Effects of Novel Retinoic Acid Metabolism Blocking Agent (VN/14-1) on Letrozole-Insensitive Breast Cancer Cells

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Abstract

Aromatase inhibitors are proving to be more effective than tamoxifen for postmenopausal estrogen receptor (ER)-positive breast cancer. However, the inevitable development of resistance to treatment is a concern. We investigated the effects of novel retinoic acid metabolism blocking agent, VN/14-1, in overcoming letrozole resistance in long-term letrozole cultured (LTLC) cells. Compared with MCF-7 cells stably transfected with aromatase (MCF-7Ca), LTLC cells were no longer sensitive to growth inhibition by aromatase inhibitors. The HER-2/phosphorylated mitogen-activated protein kinase (pMAPK) growth factor signaling pathways were activated, and ERα and coactivator amplified in breast cancer 1 (AIB1) were up-regulated ~3-fold in LTLC cells. VN/14-1 inhibited aromatase activity and growth values of in MCF-7Ca cells with IC_{50} of 8.5 and 10.5 nmol/L, respectively. In human placental microsomes, aromatase activity was inhibited with IC_{50} of 8.0 pmol/L. The IC_{50} in LTLC cells was 0.83 nmol/L, similar to letrozole (IC_{50}, 0.3 nmol/L) in MCF-7Ca cells. LTLC cells were 10-fold more sensitive to growth inhibition by VN/14-1 than MCF-7Ca cells. VN/14-1 treatment effectively down-regulated ERα, AIB1, pMAPK, HER-2, cyclin D1, cyclin-dependent kinase 4 (CDK4), and Bcl2 and up-regulated cytokeratins 8/18, Bad, and Bax. Tumor growth of LTLC cells in ovariectomized nude mice was independent of estrogens but was inhibited by VN/14-1 (20 mg/kg/d; $P < 0.002$). Decreases in ERα, cyclin D1, CDK4, and pMAPK and up-regulation of cytokeratins, Bad, and Bax with VN/14-1 in tumor samples may be responsible for the efficacy of this compound in inhibiting LTLC cell growth *in vitro* and *in vivo*. (Cancer Res 2006; 66(23): 11485-93)

Introduction

Breast cancer is the second leading cause of cancer deaths in women today (after lung cancer) and is the most common cancer among women. The role of estrogens in the progression of breast cancer in both premenopausal and postmenopausal women is well established (1). The effects of estrogens on tumor growth are mediated by the estrogen receptor (ER), mainly ERα. The binding of estrogen to ERα induces a cascade of events leading to transcription of estrogen-responsive genes, such as *cyclin D1*, which are known to stimulate mammary tumor cell proliferation (2). Although estrogens affect both premenopausal and postmenopausal breast cancer, following menopause, breast tumors become more sensitive to estrogens as the concentration of ER increases with age (3). Thus, antiestrogens, such as tamoxifen, which block ER are effective in these patients. Whereas the ovary is no longer the main source of estrogen in postmenopausal women, estrogen production is increased in peripheral sites, such as adipose tissue and breast tissue, and contributes to stimulation of breast cancer (4). Although plasma estrogen concentrations are very low in postmenopausal women, levels in breast cancers from postmenopausal patients are reported to be 10-fold higher than in plasma and normal tissue (5). Synthesis of estrogens from androgens, which is a rate-limiting step in estrogen biosynthesis pathway, is catalyzed by the enzyme aromatase. Inhibition of this conversion by selective aromatase inhibitors is now proving to be a valuable approach for reducing the growth-stimulatory effects of estrogens in estrogen-dependent breast cancer (6). Aromatase inhibitors, such as exemestane and letrozole, have advantages over tamoxifen as the latter is a weak estrogen agonist as well as antagonist (7, 8).

Greater benefits of aromatase inhibitor treatment have recently been shown in patient survival and tolerability in studies comparing aromatase inhibitors with tamoxifen as first-line and adjuvant treatments for postmenopausal patients with hormone-dependent advanced breast cancer (9–11). However, the inevitable development of drug resistance presents a significant hurdle in all cancer therapies. Although tamoxifen has proved to be a successful breast cancer therapy, patients eventually relapse, showing a hormone-independent and more invasive cancer phenotype. Several mechanisms have been proposed that may contribute to the development of resistance. These comprise activation of growth factor receptor survival pathways leading to ligand-independent activation of the ER and ER-mediated transcription (12, 13). Thus, it is possible that abnormally increased growth factor signaling pathways and/or cross-talk between these signaling pathways and steroidal receptors may play an important role in endocrine resistance and may account for loss of some estrogen dependence, resulting in resistant tumors (14–17). Indeed, acquired resistance of MCF-7 cells *in vitro*, after long-term treatment with tamoxifen, is shown to be associated with increased levels of epidermal growth factor receptor (EGFR) and mitogen-activated protein kinase (MAPK) activity (18). In addition, studies carried out in our laboratory indicate that MCF-7Ca cells (MCF-7 cells stably transfected with the human aromatase gene) deprived of estrogen loose their ability to respond to the mitogenic effects of estrogen. However, these cells still retain some sensitivity to the inhibitory effects of the ER down-regulator fulvestrant (at a dose 10-fold higher than needed to inhibit the growth of MCF-7Ca cells), indicating that ER is still functional in growth regulation. Nevertheless, these cells develop resistance to tamoxifen and several aromatase inhibitors accompanied by increased expression and activity of erbB-2 tyrosine kinase receptor and proteins in the phosphatidylinositol 3-kinase/AKT signaling pathway (19). Previous

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studies have been carried out in our laboratory to investigate the effects of resistance to aromatase inhibitor letrozole *in vivo*. The results suggested that tumor cells adapt to estrogen deprivation during letrozole treatment by activation of alternate signaling pathways to increase transcription. Adapter proteins [phosphorylated Shc and growth factor receptor binding protein 2 (Grb2)] as well as all of the signaling proteins in the MAPK cascade [phosphorylated Raf, phosphorylated MAPK/extracellular signal-regulated kinase kinase 1/2 (pMEK1/2), and phosphorylated MAPK (pMAPK)], but not AKT, were increased in tumors no longer responsive to letrozole (20, 21). The current study was undertaken to determine loss of sensitivity to aromatase inhibitor letrozole *in vitro* and to identify agents to which aromatase inhibitor refractory tumors would be responsive.

Besides endocrine therapies, another class of well-tolerated chemotherapeutic agents used in the treatment of breast cancer are retinoids. All-*trans*-retinoic acid (ATRA) and its isomers as well as other retinoids, such as fenretinide [*N*-(4-hydroxyphenyl)-retinamide (4-HPR)], are differentiation agents known to play an important role in the control of tumor cell proliferation and differentiation (22). However, the rapid metabolism of ATRA in the body is believed to be one of the major reasons for limited efficacy of ATRA. Thus, retinoic acid metabolism blocking agents (RAMBA) represent a promising approach for various diseases responsive to ATRA, including breast cancer, especially in patients heavily pretreated with hormone therapies (23).

