothesis was further supported by Luria-Delbrück fluctuation analysis. As seen in Table 2, treatment with bexarotene decreased the spontaneous development of paclitaxel resistance. In theory, if resistance was induced by drug exposure, the number of surviving colonies would be expected to have a Poisson distribution, with the variance close to the mean (29). Our data showed that the variance in the number of surviving colonies per plate was much greater than the mean, indicating that paclitaxel-resistant variants in MDA-MB-231 cells arose randomly rather than being induced by drug exposure. Because pretreatment with bexarotene followed by drug selection significantly decreased the spontaneous mutation rate compared with expansion without bexarotene, this suggests that bexarotene can maintain/increase genomic integrity of the tumor cells by interfering with the acquisition of spontaneous mutations that result in drug resistance.

The molecular mechanism by which bexarotene modulates drug-resistant genes and maintains genomic integrity is unknown at present. Several possibilities exist. First, the tumor suppressor gene p53 functions to maintain genomic integrity by preventing cells with unstable genomes from transiting through the cell cycle. The functional role of p53 as a central mediator of the DNA damage and regulation of apoptosis is well established (33, 34). In addition, wild-type p53 has been shown to repress mdr1 promoter activity, mdrl expression, and Pgp protein level, whereas mutant p53 stimulates such effects (35–37). Recent studies have indicated that a small ubiquitin-like modifier protein is involved in p53 stability, function, and transcriptional regulation and genomic stability (38, 39). The accumulated evidence suggests the important role of wild-type p53 in DNA repair, regulation of apoptosis pathways, and mdrl gene expression level. MDA-MB-231 human breast cancer cells contain mutant p53 (22). It is possible that bexarotene may interfere with mutant p53-mediated cell survival after multiple exposures to cytotoxic agents through inhibiting mutant p53 activity and/or restoring a wild-type p53 phenotype. Second, aneuploidy is one of the most common genomic abnormalities of cancer cells (40). Early studies by Duesberg et al. showed that genomic instability of cancer cells was proportional to the degree of aneuploidy (41). These investigators further showed that aneuploid cells can acquire multidrug resistance by chromosome reassortments in the absence of multidrug resistance genes (42). Thus, bexarotene may maintain/increase genomic integrity through stabilizing DNA ploidy. Third, the nuclear factor-κB has been shown to play an important role in controlling apoptotic cell death (43, 44) and the expression of the mdrl gene (45). Furthermore, we have recently shown that a novel rexinoid LG100268 inhibited nuclear factor-κB activity to increase the activity of chemotherapeutic agents (46). It is possible that bexarotene may interfere with drug-resistant gene expression through inhibition of nuclear factor-κB to prevent and overcome multidrug resistance. Fourth, the steroid and xenobiotic receptor is a member of the nuclear hormone receptor superfamily that heterodimerizes with retinoid X receptor. It was reported that paclitaxel could enhance mdrl gene expression through the steroid and xenobiotic receptor in both primary hepatocytes and colon cancer cells, thereby increasing its own clearance and leading to the development of drug resistance (47). The steroid and xenobiotic receptor was also found to be involved in cisplatin resistance by inducing glutathione S-transferase expression in endometrial cancer cells (48). Bexarotene may directly or indirectly antagonize the steroid and xenobiotic receptor activity to prevent paclitaxel- and cisplatin-induced expression of drug-resistant genes. Taken together, bexarotene may interfere with one or more of the

### Table 2. Luria-Delbrück fluctuation analysis of paclitaxel-resistant MDA-MB-231 cells

<table>
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<tr>
<th>Plate</th>
<th>Paclitaxel, colonies per plate</th>
<th>Bexarotene + Paclitaxel, colonies per plate</th>
<th>Control group</th>
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</table>

NOTE: Individual populations at 1,000 cells per flask were allowed to expand in either the culture medium or 1 μmol/L bexarotene until 80% confluence. The total cell population from each flask was seeded onto separate 96-well plates and exposed to 30 nmol/L paclitaxel. Cells grown in 1 μmol/L bexarotene during expansion period were treated with combination of 30 nmol/L paclitaxel and 1 μmol/L bexarotene. Treatment was continued for 7 days. The surviving clones were counted 3 weeks later. Control group used the bulk populations of cells (4 × 10²7 cells per plate) from mass cultures before drug selection. NA, not applicable.
above-mentioned pathways to suppress multidrug resistance gene expression. Ongoing research focuses on elucidating the mechanism of action of bexarotene in maintaining genomic integrity to prevent and overcome multidrug resistance. In the present study, we observed that cells that developed acquired drug resistance showed an increased invasiveness and angiogenic potential, whereas cells treated with the combination regimen were chemosensitive and had reduced invasive and angiogenic ability. Coexpression of drug resistance and invasion/metastasis has been observed in patient samples (49, 50). Cells that coexpress these properties are due either to selection of more aggressive cells or to increase in the metastatic potential following chemotherapeutic insults (27). It is likely that the bexarotene/cytotoxic combination interfered with increased survival and tumor progression after drug exposure through maintaining and/or increasing genomic integrity. Using cDNA microarray analysis, we have recently identified several bexarotene-regulated genes, many of which were important for drug resistance, metastasis, and angiogenesis.1 We are currently evaluating the functional roles of these genes.

Our present findings have important implications for patients with breast cancer as well as other diseases. For example, we have recently reported that the bexarotene/paclitaxel combination delayed and overcame paclitaxel resistance in non–small cell lung cancer patients (52). Ongoing research is directed toward understanding the mechanisms by which bexarotene prevents and overcomes acquired and intrinsic drug resistance. Information obtained from these studies will have significant impact on the therapeutic use of bexarotene in cancer treatment.

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Bexarotene and Drug Resistance in Breast Carcinoma


Molecular Cancer Therapeutics

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