A Selective Retinoid X Receptor Agonist Bexarotene (Targretin) Prevents and Overcomes Acquired Paclitaxel (Taxol) Resistance in Human Non–Small Cell Lung Cancer

Wan-Ching Yen, Manny R. Corpuz, Rene Y. Prudente, Tracy A. Cooke, Reid P. Bissonnette, Andres Negro-Vilar, and William W. Lamph

Department of Molecular Oncology, Ligand Pharmaceuticals, Inc., San Diego, California

ABSTRACT

Purpose: Paclitaxel is an important anticancer agent for the treatment of non–small cell lung cancer (NSCLC). However, its use in cancer therapy is limited by development of acquired drug resistance. The goal of this study was to determine the effect of bexarotene on development of acquired paclitaxel resistance in NSCLC.

Experimental Design: Human NSCLC Calu3 cells were repeatedly treated in culture with intermittent paclitaxel alone or in combination with continuous bexarotene for 3 months. Thereafter, cells were isolated and characterized for their drug sensitivity in vitro and in vivo.

Results: Repeat exposure to paclitaxel alone resulted in development of paclitaxel resistance with cross-resistance to multidrug resistance P-glycoprotein substrates, whereas the bexarotene/paclitaxel combination prevented the development of drug resistance and the cells remained chemosensitive. Furthermore, paclitaxel resistance could be overcome when the resistant cells were treated with the combination regimen. Fluctuation analysis showed that treatment with bexarotene decreased the rate of spontaneous development of paclitaxel resistance. In vivo, the bexarotene/paclitaxel combination regimens produced a statistically significant decrease in tumor growth in a Calu3 NSCLC xenograft model compared with the single agents (two-tailed, \( P < 0.05 \)). In addition, paclitaxel-resistant Calu3 tumors treated with the bexarotene/paclitaxel combination showed greater delay in tumor growth compared with those treated with paclitaxel alone.

Conclusions: Our results suggest that bexarotene may offer a novel approach to prevent and overcome paclitaxel resistance in patients with NSCLC.

INTRODUCTION

Lung cancer is the leading cause of cancer death for both men and women (1). The American Cancer Society estimates that in 2004 there will be \( \sim 173,770 \) new cases of lung cancer in the United States and \( \sim 160,440 \) people will die of this disease. Non–small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for \( \sim 80\% \) of lung cancers. Chemotherapy remains the only treatment option for patients with unresectable NSCLC; however, the current 5-year survival rate has not improved with this therapy (2). Paclitaxel is among the most promising new agents in the treatment of advanced NSCLC. Most patients with advanced NSCLC initially respond to paclitaxel (>30% response rate); however, overall survival has not improved in part because of the development of acquired paclitaxel resistance (2). Studies in human tumor cell lines reveal several mechanisms of paclitaxel resistance (3, 4). The best understood mechanism of paclitaxel resistance is the expression of the P-glycoprotein efflux pump (Pgp), encoded by the mdr1 gene, which is responsible for the multidrug resistance (MDR) phenotype (5). Pgp-mediated resistance to paclitaxel is characterized by a decrease in intracellular paclitaxel accumulation accompanied by cross-resistance to many structurally and functionally distinct anticancer agents, including anthracyclines (doxorubicin and epirubicin), Vinca alkaloids (vincristine and vinblastine), epipodophyllotoxins, and camptothecins (3, 4). Using immunohistochemistry and Tc-99m methoxyisobutylisonitrile chest single photon emission computed tomography, Shih et al. (6) showed that the level of Pgp expression and Tc-99m methoxyisobutylisonitrile chest single photon emission computed tomography. Background information and previous research suggest that Pgp expression correlates with Pgp gene expression (7). Our results suggest that bexarotene may offer a novel approach to prevent and overcome paclitaxel resistance in patients with NSCLC.

Approaches to overcoming Pgp-mediated drug efflux with Pgp inhibitors have suggested that these agents may increase drug activity in previously resistant patients (7–9). Interpretation of these results is complicated because systemic clearance of Pgp substrates was in part dependent on expression of the Pgp family of transporters (ATP-binding cassette transporters). The use of Pgp efflux inhibitors not only restores drug sensitivity of the tumor but also prolongs the clearance and enhances toxic side effects of the individual anticancer agents in patients (9, 10). Thus, new treatment methods are needed to prevent and overcome Pgp-mediated drug resistance.

Bexarotene (also known as Targretin), a selective retinoid X receptor ligand (11), has been shown to be an efficacious...
chemopreventive and chemotherapeutic agent in preclinical ro-
dent breast cancer models (12–15). Furthermore, in the rat
N-nitroso-N-methylurea-induced mammary carcinoma model,
tumors that were resistant to tamoxifen responded to both
bexarotene and the bexarotene/tamoxifen combination (16).
Mechanistically, tumor regression by bexarotene in the rat N-
nitroso-N-methylurea-induced mammary tumors involved dif-
ferentiation induction along the adipocyte lineage, leading
to terminal cell division followed by cell death (17). To addi-
tionally evaluate the role of bexarotene in treatment of solid tumors,
we studied the influence of bexarotene on the development and
treatment of paclitaxel resistance in human NSCLC. Our results
showed that the bexarotene/paclitaxel combination prevented
and overcame acquired paclitaxel resistance in NSCLC Calu3
cells. Fluctuation analysis indicated that the combination regi-
men decreased the spontaneous development of paclitaxel re-
sistance. In tumor xenografts, the bexarotene/paclitaxel combi-
nation produced a statistically significant decrease in tumor
growth when compared with vehicle control and to single agents
alone.

MATERIALS AND METHODS

Chemicals and Reagents. RPMI 1640, fetal bovine se-
rum, glutamine, and gentamicin were obtained from Life Tech-
nologies, Inc. (Grand Island, NY). Paclitaxel in sterile solution
of 6 mg/mL in 1:1 (v/v) in Cremophor, EL, and dehydrated
alcohol was obtained from Bristol Meyer Squibb (Princeton,
NJ). Bexarotene was synthesized at Ligand Pharmaceuticals,
Inc. (San Diego, CA). Paclitaxel, vincristine, doxorubicin, cis-
platin, cyclosporin A, verapamil, and 3-(4,5-dimethylthiazol-2-
yl)-2,5-diphenyltetrazolum bromide (MTT) were from Sigma
Chemicals (St. Louis, MO). Stock paclitaxel and bexarotene
solutions were dissolved in DMSO. Sufficient volumes of each
solution were added to the culture medium so that the final
concentration of the solvent was <0.1%.

Cell Line. The human NSCLC cell line Calu3 was ob-
tained from the American Type Culture Collection (Manassas,
VA). Cells were routinely cultured in RPMI 1640 supplemented
with 10% fetal bovine serum and 2 mmol/L glutamine in 95%
air-5% CO₂.

In vitro Drug Sensitivity Assay. To determine the drug
effect after a single exposure, Calu3 cells were seeded in 96-
well tissue culture plates. The cells were exposed to various
concentrations of paclitaxel for 3 days or bexarotene for 6 days.
Drug-induced growth inhibition was measured by MTT assay.
Briefly, 50 μL of MTT solution (at 5 mg/mL in sterile water)
were added to each well and incubated for 1 hour at 37°C. The
MTT-formazan formed by viable cells was dissolved in 100 μL
of DMSO, and the absorbance was measured by a microplate
reader (BioTek Instruments, Winnoski, VT) at a wavelength of
570 nm. To determine the effect of bexarotene/paclitaxel on
multiple exposures, Calu3 cells were seeded at 2 × 10⁶ cells in
T-225 flasks overnight. The 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 with the cytotoxic agent (or combination), then
washed, counted, and replated, followed by either a 7-day ex-
posure to bexarotene or to control medium. At the end of each
treatment cycle, the cells were trypsinized, when possible,
counted, and then replated onto a new flask and exposed to the
same treatment regimen again. This procedure was repeated 10
times. For the paclitaxel-alone regimen (scheme 1; Fig. 1), the
cells were exposed to 30nmol/L paclitaxel for 3 days followed
by 7 days in control medium. For the combination of intermit-
tent paclitaxel with continuous bexarotene (scheme 2; Fig. 1),
the cells were exposed to 30nmol/L paclitaxel and 1μmol/L LGD1069 for 7 days. Control cells were treated similarly with fresh medium containing 0.1% solvent or cell growth in
the presence of 1μmol/L LGD1069 either given continuously was also
evaluated as an additional control (data not shown).

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 (Molecular
Probes, Eugene, OR). Calu3 control and drug-treated cells were
seeded on opaque 96-well plates at 20,000 cells per well over-
night. Thereafter, cells were incubated with 50 μL of 240
μmol/L Pgp inhibitor verapamil, 120 μmol/L cyclosporin A, or
culture medium for 30 minutes at 37°C. After the first incuba-
tion, each well received 1μmol/L Calcein AM and incubated for
an additional 30 minutes at 37°C. The final concentration of
verapamil, cyclosporin A, and Calcein AM were 60, 30, and
0.25 μmol/L, respectively. At the end of second incubation,
cells were washed three times with 200 μL of cold culture
medium. The intracellular accumulation of free Calcein was
measured with a fluorescence microplate reader (BioTek Instru-

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Fig. 1 Treatment scheme for the bexarotene (LGD1069)/paclitaxel
combination. 1. paclitaxel alone regimen: the cells were exposed to 30
nmol/L paclitaxel for 3 days followed by 7 days in control medium.
2, intermittent paclitaxel with continuous LGD1069: the cells were ex-
posed to 30 nmol/L paclitaxel and 1 μmol/L LGD1069 for 3 days
followed by 1 μmol/L LGD1069 for 7 days. Control cells were treated
similarly with fresh medium containing 0.1% solvent or cell growth in
the presence of 1 μmol/L LGD1069 either given continuously was also
evaluated as an additional control (data not shown).
ments, Winnoski, VT) with excitation/emission wavelength 485/530 nm.

RNA Preparation and Quantitative Real-Time PCR. Total RNA was isolated from 2 to 5 × 10^6 cells with RNase-free water and stored at −80°C. Total RNA (1 μg) was reverse transcribed into cDNA in a 50-μL reaction volume containing 1 × reverse transcription buffer, 5.5 mM MgCl₂, 2 mM dNTPs, triphosphates, 2.5 μM random hexamer, 0.4 unit of RNase inhibitor, and 1.25 units of murine leukemia virus reverse transcriptase (Applied Biosystems, Foster City, CA). Human brain and liver cDNA were used as standards (Ambion, Austin, TX). One μg of human RNA was used for reverse transcription reaction. The resulting cDNA was diluted in RNase-free water, aliquoted, and stored at −80°C. The real-time PCR was done with a dual-fluorescent nonextendable probe containing a 5′-FAM (6-carboxyfluorescein) reporter dye and a 3′-TAMRA (6-carboxy-tetramethylrhodamine). Fifty ng of each cDNA were used for real-time PCR in a final volume of 50 μL containing 1 × Taqman buffer (Applied Biosystems), 300 mM of each forward and reverse primer, and 100 mM 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 seconds at 60°C. The level of expression of the target gene was normalized to the expression level of the house keeping gene 36B4.

Primers and Probes for Real-Time PCR. The primers and probes used in this study are as follows: mdrl (GenBank accession no. M14758) forward 5′-agagaagatccctagctacatt-3′, reverse 5′-ccagcctgctctctgctc-3′, and probe FAM-atgaaactgc-gcagatccgcatgtccctt-3′ (20); abcb11 (GenBank accession no. AF098081) forward 5′-ccagtcccagtauactgacgtgaca-3′, reverse 5′-ccttggcagtcccggtg-3′, and probe FAM-acggccatgctc-gagtggctaca-3′ (20); abcc2 (GenBank accession no. NM_000392) forward 5′-gagcgttgccggagtaatccccaggcctctatagc-TAMRA (20); and abcc3 (GenBank accession no. NM_017458) forward 5′-gacatgcttgcgaggacctt-3′, reverse 5′-gttcgatatacc-gtgcaccattgtc-3′, and probe FAM-tctgtacacaccattgtctgctgtattggatcag-TAMRA;

Fluctuation Analysis. Thirty 25-cm² tissue culture flasks were seeded with Calu3 cells at low density (1000 cells per flask). The flasks were divided into two groups with one group of cells grown in culture medium and the other group in 1 μM/mL bexarotene. Cells were allowed to grow to near confluence (average 2 × 10^6 cells per flask). The total cell population from each flask was seeded onto separate 96-well plates overnight and treated with 100 mM/mL paclitaxel for 7 days. Preliminary experiments showed that this drug concentration resulted in 99% cell kill. Cells grown in 1 μM/mL bexarotene during the expansion period were treated with the combination of 100 mM/mL paclitaxel and 1 μM/mL bexarotene. Drug-containing medium was changed every other day for 7 days and then replaced by drug-free medium. Surviving colonies were allowed to grow for another 3 weeks, counted, and were then individually harvested and propagated in drug-free medium for additional studies. In a control experiment, the bulk population of Calu3 cells (1.5 × 10^7 at 1 × 10^6 cells per plate) without expansion of the population before drug treatment were treated directly with paclitaxel. Mutation rate was calculated by the method of Catchside (21).

In vivo Animal Studies. For human xenograft tumor model, Calu3 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 the right and left axial regions of 6-week-old male athymic nude mice (Harlan, Madison, WI) with a 25-gauge needle containing 0.5 × 10^6 cells/100 μL. Animals were randomized, and treatment began when tumors were palpable (4 to 5 days after tumor injection). Each group consisted of 8 to 10 animals bearing two tumors per animal. Bexarotene was suspended in an aqueous solution containing 10% (v/v) polyethylene glycol (M, 400)/Tween 80 (99.5:0.5) and 90% of 1% (w/v) carboxymethyl cellulose (Sigma Chemicals, St. Louis, MO) and dosed orally once daily at 100 mg/kg. This dose of bexarotene was previously determined as the maximum-tolerated dose, the dose that caused <10% weight loss over the course of the study (12, 15). Paclitaxel was prepared fresh each time from concentrated stock solution with sterile saline and was given at 20 mg/kg i.p. once a week. The reported maximum-tolerated dose for paclitaxel was 25 mg/kg (22). Our preliminary study indicated that this dose resulted in a >10% decrease in body weight after 4 weeks of dosing; consequently, the dose was reduced to 20 mg/kg and used in this study for chronic treatment. Animals receiving no drugs were given vehicle for bexarotene orally every day and saline i.p. every week. Animals receiving bexarotene only were given saline i.p. once a week; animals receiving paclitaxel 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 with the formula, \( V = \frac{a \times b^2}{2} \), where \( a \) was the longest and \( b \) was the shortest axis of the tumor. Animal weights were recorded once weekly. The animals used in this study were housed in a United States 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 were plotted as a percentage of untreated control. Values for IC_{50} (the drug concentration needed to produce 50% growth inhibition) were determined by nonlinear least square regression (JMP, Cary, NC). Differences in mean values between groups were analyzed by unpaired Student’s t test with two-tailed comparison. Multiple comparisons

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used one-way ANOVA test with posthoc t test comparison. Differences of $P < 0.05$ are considered significantly different. Software for statistical analysis was by SigmaStat (SPSS, Inc., Chicago, IL).

RESULTS

In vitro Growth Inhibition by Bexarotene and Paclitaxel. When used as a single agent, paclitaxel produced a sigmoidal concentration-dependent growth inhibition in Calu3 cells. The concentration needed to inhibit 50% of cell growth, IC$_{50}$, was 21.1 ± 3.3 nmol/L (mean ± SD, n = 3). On the other hand, bexarotene showed limited growth inhibitory activity up to 10 μmol/L. Bexarotene did not interfere with nor enhance paclitaxel activity in the combination with paclitaxel after single exposure or multiple exposures (Fig. 2A). Furthermore, after repeated treatment for six 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 controls (Fig. 2A). However, surviving cells regrew within 80 days (Fig. 2B) after repeated treatment with paclitaxel alone (scheme 1; Fig. 1). Treatment with intermittent paclitaxel and continuous bexarotene (scheme 2; Fig. 1) delayed regrowth of the cells for additional 2 months compared with cells treated with paclitaxel alone (Fig. 2B). Repeated exposure to bexarotene for 10 cycles did not alter cell growth (data not shown).

Involvement of Pgp-mediated MDR in Paclitaxel-resistant Cells. Surviving cells obtained from paclitaxel alone or from the combination at the end of treatment cycles were evaluated for resistance to other cytotoxic agents. The surviving cells isolated from paclitaxel-treated cultures showed cross-resistance to Pgp substrates vincristine and doxorubicin but responded to non-Pgp substrate cisplatin (Table 1). On the other hand, cells recovered after treatment with the bexarotene/paclitaxel combination remained chemosensitive to both Pgp and non-Pgp substrates (Table 1). These results suggest the presence of a Pgp-mediated MDR phenotype in the paclitaxel-resistant cells.

To verify the functional presence of the Pgp membrane pump, the efflux activity of Pgp was measured by the intracellular accumulation of a Pgp substrate, Calcein. As seen in Fig. 3A, paclitaxel-resistant Calu3 cells showed a 5-fold increase in efflux activity compared with parental cells. The efflux activity profile for cells recovered after treatment with bexarotene alone and the combination was similar to that of parental cells, indicating that long-term exposure to bexarotene did not alter the efflux activity of Pgp (Fig. 3A). Addition of the Pgp inhibitor verapamil or cyclosporin A had no effect on the efflux activity in the parental cells, in cells treated with bexarotene alone, or in the combination but significantly decreased the efflux activity of Pgp in paclitaxel-resistant cells (data not shown). These results confirmed the presence of Pgp-mediated efflux in paclitaxel-resistant cells and showed lack of Pgp activity in the parental and bexarotene-treated cells. 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: mdr1 (Pgp), abcb11 (MDR/TAP), abcc1 (MRP1), abcc2 (MRP2), abcc3 (MRP3), bcrp (breast cancer-resistant protein), and mvp (lung-resistant protein). Our data showed that mdr1 mRNA expression was low in Calu3 parental cells, in cells treated with bexarotene alone, or in the combination but was increased 300-fold in the paclitaxel-resistant cells (Fig. 3B). A 4-fold increase in abcc2 was also seen in the paclitaxel-resistant cells. Long-term exposure to bexarotene alone or in combination caused a modest increase in abcc1. The expression of abcc3, bcrp, and mvp was not altered in the paclitaxel-resistant cells. Finally, abcb11 was not detectable in either the control or treated cells. Flow cytometric analysis additionally confirmed the presence of Pgp protein in paclitaxel-resistant cells (data not shown). The extent of the efflux activity

![Fig. 2](image-url) Effect of the bexarotene (LGD1069)/paclitaxel combination on growth of Calu3 cells after multiple exposures. A, comparison of total cell numbers in vehicle control, LGD1069 alone, paclitaxel alone, and LGD1069/paclitaxel combination for the first six cycles. B, effect of LGD1069 on the development of paclitaxel-resistant Calu3 cells. Calu3 cells were subject to treatments as described in Fig. 1. At the end of each treatment cycle (10 days), cells were harvested by trypsinization. The number of viable cells was determined by trypan blue exclusion and were reexposed to the same treatment. The procedure was repeated 10 times. The shaded area indicates the treatment period.
of Pgp was consistent with the level of mdr1 mRNA and the degree of MDR phenotype (compare Fig. 3 and Table 1). Taken together, these results showed that increased mdr1 mRNA expression was responsible for the MDR phenotype in paclitaxel-resistant cells.

Effect of Bexarotene/Paclitaxel Combination on the Growth of Paclitaxel-resistant Cells. Because the combination of intermittent paclitaxel and continuous bexarotene prevented the development of paclitaxel resistance, we sought to determine whether paclitaxel resistance can be overcome by treatment with the bexarotene/paclitaxel combination. Paclitaxel-resistant cells were repeatedly treated with intermittent paclitaxel and continuous bexarotene (similar to scheme 2; Fig. 1). Treatment with paclitaxel alone or bexarotene alone had no effect on growth of the paclitaxel-resistant cells. However, the paclitaxel-resistant cells were resensitized to the cytotoxic activity of paclitaxel after multiple exposures to the bexarotene/paclitaxel combination (Fig. 4). The reversal of drug resistance became apparent after repeated treatment for four cycles; the remaining viable fraction of cells continued to decrease with increasing treatment cycles, reaching <1% of untreated control after eight treatment cycles.

Effect of Bexarotene on Mutation Rate. Table 2 summarizes the results of fluctuation analysis where surviving colonies of Calu3 cells were selected with paclitaxel in a single step. Two surviving clones were found in the bulk population of $1.5 \times 10^7$ cells in the control group, indicating that preexisting paclitaxel resistant variants in the Calu3 cell population was minimal. The results showed that average number of colonies per plate in paclitaxel-treated cells was 3.1, whereas the average number of colonies per plate in the bexarotene-treated cultures decreased 4-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 paclitaxel-treated cells was calculated to be $1.7 \times 10^{-7}$ per cell generation, whereas pretreatment with bexarotene followed by the bexarotene/paclitaxel combination resulted in a 5-fold reduction in the mutation rate ($P < 0.05$ by $t$ test). The mutation rate calculated by Ma-Sandri-Sarkar maximum-likelihood method (23) was similar (data not shown). A total of 12 clones from paclitaxel-treated cells and 8 clones from the bexarotene-treated cultures was isolated, propagated, and tested for the sensitivity to pacli-

![Fig. 3](image-url) Analysis of efflux activity of Pgp and mdr1 expression in Calu3 cells. A, Pgp activity was quantified by the fluorescence intensity of free Calcein inside the cells (mean ± SD, $n = 3$). B, mdr1 and other gene expression were analyzed by real-time PCR. The expression of a housekeeping gene was used to normalize gene expression level (mean ± SD, $n = 3$). *, statistically significant from parental cells at $P < 0.05$.

![Fig. 4](image-url) Effect of bexarotene (LGD1069)/paclitaxel combination on paclitaxel-resistant Calu3 cells after multiple exposures. Paclitaxel-resistant Calu3 cells derived from paclitaxel alone were repeatedly treated with vehicle, paclitaxel alone, LGD1069 alone, or in the combination. For comparison, parental cells were treated with drug for 3 days. Numbers of viable and dead cells were determined by trypan blue exclusion at the end of each treatment cycle (mean ± SD, $n = 3$).
The present study shows that bexarotene can prevent the development of paclitaxel resistance in the human NSCLC Calu3 cells. Cells treated with paclitaxel alone developed a MDR phenotype with cross-resistance to Pgp substrates vincristine and doxorubicin but remained sensitive to non-Pgp substrate cisplatin. On the other hand, the bexarotene/paclitaxel combination prevented the development of paclitaxel resistance and cells remained sensitive to chemotherapeutic agents. The lack of a MDR phenotype in the cells recovered after combination treatment was additionally confirmed by the absence of mdr1 gene expression and lack of Pgp efflux activity in these cells. The paclitaxel-resistant cells when treated with the bexarotene/paclitaxel combination were resensitized to paclitaxel. The ability of bexarotene/paclitaxel to interfere with development of paclitaxel resistance was due to reduction in mutation rate by bexarotene. The in vivo xenograft model showed bexarotene/paclitaxel combination produced a statistically significant decrease in tumor growth when compared with vehicle control and to single agents. Furthermore, xenograft tumors that were initially resistant to paclitaxel could be resensitized to paclitaxel by the combination.

DISCUSSION

Genetic instability of cancer cells is thought to be one of the major factors giving rise to drug-resistant mutant or variant subpopulations (24, 25). One mechanism of developing resistance to chemotherapeutic agents involves increased efflux activity of the Pgp, which is associated with overexpression of the mdr1 gene. Our data showed that long-term exposure to the bexarotene/paclitaxel combination can influence mdr1 gene expression and lack of Pgp efflux activity in these cells. The paclitaxel-resistant cells when treated with the bexarotene/paclitaxel combination were resensitized to paclitaxel. The ability of bexarotene/paclitaxel to interfere with development of paclitaxel resistance was due to reduction in mutation rate by bexarotene. The in vivo xenograft model showed bexarotene/paclitaxel combination produced a statistically significant decrease in tumor growth when compared with vehicle control and to single agents. Furthermore, xenograft tumors that were initially resistant to paclitaxel could be resensitized to paclitaxel by the combination.

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<th>Clone from paclitaxel-treated cells</th>
<th>Paclitaxel Vincristine Doxorubicin Cisplatin</th>
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NOTE. The surviving clones obtained from single step paclitaxel selection were tested for drug sensitivity by MTT assay. Values are average of three experiments.
Bexarotene Prevents and Overcomes Paclitaxel Resistance

Expression and development of the MDR phenotype. We hypothesize that bexarotene may increase and/or maintain genomic integrity of cells to prevent the cancer cell from modifying its genome, resulting in resistance to chemotherapeutic insult, thereby preventing and overcoming the development of acquired paclitaxel resistance. This hypothesis was additionally supported by Luria-Delbrück fluctuation analysis. As seen in Tables 2 and 3, treatment with bexarotene decreased the spontaneous development of paclitaxel resistance and the degree of MDR phenotype in the surviving clones. Although fluctuation analysis was originally designed for analysis of mutations in bacteria (26), this method has become an important tool to measure the spontaneous mutation rate in cancer cells and to study the nature and rate of acquired drug resistance. In theory, if resistance was acquired by the induced event, the number of surviving colonies would be expected to have a Poisson distribution, with the variance close to the mean (26). 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 Calu3 cells arose randomly rather than being induced by drug exposure. Because pretreatment with bexarotene followed by drug selection significantly decreased the spontaneous mutation rate as compared with paclitaxel selection alone, this additionally suggested 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 of bexarotene in modulating mdr1 gene expression and to maintain genomic integrity is unknown at present. Several possibilities exist. First, we have recently shown that bexarotene and other rexinoids inhibited the nuclear factor (NF)-κB activity to increase the activity of chemotherapeutic agents (27). NF-κB has been shown to play an important role in controlling apoptotic cell death (28). Inhibition of NF-κB activity in NSCLC cell lines increased the sensitivity to chemotheraphy-induced apoptosis (29). NF-κB also controls the expression of the mdr1 gene. In human colon cancer cells, inhibition of NF-κB reduced mdr1 mRNA and Pgp expression (30). It is possible that bexarotene may interfere with mdr1 gene expression through inhibition of NF-κB to prevent and overcome paclitaxel-mediated drug resistance. Second, the retinoid X receptor is the obligate heterodimeric partner for a number of nuclear hormone receptors and is required for their function. The activity of many of these heterodimeric partners has been shown to be important in both the prevention and progression of the malignant potential. Ligands for the retinoic acid receptors, peroxisome proliferator-activated receptors, and vitamin D receptor have all been shown to influence the growth and differentiation of cancer cells (31, 32). Importantly, it has been reported that paclitaxel could enhance mdr1 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 (33). Steroid and xenobiotic receptor is a member of the nuclear hormone receptor superfamily that heterodimerizes with retinoid X receptor. Bexarotene may directly or indirectly antagonize steroid and xenobiotic receptor to prevent paclitaxel-induced mdr1 expression. Third, the tumor suppressor gene p53 functions to maintain genomic integrity by preventing cells with unstable genomes from transiting through the cell cycle. Wild-type P53 has been shown to repress mdr1 promoter activity, mdr1 expression, and Pgp protein level, whereas mutant P53 stimulates such effects (34–36). These results suggest the important role of wild-type P53 in regulation of mdr1 gene and Pgp protein levels. It is possible that bexarotene may interfere with mutant P53-mediated mdr1 up-regulation after multiple exposures to paclitaxel. Recently, rexinoids have been shown to trigger cyclin D1 proteolysis, causing G1 arrest and allowing subsequent repair of genomic DNA damage in immortalized human bronchial epithelial cells. The degradation of cyclin D1 may set up a situation that mimics the activity of wild-type P53 to maintain/increase genomic integrity (37). Fourth, aneuploidy is one of the most common genomic abnormalities of cancer cells (38). Early studies by Duesberg et al. (39) showed that genomic instability of cancer cells was proportional to the degree of aneuploidy. These investigators additionally showed that aneuploid cells can acquire MDR by chromosome reassortments in
the absence of MDR genes (40). Thus, bexarotene may maintain/increase genomic integrity through stabilizing DNA ploidy. Taken together, bexarotene may interfere with one or more of the above-mentioned pathways to suppress mdr1 expression. Ongoing research focuses on elucidating the mechanism of action of bexarotene in maintaining genomic integrity to prevent and overcome MDR.

In summary, we showed that bexarotene can prevent and overcome acquired paclitaxel resistance in human NSCLC. Such effects were likely due to the ability of bexarotene to modulate mdr1 expression through maintaining/increasing genomic integrity, thereby preventing the cancer cell from modifying its genome resulting in resistance to chemotherapeutic insult. The benefit of the bexarotene/paclitaxel combination was additionally shown in both paclitaxel sensitive and resistant Calu3 xenograft tumors. These findings have important implications for patients with NSCLC. For example, the results from a recent phase I/II clinical trials show that addition of Targretin capsules to cisplatin/vinorelbine chemotherapy extends survival in late-stage NSCLC patients (41). Thus, additionally understanding the mechanisms by which bexarotene prevents and overcomes acquired drug resistance will have significant impact on the therapeutic use of bexarotene in cancer treatment. In addition, it will also be interesting to determine the effect of bexarotene on the development of non-Pgp-mediated drug resistance such as resistance to cisplatin. To this end, additional research will be directed toward the following: (a) to determine the molecular mechanisms of the bexarotene/paclitaxel combination in drug resistance; (b) to identify gene targets associated with combination therapy; and (c) to evaluate the combination of bexarotene and chemotherapeutic agents in other tumor types, including breast, prostate, and colon cancer.

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A Selective Retinoid X Receptor Agonist Bexarotene (Targretin) Prevents and Overcomes Acquired Paclitaxel (Taxol) Resistance in Human Non–Small Cell Lung Cancer

Wan-Ching Yen, Manny R. Corpuz, Rene Y. Prudente, et al.


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