Several novel potent RAMBAs that are structural analogues of ATRA and 13-*cis*-retinoic acid have been designed and synthesized in our laboratory (24). They have been shown to compete with ATRA, thus preventing ATRA metabolism and leading to increased levels of endogenous ATRA. VN/14-1 (Fig. 1) was found to be the most potent and effective compound among several RAMBAs studied in human breast cancer MCF-7 and T47D models both *in vitro* and *in vivo* (25). This novel compound was found to possess various biological properties, including induction of differentiation, apoptosis, as well as cell cycle arrest (25). Therefore, we investigated the possible effects of VN/14-1 on these processes in breast cancer cells that are no longer responsive to aromatase inhibitor letrozole. Our studies suggest that VN/14-1 might be a promising treatment following development of resistance to aromatase inhibitors in breast cancer patients.

Materials and Methods

Materials

DMEM, penicillin/streptomycin (10,000 IU each), 0.25% trypsin-1 mmol/L EDTA solution, Dulbecco's PBS, and geneticin (G418) were obtained from Life Technologies (Grand Island, NY). Fetal bovine serum (FBS) and dextran-coated charcoal-treated serum were obtained from Hyclone (Logan, UT). Androstenedione, DMSO, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and Tween 20 (polyoxyethylene sorbitan monolaurate) were obtained from Sigma Chemical Co. (St. Louis, MO). Tritiated androst-4-ene-3,17-dione ([1β-^{3}H]4A; specific activity 25.3 Ci/mmol) for aromatase enzyme studies was purchased from PerkinElmer Life Sciences (Boston, MA). Antibodies against cytokeratins 8 and 18 were from Santa Cruz Biotechnology (Santa Cruz, CA). MCF-7 human breast cancer cells stably transfected with the human placental aromatase gene (MCF-7Ca) were kindly provided by Dr. S. Chen (Duarte, CA; ref. 26). ECL chemiluminescence kit and Hybond-ECL nitrocellulose membranes were purchased from Amersham Biosciences (Piscataway, NJ). Letrozole (CGS20267, Femara) was kindly provided by Dr. D. Evans (Novartis Pharma, Basel, Switzerland; Fig. 1). We have previously reported the synthesis of VN/14-1 (Fig. 1; ref. 25).

Cell Lines

The MCF-7 human breast cancer cell line stably transfected with the human placental aromatase gene (designated as MCF-7Ca; ref. 27) was routinely maintained in T75 or T150 tissue culture flasks in a humidified incubator (5% CO_2, 95% air) at 37°C in DMEM with 5% FBS, 1% penicillin/streptomycin solution, and 700 μg/mL G418.

The long-term letrozole cultured (LTLC) cells were obtained by culturing early passage of MCF-7Ca cells (passage 7) in steroid-depleted medium (phenol red–free improved MEM) supplemented with 5% dextran-coated charcoal-treated serum, 1% penicillin/streptomycin, 700 μg G418, 25 nmol/L of aromatase substrate androstenedione, and 1 μmol/L of aromatase inhibitor letrozole.

Cell Proliferation (Growth) Inhibition—MTT Assay

The MTT assay is a colorimetric assay used to measure cell proliferation. Growth studies were done on synchronized cells. This was achieved by transferring the parental MCF-7Ca cells into steroid-free medium and the LTLC to low-serum Opti-MEM medium 72 hours before plating. Cells (1×10^4 per well) were plated into 24-well plates (Corning Costar) and allowed to attach for 24 hours. The cells were then washed with Dulbecco's PBS and treated with steroid-free medium containing vehicle or indicated concentrations of estrogens, androstenedione, aromatase inhibitors, or RAMBAs. The medium was changed every 3 days, and the cells were counted on the 10th day using the MTT powder (Sigma Chemical). MTT (500 μg) was added to each well in serum-free medium, and cells were incubated for 2 hours. Medium was then removed and the formazan dye trapped in the living cells was dissolved in DMSO and absorbance was measured in a spectrophotometer at 560 nm. The results are expressed as a percentage of the cell number in the vehicle-treated control wells (26–28). IC_{50} values were determined using SigmaPlot 2000.

Preparation of Human Placental Microsomes

The human placental microsomal assay was used for measuring the inhibition of aromatase activity (29). Microsomes were isolated from human term placenta and stored in 0.1 mol/L phosphate buffer (pH 7.4) in −70°C until required as described elsewhere (30).

Radiometric ^3H_2O Release Assay for Measuring the Aromatase Activity

Microsomal and cellular assay. This assay was done as reported earlier (31, 32). Tritiated water (^3H_2O) formed during the aromatization of [1β-^{3}H]4A to estrogen was measured after incubation with microsomes or cells and extraction of steroids from the medium with organic solvent (33). The protein concentration of the homogenate was measured using the

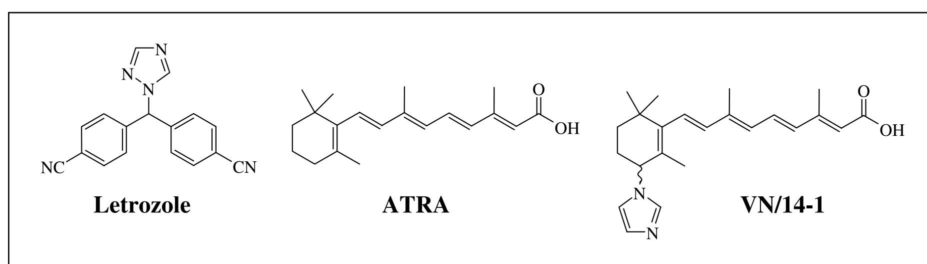

Figure 1. Structures of letrozole, ATRA, and VN/14-1.

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Bradford method (Bio-Rad, Hercules, CA), and 25 μg protein was used in the assay (33, 34).

Western Imunoblotting

Cells were harvested on the 10th day of treatment. The cells were washed with ice-cold Dulbecco's PBS, scraped, and processed, and the supernatant was separated and stored at −80°C. The protein concentration in the cell lysates was measured using Bio-Rad method. Western immunoblotting was done on the whole-cell lysates as described previously (19).

Tumor Growth in Ovariectomized Female Athymic Nude Mice

All animal studies were done according to the guidelines approved by the Institution of Animal Care and Use Committee of the University of Maryland School of Medicine (Baltimore, MD). Female ovariectomized BALB/c athymic nude mice 4 to 6 weeks of age were obtained from the National Cancer Institute (NCI; Frederick, MD). The animals were housed in a pathogen-free environment under controlled conditions of light and humidity and received food and water *ad libitum*.

LTLC cells were routinely maintained in improved MEM with 5% charcoal-stripped FBS, 1% penicillin/streptomycin solution, 700 μg/mL G418, 1 μmol/L letrozole, and 25 nmol/L androstenedione. Subconfluent cells were scraped into Dulbecco's PBS, collected by centrifugation, and resuspended in Matrigel (10 mg/mL) at 5.0×10^7 cells/mL. Each animal received s.c. inoculations in one site per flank with 100 μL of cell suspension. Animals were randomly grouped into three. One group (n = 7) was injected daily s.c. with 4-androstenedione (100 μg/d) for the duration of treatment. The second group (n = 7) was injected daily s.c. with 4-androstenedione (100 μg/d) along with letrozole (10 μg/d) for the duration of treatment. The third group (n = 14) was injected with vehicle (0.3% hydroxypropylcellulose). Tumors were measured twice weekly with calipers, and tumor volume was calculated by the following formula: $4/3\pi r_1^2 \times r_2$, where r_1 is the smaller radius and r_2 is the larger radius. Treatments began when the tumors reached a measurable size (~100 mm^3), which was ~6 weeks after cell inoculation. Mice from the vehicle group were then regrouped in two. One group (n = 7) was injected s.c. 20 mg/kg/d with VN/14-1. The second group (n = 7) continued to receive the vehicle and served as control group. Letrozole, 4-androstenedione, and VN/14-1 were prepared in sterile conditions as suspensions in 0.3% hydroxypropylcellulose.

Statistical Analysis

All experiments were carried out thrice in replicates of six, and the results are expressed as mean ± SE where applicable. The effects of treatment were compared with MCF-7Ca control cells using either GraphPad Prism 4.0 software or Student's t test on SigmaPlot 2000, and Ps < 0.05 were considered statistically significant.

Results

Progression of hormone-dependent, letrozole-sensitive MCF-7Ca cells to hormone-independent, letrozole-insensitive LTLC cells. Early passage of MCF-7Ca cells (passage 7) was cultured in steroid-depleted medium (phenol red–free improved MEM) supplemented with 5% dextran-coated charcoal-treated serum, 1% penicillin/streptomycin, 700 μg G418, 25 nmol/L of aromatase substrate androstenedione, and 1 μmol/L letrozole. The cells became quiescent for 6 to 8 weeks before they began to proliferate slowly in presence of 1 μmol/L letrozole. These cells were designated the LTLC cells. Growth studies were done at various time points to evaluate the effects of a range of concentrations of letrozole (10^{-12} to 10^{-4} mol/L) on proliferation of LTLC cells versus the parental MCF-7Ca cells. We observed a gradual loss of sensitivity of LTLC cells to letrozole compared with the parental cells that show a dose-dependent inhibition of growth following treatment with letrozole (Fig. 2*A*). By 50 to 52 weeks, 1 μmol/L letrozole (the concentration in which the cells were growing) no longer inhibited the growth of these cells (Fig. 2*A*). However, higher concentrations (10 and 100 μmol/L) of letrozole were inhibitory, although to a significantly less extent than in the parental MCF-7Ca cells (Fig. 2*A*). This clearly indicates that the cells have become less responsive to letrozole compared with the parental MCF-7Ca cells.

As shown previously, the rate of proliferation of MCF-7Ca cells slows down when cultured in estrogen-deprived medium but is increased in response to estradiol (E_2; ref. 33). MCF-7Ca cells show maximum growth stimulation in response to 1 nmol/L E_2 and 25 nmol/L androstenedione (33, 35). To examine the response of LTLC cells to these hormones, growth studies were carried out on these cells and results were compared with those of the parental MCF-7Ca cells. Cells were synchronized by transferring them to steroid-depleted medium for 3 days. After prolonged estrogen deprivation caused by long-term letrozole treatment, LTLC cells had acquired the ability to grow in an estrogen-deprived environment and did not respond to treatment with E_2 or androstenedione, indicating that their growth was no longer dependent on estrogen (Fig. 2*B*). The LTLC cells were not only insensitive to letrozole but were also found to be no longer sensitive to growth inhibition by other clinically used aromatase inhibitors, such as exemestane and anastrazole (data not shown), indicating cross-resistance to other aromatase inhibitors.

Mechanism of resistance. Studies have shown the involvement of growth factor pathways in proliferation of breast cancer cells after prolonged estrogen deprivation (36). It is known that estrogens can stimulate growth factor production, which in turn can regulate the process of ER-mediated transcription. Therefore, we examined the expression of the growth factor receptor erbB-2, an EGFR that is activated in a ligand-independent manner. HER-2 protein is found to be overexpressed in 20% to 30% of metastatic breast cancer patients and is a negative prognostic factor (37, 38). HER-2 protein was found to be increased 4.5-fold in the LTLC cells compared with the MCF-7Ca cells (Fig. 2*C*). It has been shown that overexpression of HER-2 in MCF-7 breast cancer cells results in MAPK hyperactivity. MAPK hyperactivity promotes increased association of ER with coactivators and reduces association with corepressors, thus favoring estrogen-inducible gene transcription (39, 40). Therefore, we also examined the expression of ERα and its coactivator protein amplified in breast cancer 1 (AIB1). The levels of ERα and AIB1 proteins were both increased ~3-fold compared with the MCF-7Ca cells (Fig. 2*C*). In addition, evidence of HER-2/MAPK growth factor signaling pathways driving the growth of LTLT-Ca cells (20) prompted us to investigate whether this signaling pathway was also responsible for insensitivity of LTLC cells to letrozole *in vitro*. As reported for the LTLT-Ca cells, LTLC cells also showed up-regulation of HER-2 as well as overexpression of Grb2, pMEK1/2, and pMAPK1/2 proteins (Fig. 2*C*) compared with the parental MCF-7Ca cells (21).

Effect of VN/14-1 on growth of MCF-7Ca and LTLC cells. The effect of VN/14-1 on growth of cells that had become less responsive to letrozole and other aromatase inhibitors was examined and compared with the parental MCF-7Ca cells. VN/14-1 inhibited the growth of MCF-7Ca with IC_{50} of 10.5 nmol/L, whereas letrozole inhibited growth with an IC_{50} of 0.4 nmol/L (Fig. 3*A*). Interestingly, LTLC cells were exquisitely sensitive to VN/14-1 and growth was inhibited with an IC_{50} of 0.83 nmol/L (Fig. 3*B*), whereas letrozole was essentially ineffective with an IC_{20} of ~100 μmol/L. Thus, LTLC cells were significantly more

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sensitive to VN/14-1 than the parental cells. More importantly, the potency of VN/14-1 was ∼10,000-fold greater than that of letrozole in LTLC cells.

Effect of VN/14-1 on aromatase inhibition in MCF-7Ca cells and human placental microsomes. The effect of the chemopreventive synthetic retinoid 4-HPR on aromatase activity inhibition in microsomes of JEG3 cells and in MCF-7 cells has been shown previously (41). Because VN/14-1 also possesses retinoidal properties and, in addition, also inhibits retinoic acid metabolism by blocking CYP-mediated catabolism of ATRA, we investigated its effect on CYP-19 (aromatase) activity. We found that VN/14-1 was a potent inhibitor of intracellular aromatase activity in MCF-7Ca cells (Fig. 4*A*), with an IC_{50} value of 8.5 nmol/L. Thus, VN/14-1 is comparable with other potent aromatase inhibitors, such as letrozole, anastrazole, and exemestane, whose IC_{50} values range from 1 to 50 nmol/L (42).

To confirm whether VN/14-1 has a direct effect on aromatase, the enzyme assay was repeated using human placental microsomes. In microsomes, VN/14-1 inhibited aromatase with an IC_{50} of 8.0 pmol/L. Although letrozole was ∼10 times less potent in microsomes than in cells (IC_{50}, 0.6 pmol/L; Fig. 4*B*), it is a potent aromatase inhibitor in addition to being a potent RAMBA (25).

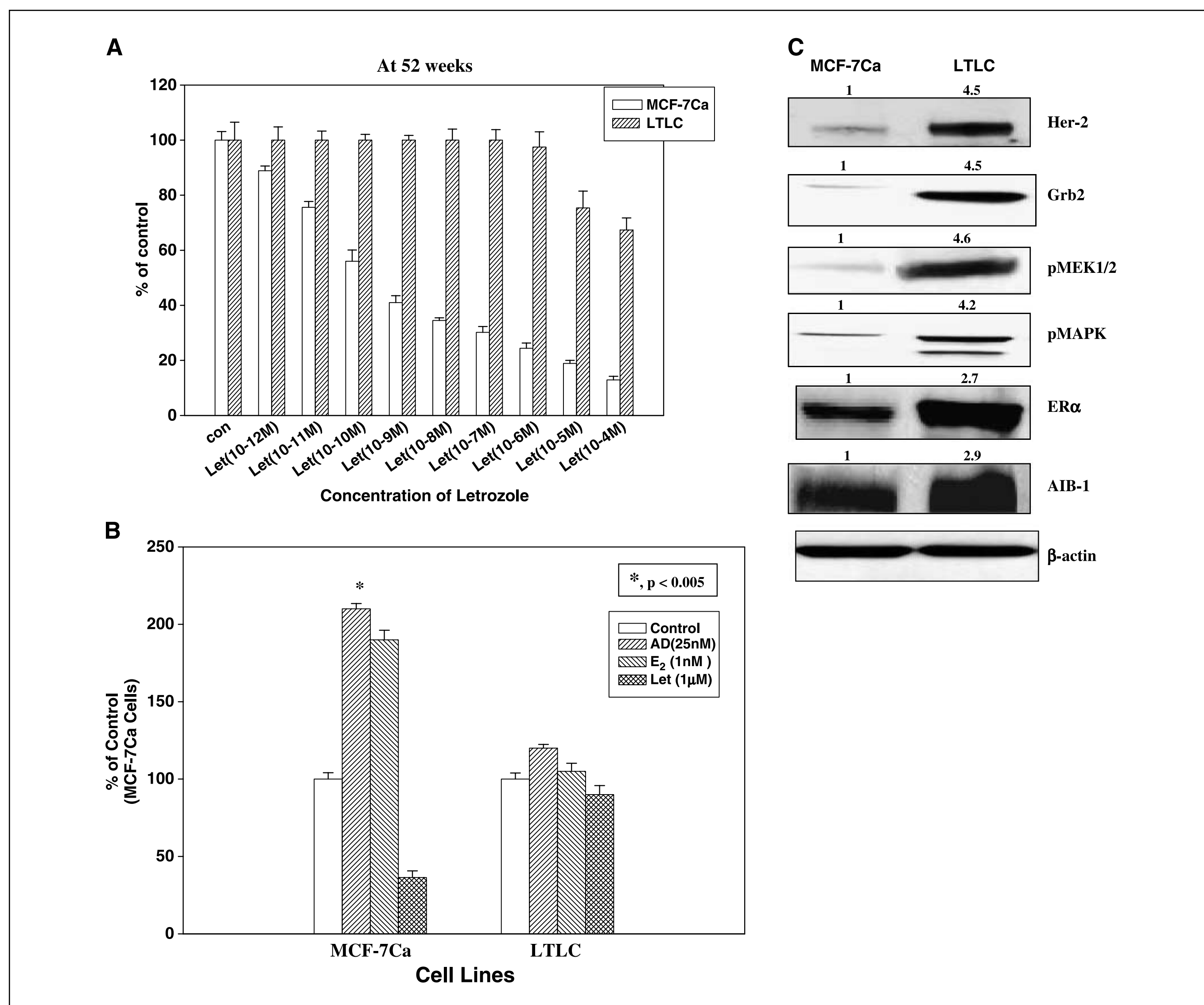

Figure 2. *A,* effect of letrozole (*Let*) on the growth of MCF-7Ca and LTLC human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating, and LTLC cells were cultured in reduced serum medium Opti-MEM 3 days before plating. Triplicate wells were then treated with the indicated concentrations of letrozole for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Columns,* mean of three experiments; *bars,* SE. *B,* effect of E_2 and androstenedione on the growth of MCF-7Ca and LTLC human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating, and LTLC cells were cultured in reduced serum medium Opti-MEM 3 days before plating. Triplicate wells were then treated with the indicated concentrations of E_2 and androstenedione for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Columns,* mean of triplicate experiments; *bars,* SE. For MCF-7Ca cells, E_2 or androstenedione treatment significantly increased cell viability ($P < 0.005$) *C,* growth factor receptor pathway adopted by the LTLC human breast cancer cells *in vitro*. Western immunoblotting analysis of whole-cell lysates from MCF-7Ca and LTLC cells cultured *in vitro* for HER-2, Grb2, MEK1/2, pMAPK, ERα, and AIB1 proteins. Experimental protocol was as described in Materials and Methods. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Representative of three independent experiments.

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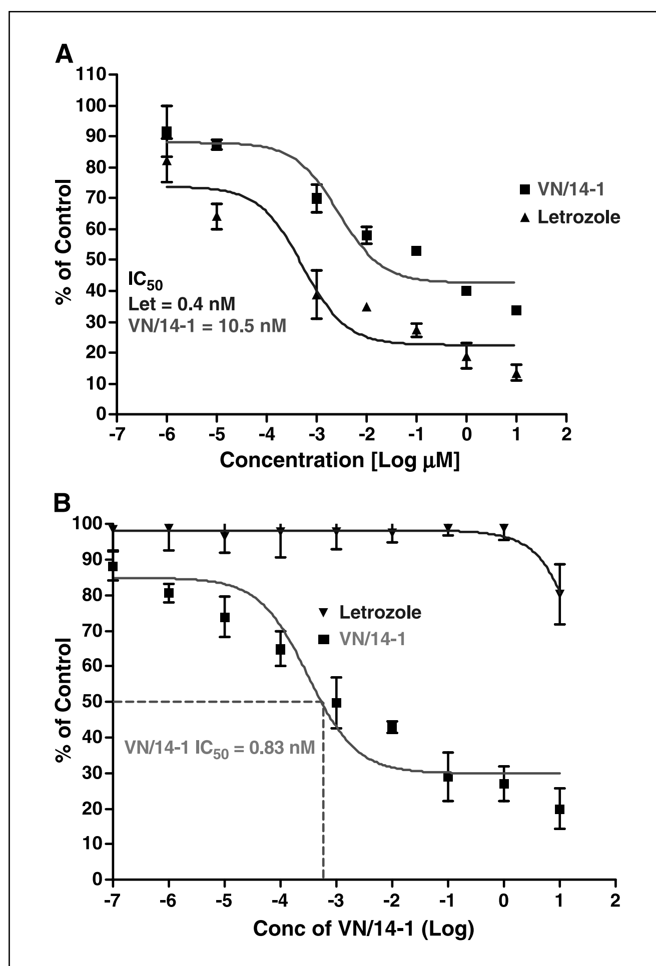

Figure 3. *A,* effect of letrozole and VN/14-1 on the growth of MCF-7Ca human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating. Triplicate wells were then treated with the indicated concentrations of letrozole or VN/14-1 for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE. *B,* effect of VN/14-1 on the growth of LTLC human breast cancer cells *in vitro*. LTLC cells were cultured in reduced serum medium for 3 days before plating. Triplicate wells were then treated with the indicated concentrations of VN/14-1 for 9 days, and the media were refreshed every 3 days. Cell proliferation was measured on day 10 using the MTT assay as described in Materials and Methods. Cell viability is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE. Treatment with VN/14-1 caused a dose-dependent inhibition in LTLC cells with IC_{50} of 0.83 nmol/L.

Effect of VN/14-1 on growth factor signaling pathways. As seen from the growth study results, LTLC cells were more sensitive to growth-inhibitory effects of VN/14-1 compared with the parental MCF-7Ca cells. Because the growth of the LTLC cells seems to be driven by the MAPK survival pathway, we investigated the effects of VN/14-1 on HER-2 and pMAPK proteins in LTLC cells. VN/14-1 caused a significant down-regulation of pMAPK at 1 µmol/L (1.2-fold) and HER-2 at 10 µmol/L (3.35-fold; Fig. 5).

Down-regulation of ERα and AIB1 following treatment with VN/14-1. To explore the mechanism of VN/14-1 in MCF-7Ca as well as LTLC cells, we investigated the effect of VN/14-1 on the ER expression by examining ERα protein and coactivator AIB1 by Western blotting. As indicated above, ERα protein was found to be increased 2.72-fold in the LTLC cells compared with the parental MCF-7Ca cells. VN/14-1 caused 1.4-fold decrease in ERα expression (Fig. 5). AIB1 was also up-regulated ~3-fold in the LTLC cells but was almost completely inhibited by VN/14-1 treatment (Fig. 5).

Down-regulation of cell cycle proteins (cyclin D1 and cyclin-dependent kinase 4) after treatment with VN/14-1. Treatment with retinoids inhibits cell cycle progression usually by causing arrest in the G_1 phase by affecting different cell cycle proteins, such as cyclins and cyclin-dependent kinases (CDK; refs. 43–47). Cyclin D1 is overexpressed in about one third of breast cancer cell lines (46). CDK2 and CDK4 are also known to be up-regulated, resulting in increase kinase activities (47). Because *cyclin D1* is an estrogen-responsive gene and because the ER was down-regulated

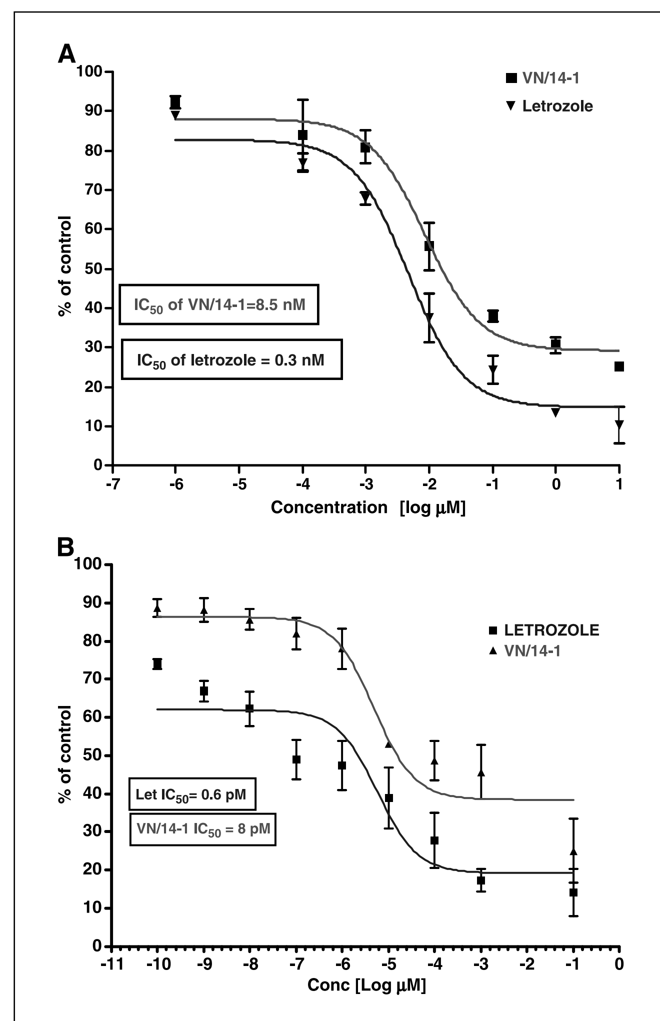

Figure 4. *A,* effect of letrozole and VN/14-1 on aromatase activity in MCF-7Ca human breast cancer cells *in vitro*. MCF-7Ca cells were cultured in steroid-free medium for 3 days before plating. Triplicate wells were then treated with the indicated concentrations of letrozole or VN/14-1 for 24 hours. Tritiated water formed was measured as described in Materials and Methods. Aromatase enzyme activity is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE. *B,* effect of letrozole and VN/14-1 on aromatase activity in human placental microsomes *in vitro*. Microsomes were extracted from human placenta as described in Materials and Methods. Triplicate wells were then treated with the indicated concentrations of letrozole or VN/14-1 for 30 minutes in the presence of oxygen. Tritiated water formed was measured as described in Materials and Methods. Aromatase enzyme activity is expressed as the percentage of the cells compared with the control wells. *Points,* mean of triplicate experiments; *bars,* SE.

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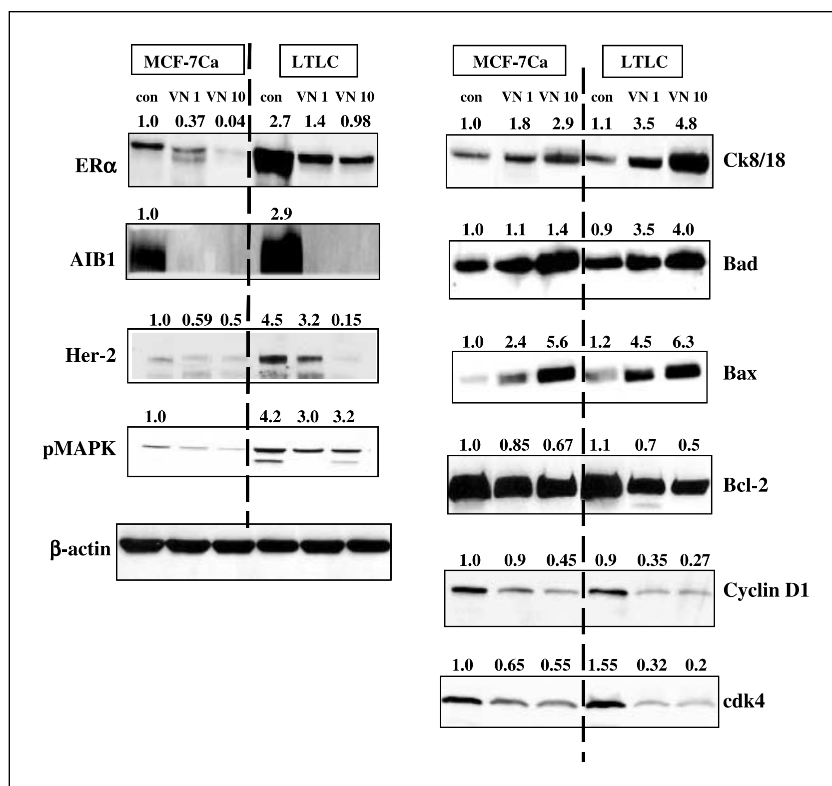

Figure 5. Effect of VN/14-1 on protein expression of ERα, AIB1, HER-2, pMAPK, cytokeratins 8 and 18, Bad, Bax, Bcl2, cyclin D1, and CDK4 in MCF-7Ca and LTLC cells. Western immunoblotting analysis of whole-cell lysates from MCF-7Ca and LTLC cells cultured *in vitro*. *Left,* ERα at 66 kDa, AIB1 at 160 kDa, HER-2 at 180 kDa, and pMAPK at 4,442 kDa; *right,* cytokeratins 8 and 18 at 47 kDa, Bad at 23 kDa, Bax at 20 kDa, Bcl2 at 28 kDa, cyclin D1 at 36 kDa, and CDK4 at 30 kDa. *Left* and *right, lane 1,* MCF-7Ca cells (control); *lanes 2* and *3,* 1 and 10 μmol/L VN/14-1 in MCF-7Ca cells; *lane 4,* LTLC cells (control); *lanes 5* and *6,* VN/14-1 treatments at 1 and 10 μmol/L in LTLC cells. Experimental protocol was as described in Materials and Methods. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Blots are representative of three independent experiments.

after treatment with VN/14-1, we examined the level of this protein after treatment with VN/14-1. The level of cyclin D1 was decreased by 0.8- and 0.45-fold in MCF-7Ca and 0.35- and 0.27-fold in LTLC cells after treatment with 1 and 10 μmol/L VN/14-1, respectively (Fig. 5). The level of CDK4 was increased by ~1.7-fold in LTLC cells compared with the parental MCF-7Ca cells, indicating increased kinase activity. Its levels were also down-regulated in both the cell lines by 0.65- and 0.55-fold in MCF-7Ca and 0.32- and 0.20-fold in LTLC cells compared with the untreated cells, respectively (Fig. 5). These results suggest that LTLC cells are more sensitive to the effect of VN/14-1 compared with the parental MCF-7Ca cells. Down-regulation of cyclin D1 and CDK4 after VN/14-1 treatment indicates that VN/14-1 causes arrest of the cells in G_1 phase of the cell cycle, thus preventing the proliferation of the tumor cells.

Effect of VN/14-1 on differentiation proteins, cytokeratin 8, and cytokeratin 18. Cytokeratins have been identified as one of the differentiation marker proteins also known as structural marker proteins for epithelial cells (48). Elevated levels of cytoskeletal proteins indicate a favorable prognosis and are useful predictors for overall survival of breast cancer patients (49, 50). Recent studies have shown that retinoids enhance the expression of cytokeratin in breast cancer cells. Therefore, we examined the expression of cytokeratins 8 and 18 after treatment with VN/14-1. Cell lysates of MCF-7Ca and LTLC cells were obtained on the 10th day after treatment with 1 and 10 μmol/L VN/14-1 and then probed by Western blotting for cytokeratins 8 and 18 using antibody at 1:2,500 dilution in 10% milk-PBS-Tween 20 for 1 hour at room temperature. Treatment with 1 and 10 μmol/L VN/14-1 showed dose-dependent increases of 2.0- and 6.2-fold in MCF-7Ca cells and 4.1- and 9.9-fold in LTLC cells of cytokeratins 8 and 18, respectively (Fig. 5).

Effect of VN/14-1 on apoptotic proteins (Bad, Bax, and Bcl2). The mechanisms underlying the anticarcinogenic activity of retinoids seem to be associated with the ability of retinoids to modulate growth, differentiation, and apoptosis in different malignancies, including breast cancer (43, 44, 46). Therefore, we investigated the apoptotic proteins Bad, Bax, and Bcl2 after treatment with VN/14-1. The expression of proapoptotic protein Bad showed dose-dependent increases of about 1- to 2-fold after treatment with 1 and 10 μmol/L VN/14-1 in both cell lines. Another proapoptotic protein Bax showed much greater increases of 2.4- and 5.56-fold in MCF-7Ca cells and 4.5- and 6.3-fold increase in LTLC cells after treatment with 1 and 10 μmol/L VN/14-1, respectively. The antiapoptotic protein Bcl2 was down-regulated by 0.85- and 0.67-fold in MCF-7Ca cells and 0.68- and 0.5-fold in LTLC cells after treatment. Thus, these results indicate that VN/14-1 is causing apoptosis in both the cell lines but to a greater extent in LTLC cells (Fig. 5).

Effect of VN/14-1 in female athymic ovariectomized nude mice. To confirm our *in vitro* findings, we inoculated LTLC cells in athymic ovariectomized nude mice. The hormone-independent nature of these cells was evident when one group was injected with 100 μg/d androstenedione from day 1 of inoculation. Unlike MCF-7Ca tumors that require estrogens to grow, LTLC tumors grew without hormones (Fig. 6*A*). After a period of 6 weeks, when the tumors had reached a size of 100 mm^3, the vehicle-treated group was divided into two. One group continued to receive vehicle (control group), whereas the other group received 20 mg/kg/d VN/14-1 s.c. five times weekly. The dose of VN/14-1 was selected based on previous studies with this novel compound done in our laboratory (25). Tumors of the mice treated with letrozole and androstenedione grew like the control tumors, indicating that the LTLC tumors were insensitive to the effects of letrozole as shown

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in vitro. Thus, VN/14-1 inhibited the LTLC tumor growth significantly. Tumor growth resumed, but even after 60 days, tumors had not doubled in volume and were significantly smaller than the control group, androstenedione alone, as well as letrozole plus androstenedione group.

Effect of VN/14-1 on LTLC tumor protein expressions. Expression of the same proteins was examined in tumor samples as studied earlier *in vitro*. ERα, AIB1, cyclin D1, and CDK4, including pMAPK, MEK1/2, and phosphorylated ribosomal protein S6 kinase (90-kDa protein; p90RSK), were down-regulated in the tumors of VN/14-1 treatment group. Similarly Bad, Bax, and cytokeratins 8/18 were up-regulated (Fig. 6*B*). However, HER-2 protein was not affected unlike our *in vitro* finding where treatment with VN/14-1 caused down-regulation of HER-2. This may be due to differences in doses of VN/14-1 *in vitro* and *in vivo*.

Discussion

Aromatase inhibitors are now showing greater efficacy than antiestrogen tamoxifen. However, as resistance eventually develops to all forms of treatment, it is relevant to investigate other strategies to control tumor proliferation. In this study, we investigated the growth effects of a novel RAMBA on breast cancer cells using cells that were made refractory to aromatase inhibitor letrozole. Aromatase-expressing MCF-7Ca cells were cultured in the presence of 1 μmol/L letrozole (concentration ~1,000 times higher

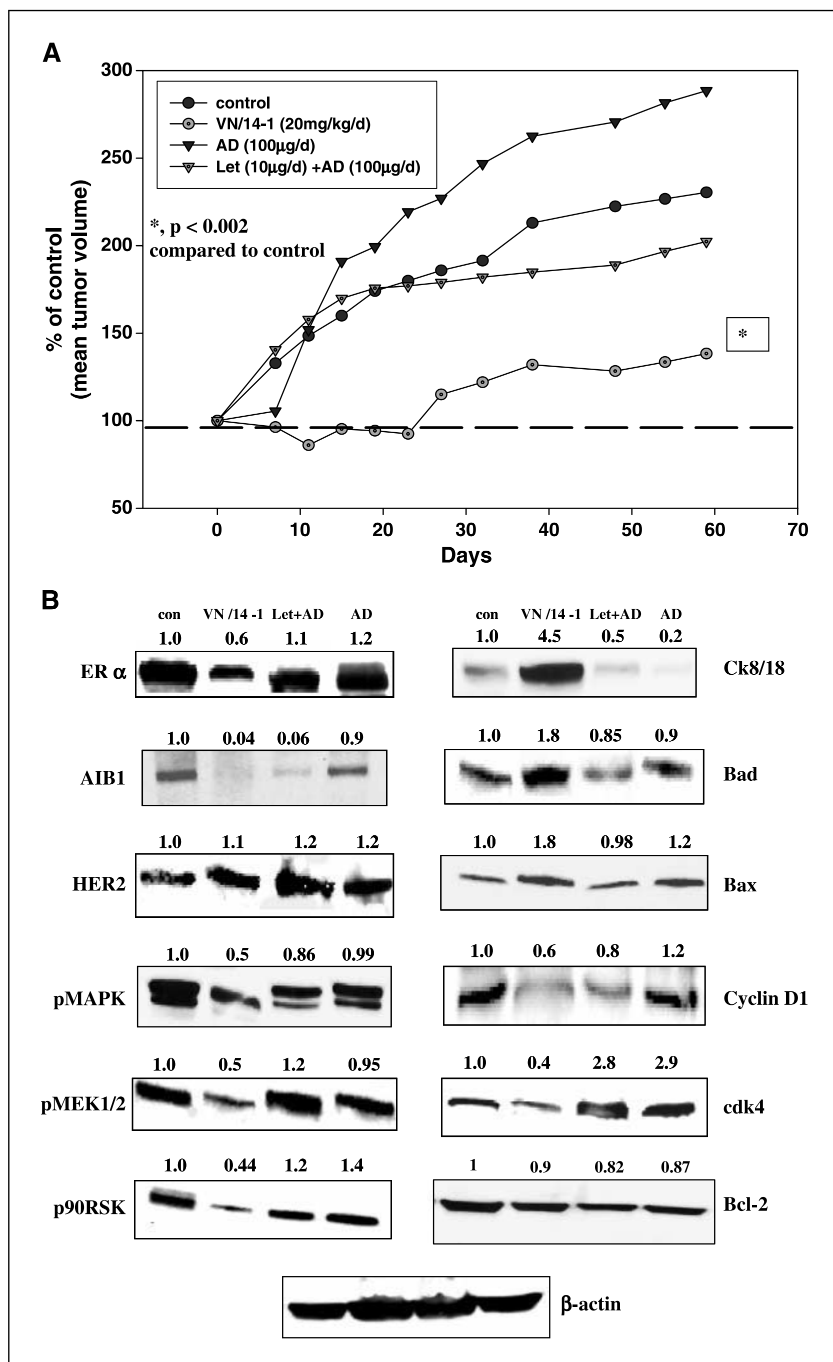

Figure 6. *A,* effect of letrozole plus androstenedione (*AD*), androstenedione alone, and VN/14-1 in LTLC xenograft model. Female ovariectomized nude mice were inoculated with LTLC cells as described in Materials and Methods. Mice were randomly grouped (n = 10 control; n = 5 in other two groups) and treated with vehicle only for control, androstenedione (100 μg/mouse/d), and letrozole (10 μg/mouse/d) plus androstenedione (100 μg/mouse/d). After the tumors were 100 mm^3, control mice were regrouped (n = 5). One group continued on vehicle and other group switched to VN/14-1 (20 mg/kg/mouse/d). Treatment was continued for 70 days. The mice were sacrificed at the end of the study, and tumor samples were collected for further studies. *B,* effect of letrozole plus androstenedione, androstenedione alone, and VN/14-1 on protein expression of ERα, AIB1, HER-2, pMAPK, pMEK1/2, p90RSK, cytokeratins 8 and 18, Bad, Bax, Bcl2, cyclin D1, and CDK4 in LTLC tumor samples. Western immunoblotting analysis of LTLC tumor lysates. *Left,* ERα at 66 kDa, AIB1 at 160 kDa, HER-2 at 180 kDa, pMAPK at 4,442 kDa, pMEK1/2 at 45 kDa, and p90RSK at 90 kDa; *right,* cytokeratins 8 and 18 at 47 kDa, Bad at 23 kDa, Bax at 20 kDa, Bcl2 at 28 kDa, cyclin D1 at 36 kDa, and CDK4 at 30 kDa. Experimental protocol was as described in Materials and Methods. Blots were stripped and probed for β-actin to verify equal amount of protein loaded in each lane. Blots are representative of three independent experiments.

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than its IC_{50} value) and the aromatase substrate androstenedione. The latter is converted by aromatase in the cells into E_2. As previously reported, letrozole inhibited the growth of the cells initially for a period of 6 to 8 weeks. The growth-inhibitory effects of letrozole (10^{-12} to 10^{-4} mol/L) were evaluated at several time points. Eventually, after 50 to 52 weeks, the cells then began to proliferate slowly and were no longer inhibited by 1 μmol/L letrozole, although they were marginally inhibited by 10 and 100 μmol/L letrozole. Thus, these cells (designated as LTLC) were less sensitive than the parental MCF-7Ca cells to the growth-inhibitory effects of letrozole. The LTLC cells were also insensitive to antiestrogens tamoxifen and Faslodex (data not shown). Compared with the LTLT-Ca cells previously reported (33), LTLC cells retain some sensitivity to letrozole. LTLT-Ca cells were isolated from MCF-7Ca tumors of mice with letrozole for 56 weeks. The LTLT-Ca cells, like the LTLC cells, also showed up-regulation of the proteins in MAPK pathway. However, the level of ER, which is up-regulated in the LTLC cells, was up-regulated initially but subsequently decreased in tumors that were unresponsive to letrozole. This suggests that the LTLT-Ca cells were subjected to more severe estrogen deprivation than the LTLC cells. We have also reported studies of MCF-7Ca cells that were deprived of estrogen in culture (UMB-1Ca; refs. 19, 33). These cells were only sensitive to higher concentrations of letrozole like the LTLC cells (Fig. 2*A*; refs. 19, 33).

The results in the present study indicate that after prolonged estrogen deprivation caused by letrozole treatment *in vitro*, MCF-7Ca cells have increased expression of proteins in the estrogen signaling pathway (increase ER, AIB1, cyclin D1, and CDK4). The cells had up-regulated proteins in the MAPK growth factor pathway for survival. LTLC cells were less responsive to the inhibitory effects of letrozole as well as other aromatase inhibitors: anastrazole (nonsteroidal aromatase inhibitor) and exemestane (steroidal aromatase inhibitor). Thus, MCF-7Ca cells that have developed resistance to letrozole also tend to be unresponsive to other aromatase inhibitors (data not shown).

Using the LTLC breast cancer cells that were no longer responsive to the growth-inhibitory effect of letrozole, the goal was to identify agent(s) that would inhibit the growth of these cells. As reported previously, we have discovered several RAMBAs with multiple biological and inhibitory activities against several human breast and prostate cancer cells (25). Our results presented here show that the lead compound in this series of novel RAMBAs, VN/14-1, was a remarkably potent inhibitor of the growth of LTLC and MCF-7Ca cells. However, the other RAMBAs (VN/12-1, VN/50-1, and VN/66-1) were ineffective in the LTLC cells (data not shown). Our results indicate that VN/14-1 is also a potent aromatase inhibitor and down-regulates ERα and steroid coactivator AIB1. In LTLC cells, estrogen signaling was significantly increased. Figure 5 shows increased ERα, its coactivator AIB1, *cyclin D1* (estrogen-responsive gene), and CDK4. Treatment with VN/14-1 caused marked down-regulation of ERα, the related coactivator AIB1, as well as cyclin D1 and CDK4 in the MCF-7Ca cells as well as LTLC cells. Our findings suggest that, similar to other retinoids, VN/14-1 mediates its effects in part through interference with coactivator AIB1 and ER signal transduction, thus affecting estrogen-responsive genes, such as *cyclin D1* (51).

In addition, VN/14-1 also showed several other significant effects on cell differentiation, cell cycle, and apoptosis. Hormone independence and letrozole insensitivity of LTLC cells were further confirmed in the xenograft model. Parental MCF-7Ca xenografts are hormone dependent and need androstenedione supplementation to grow (35, 52). However, LTLC cells grew without any supplementation (androstenedione or estrogens) as well as in the presence of letrozole. It should be noted that letrozole (10 μg/d) has shown to be the most effective inhibitor of MCF-7Ca tumor growth (53). Treatment with 20 mg/kg/d VN/14-1 caused significant tumor growth suppression ($P < 0.002$). In addition, similar *in vitro* and *in vivo* findings (alteration in the levels of apoptotic proteins, cell cycle proteins, differentiation proteins, ERα, and AIB1) further strengthen the effectiveness of this compound. Down-regulation of HER-2 at 10 μmol/L and pMAPK at 1 μmol/L proteins *in vitro* as well as pMEK1/2, pMAPK, and p90RSK *in vivo* following VN/14-1 treatment suggests interference of VN/14-1 in this signaling pathway. This may partly explain why VN/14-1 is more effective in LTLC cells, although the exact reason for VN/14-1 being more effective in LTLC cells than in MCF-7Ca cells is unclear at this time.

In conclusion, we induced letrozole insensitivity by prolonged treatment of MCF-7Ca cells with letrozole *in vitro* (LTLC). These cells grew without hormone supplementation and showed up-regulation of proteins in the estrogen and MAPK signaling pathways. VN/14-1 has potent antiproliferative effects against estrogen-dependent MCF-7Ca cells. VN/14-1 was found to be a potent inhibitor of the aromatase activity as well as of growth in the parental MCF-7Ca cells. We observed that the anticancer effects of VN/14-1 seem to be due to its multiple biological properties. These include significant down-regulation of proteins in the MAPK pathway in the LTLC cells as well as marked ER and AIB1 down-regulation in both the cell lines. In addition, VN/14-1 caused induction of apoptosis, differentiation, and cell cycle arrest *in vitro* and *in vivo*. Furthermore, treatment with VN/14-1 induced significant arrest of growth of LTLC tumors in the xenograft model. These multiple anticancer properties of this novel RAMBA, VN/14-1, can be exploited clinically. The compound has potential as a new therapeutic agent for hormone-dependent breast cancer as well as following resistance to aromatase inhibitors.

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References

1. Jenson E, Jordan C. The estrogen receptor, a model for molecular medicine. Clin Cancer Res 2003;9:1980–9.
2. Yue W, Mor G, Naftolin F, et al. Aromatase inhibitors in breast cancer. In: Robertson J, Nicholson R, Hayes D, editors. Endocrine therapy of breast cancer. London: Martin Dunitz Ltd.; 2002. p. 75–106.
3. McGuire W. An update on estrogen and progesterone receptors in prognosis for primary and advanced breast cancer. In: Iacobelli S, King RJ, Lindner HR, Lippman ME, editors. Hormones and cancer. New York: Raven Press; 1980. p. 337–44.
4. Reed M, Owen A, Lai L, et al. *In situ* estrone synthesis in normal breast and breast tumor tissues: effect of

treatment with 4-hydroxyandrostenedione. Int J Cancer 1989;44:233–7.
5. van Landegham A, Portman J, Nabauurs M. Endogenous concentration and subcellular distribution of estrogens in normal and malignant human breast tissue. Cancer Res 1985;45:2900–6.
6. Brodie A, Garrett W, