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

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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 regimen 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 ~173,770 new cases of lung cancer in the United States and ~160,440 people will die of this disease. Non–small cell lung cancer (NSCLC) is the most common type of lung cancer, accounting for ~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 *mdr*1 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 tumor uptake was strongly correlated with response to paclitaxel in NSCLC. No significant differences were found for other prognostic factors such as age, sex, tumor stage, and tumor type between patients with good and poor response. Their findings suggested a possible role of Pgp expression in paclitaxel failure in 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

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chemopreventive and chemotherapeutic agent in preclinical rodent 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 differentiation induction along the adipocyte lineage, leading to terminal cell division followed by cell death (17). To additionally 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 regimen decreased the spontaneous development of paclitaxel resistance. In tumor xenografts, the bexarotene/paclitaxel combination produced a statistically significant decrease in tumor growth when compared with vehicle control and to single agents alone.

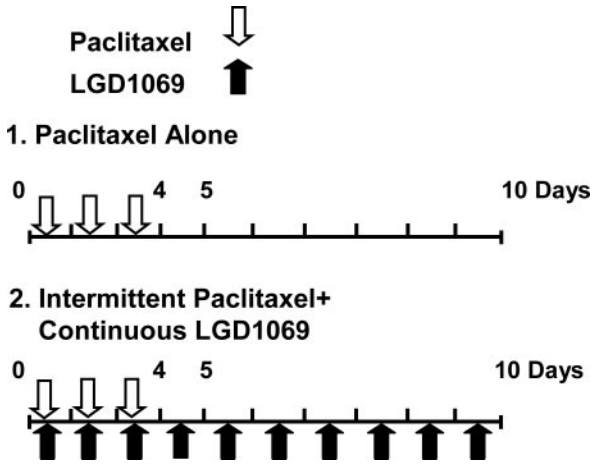

Fig. 1 Treatment scheme for the bexarotene (LGD1069)/paclitaxel combination. 1, paclitaxel alone regimen: the cells were exposed to 30 nmol/L paclitaxel for 3 days followed by 7 days in control medium. 2, intermittent paclitaxel with continuous LGD1069: the cells were exposed to 30 nmol/L paclitaxel 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).

MATERIALS AND METHODS

Chemicals and Reagents. RPMI 1640, fetal bovine serum, glutamine, and gentamicin were obtained from Life Technologies, 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, cisplatin, 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 stock 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 obtained 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% CO_2.

***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 2×10^6 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 exposure 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 30 nmol/L paclitaxel for 3 days followed by 7 days in control medium. For the combination of intermittent paclitaxel with continuous bexarotene (scheme 2; Fig. 1), the cells were exposed to 30 nmol/L paclitaxel 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 or in the presence of 1 μmol/L bexarotene given continuously (data not shown in Fig. 1). Drug-induced growth inhibition was measured by trypan blue exclusion. To determine the sensitivity of paclitaxel-resistant variants to vincristine, doxorubicin, and cisplatin, cells were seeded in 96-well tissue culture plates and treated with each agent at various concentrations 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 (Molecular Probes, Eugene, OR). Calu3 control and drug-treated cells were seeded on opaque 96-well plates at 20,000 cells per well overnight. 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 incubation, each well received 1 μmol/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-

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 $\times$ 10^6 cells with RNeasy Mini kit (Qiagen, Valencia, CA), according to the manufacture'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 1× reverse transcription buffer, 5.5 mmol/L $MgCl_2$, 2 mmol/L deoxynucleoside triphosphates, 2.5 μmol/L 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 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 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: *mdr*1 (GenBank accession no. M14758) forward 5′-aggaagccaatgcctatgacttta-3′, reverse 5′-caactgggccctctctctc-3′, and probe FAM-atgaaactgcctcataaatttgacaccctgg-TAMRA (18); human *36B4* (GenBank accession no. M17885) forward 5′-gcagatccgcatgtccctt-3′, reverse 5′-tgtttttccaggtgccctcg-3′, and probe FAM-aggctgtggtgctgatgg-TAMRA; human *abcb11* (GenBank accession no. NM_003742) forward 5′-gacatgcttgcgaggacctt-3′, reverse 5′- gagcgttgccggatgg-3′, and probe FAM-agcccttaaactatcctggtagctccctctgct-TAMRA; *abcc1* (GenBank accession no. NM_004996) forward 5′-tcatggtgcccgtcaatg-3′, reverse 5′-cgattgtctttgctcttcatgtg-3′, and probe FAM- acctgatacgtcttggtcttcatcgcca-TAMRA; *abcc2* (GenBank accession no. NM_000392) forward 5′-gttcgatataccaatccaagcctc-3′, reverse 5′-ccagaatagggacaggaaccag-3′, and probe FAM-tctgtacacaccattgtctgctgtattggatcag-TAMRA; *abcc3* (GenBank accession no. NM_003786) forward 5′-gcaccattgtcgtggctaca-3′, reverse 5′-gcaggacacccaggaccat-3′, and probe FAM-catcctctcccacctgtccaagctca-TAMRA (19); *bcrp* (GenBank accession no. AF098951) forward 5′-agatgggtttccaagcgttcat-3′, reverse 5′-ccagtcccagtacgactgtgaca-3′, and probe FAM-tgctgggtaatccccaggcctctatagc-TAMRA (20); and *mvp* (GenBank accession no. NM_017458) forward 5′-agctcgcaaggaacttttgga-3′, reverse 5′-ccttggcagtcccggtg-3′, and probe FAM-acggccatgctcagagcctcca-TAMRA. Brain standard was used for *36B4*, *abcc1*, *bcrp*, *mvp*, and liver standard for *36B4*, *mdr*1, *abcb11*, *abcc2*, and *abcc3*. The probes were obtained from Integrated DNA Technologies (Coralville, IA).

Fluctuation Analysis. Thirty 25-cm^2 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 μmol/L bexarotene. Cells were allowed to grow to near confluence (average 2 $\times$ 10^6 cells per flask). The total cell population from each flask were seeded onto separate 96-well plates overnight and treated with 100 nmol/L paclitaxel for 7 days. Preliminary experiments showed that this drug concentration resulted in 99% cell kill. Cells grown in 1 μmol/L bexarotene during the expansion period were treated with the combination of 100 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 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 $\times$ 10^7 at 1 $\times$ 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 $\times$ 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_r 400)/Tween 80 (99.5:0.5) and 90% of 1% (w/v) carboxymethylcellulose (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, $1/2ab^2$, 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

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. 2*A*). 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. 2*A*). Cells treated with continuous bexarotene grew similarly as vehicle-treated controls (Fig. 2*A*). However, surviving cells regrew within 80 days (Fig. 2*B*) 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. 2*B*). 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. 3*A*, 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. 3*A*). 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: *mdr*1 (Pgp), *abcb11* (MDR/TAP),

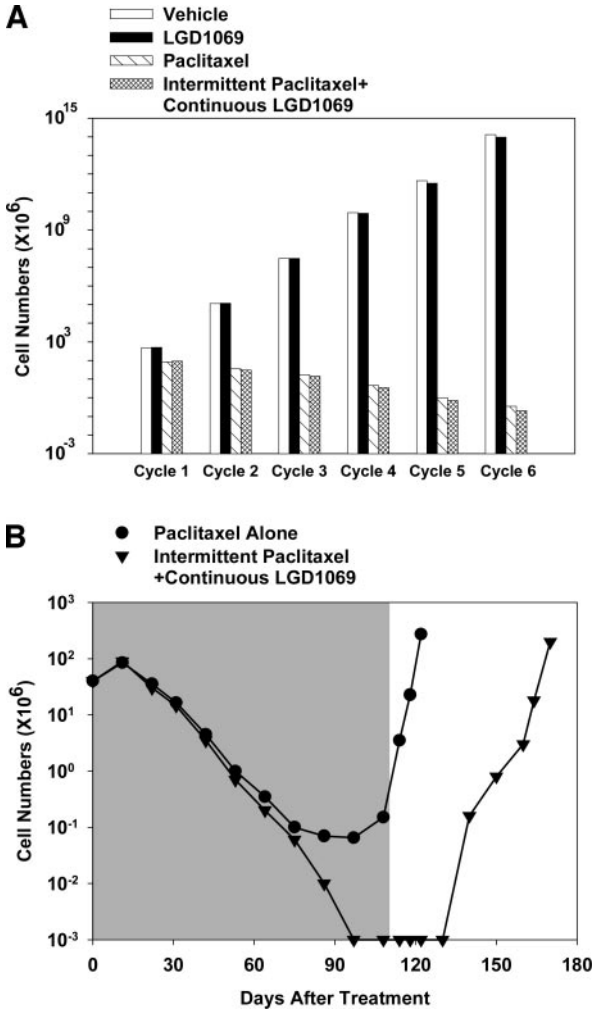

Fig. 2 Effect of the bexarotene (LGD1069)/paclitaxel combination on growth of Calu3 cells after multiple exposures. *A*, comparison of total cell numbers in vehicle control, LGD1069 alone, paclitaxel alone, and LGD1069/paclitaxel combination for the first six cycles. *B*, effect of LGD1069 on the development 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.

abcc1 (MRP1), *abcc2* (MRP2), *abcc3* (MRP3), *bcrp* (breast cancer-resistant protein), and *mvp* (lung-resistant protein). Our data showed that *mdr*1 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. 3*B*). 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

Table 1 Comparison of drug sensitivity among Calu3 parental cells and cells derived from paclitaxel resistance and combination (mean ± SD, $n = 3$ from three separate experiments)

Cells	Paclitaxel RF	Vincristine RF	Doxorubicin RF	Cisplatin RF
Parental cells	1	1	1	1
Paclitaxel alone	26.0±1.3	25.7±2.0	6.8±2.0	1.0±0.1
Combination	1.0±0.2	0.9±0.1	0.9±0.2	0.9±0.1

NOTE. Cells were treated with 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. The ratio of IC_{50} of resistant variant to that of parent cells was expressed as resistant factor (RF).

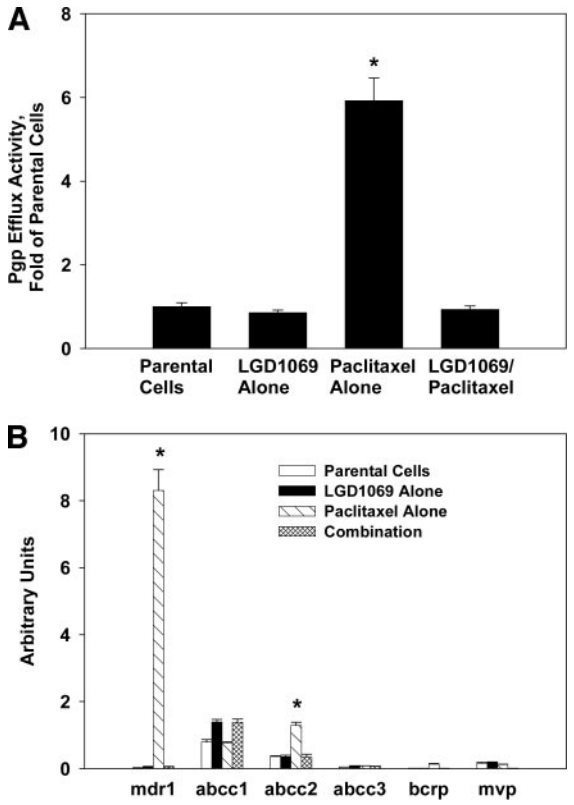

Fig. 3 Analysis of efflux activity of Pgp and *mdr*1 expression in Calu3 cells. *A*, Pgp activity was quantified by the fluorescence intensity of free Calcein inside the cells (mean ± SD, $n = 3$). *B*, *mdr*1 and other gene expression were analyzed by real-time PCR. The expression of a housekeeping gene was used to normalize gene expression level (mean ± SD, $n = 3$). *, statistically significant from parental cells at $P < 0.05$.

of Pgp was consistent with the level of *mdr*1 mRNA and the degree of MDR phenotype (compare Fig. 3 and Table 1). Taken together, these results showed that increased *mdr*1 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 1.5×10^7 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 1.7×10^{-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-

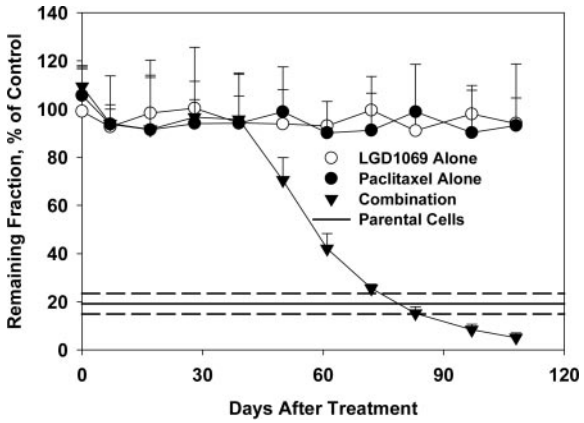

Fig. 4 Effect of bexarotene (LGD1069)/paclitaxel combination on paclitaxel-resistant Calu3 cells after multiple exposures. Paclitaxel-resistant Calu3 cells derived from paclitaxel alone were repeatedly treated with vehicle, paclitaxel alone, LGD1069 alone, or in the combination. For comparison, parental 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, $n = 3$).

Table 2 Luria-Delbrück fluctuation analysis of paclitaxel-resistant Calu3 cells

	Fluctuation group		Control group
	Paclitaxel	Bexarotene + paclitaxel	Paclitaxel
Plate	Colonies/plate	Colonies/plate	Colonies/plate
Total colonies	46	12	2
Mean	3.1*	0.8	0.1
Variance	9.4	1.2	0.1
Mutation rate	1.7×10^{-7}	3.4×10^{-8}	NA

NOTE. Individual populations at 1000 cells per flask were allowed to expand in either the culture medium or in 1 μmol/L bexarotene until confluence. The total cell population from each flask was seeded onto separated 96-well plates and exposed to 100 nmol/L paclitaxel. Cells grown in 1 μmol/L bexarotene during expansion period were treated with combination of 100 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 (15×10^6 cells at 1×10^6 cells per plate) from mass cultures prior to drug selection.

* $P < 0.05$ *versus* control group.

Abbreviation: NA, not applicable.

taxel and other Pgp substrates. These clones included one from each of the 15 original populations. As seen in Table 3, 9 of 12 clones from paclitaxel-treated cells showed a stable MDR phenotype, whereas only 3 of 8 clones had a stable MDR phenotype from the bexarotene-treated cultures. Furthermore, the degree of MDR phenotype from the paclitaxel-treated clones was greater than those isolated after bexarotene treatment. All resistant variants were sensitive to non-Pgp substrate cisplatin.

Effect of Bexarotene/Paclitaxel Combination *In vivo*. To evaluate the antitumor efficacy of the bexarotene/paclitaxel combination *in vivo*, Calu3 cells were established as xenograft tumors in athymic nude mice. As seen in Fig. 5*A*, Calu3 tumors grew continuously throughout the course of study in both vehicle-treated control and drug-treated animals. When compared with vehicle control, bexarotene given alone at 100 mg/kg daily had no significant effect on tumor growth after the 4-week treatment period. In contrast, paclitaxel at 20 mg/kg once weekly decreased tumor volume by 30% ($P < 0.05$ *versus* control). More importantly, the combination of both agents decreased tumor volume by 38% relative to paclitaxel alone ($P < 0.05$) and by 55% relative to vehicle control ($P < 0.05$; Fig. 5*A*). To determine whether the combination regimen can overcome paclitaxel resistance *in vivo*, animals were implanted with the paclitaxel-resistant Calu3 cells and were treated with single agents or combination. Paclitaxel-resistant tumor growth in animals treated with single agents was similar to that of vehicle controls, whereas the combination regimen produced a 40% decrease in tumor growth compared with paclitaxel-treated animals ($P < 0.05$ *versus* paclitaxel alone; Fig. 5*B*). The benefit of the bexarotene/paclitaxel combination became apparent beyond 30 days as the tumor volume in mice receiving the combination therapy began to diverge from the tumor volume in mice treated with paclitaxel alone. Collectively, these results showed that the bexarotene/paclitaxel combination produced a greater antitumor effect than the single agents in the Calu3 xenograft model. These data additionally showed that the combination regimen could overcome paclitaxel resistance *in vivo*.

DISCUSSION

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 *mdr*1 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.

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 *mdr*1 gene. Our data showed that long-term exposure to the bexarotene/paclitaxel combination can influence *mdr*1 gene ex-

Table 3 Drug sensitivity of paclitaxel-resistant Calu3 variants derived from single step paclitaxel selection

	Paclitaxel	Vincristine	Doxorubicin	Cisplatin
Clone from paclitaxel-treated cells				
P-2	1.3	1.1	0.8	0.9
P-4	2.6	1.7	1.6	0.9
P-5	7.5	3.4	3.3	0.9
P-6	11.5	6.6	4.0	1.0
P-7	8.2	5.4	3.0	0.9
P-8	32.2	31.7	8.5	1.0
P-9	29.1	16.3	5.9	0.8
P-10	12.2	6.5	3.0	0.9
P-11	13.9	7.6	3.5	0.9
P-12	13.4	5.5	4.1	0.9
P-13	1.3	1.0	0.8	1.0
P-15	14.1	7.8	2.1	0.8
Clone from bexarotene/paclitaxel-treated cells				
PL-2	0.9	0.8	1.0	1.0
PL-4	7.7	4.9	1.9	0.9
PL-8	1.3	1.0	0.9	0.8
PL-9	11.9	5.3	4.0	0.7
PL-10	1.1	1.1	0.9	0.7
PL-11	5.6	6.2	2.9	0.9
PL-12	0.9	0.9	1.0	0.9
PL-14	1.3	1.1	1.0	0.8

NOTE. The surviving clones obtained from single step paclitaxel selection were tested for drug sensitivity by MTT assay. Values are average of three experiments.

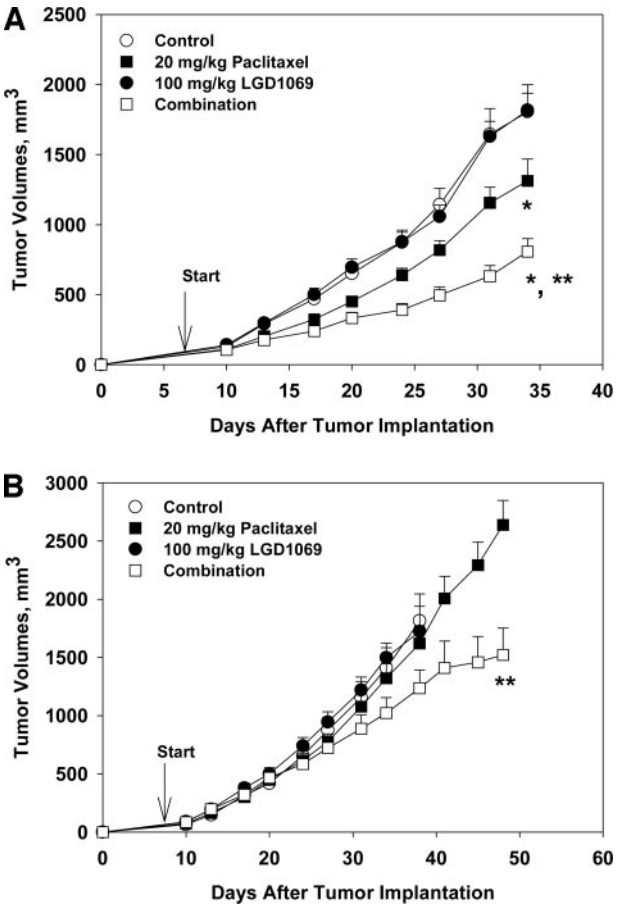

Fig. 5 Antitumor effect of the bexarotene (LGD1069)/paclitaxel combination in (*A*) Calu3 xenograft tumors from parental cells and (*B*) Calu3 xenograft tumors from paclitaxel-resistant Calu3 cells. Nude mice bearing Calu3 tumors were treated with vehicle, LGD1069, paclitaxel, or combination for 4 to 6 weeks. Drug effect on tumor growth was determined twice a week (mean ± SE, $n = 8–10$). *, statistically significant from vehicle control at $P < 0.05$; **, statistically significant from paclitaxel alone at $P < 0.05$.

pression 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 *mdr*1 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 chemotherapy-induced apoptosis (29). NF-κB also controls the expression of the *mdr*1 gene. In human colon cancer cells, inhibition of NF-κB reduced *mdr*1 mRNA and Pgp expression (30). It is possible that bexarotene may interfere with *mdr*1 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 *mdr*1 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 *mdr*1 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 *mdr*1 promoter activity, *mdr*1 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 *mdr*1 gene and Pgp protein levels. It is possible that bexarotene may interfere with mutant P53-mediated *mdr*1 up-regulation after multiple exposures to paclitaxel. Recently, rexinoids have been shown to trigger cyclin D1 proteolysis, causing G_1 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 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 *mdr*1 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 *mdr*1 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

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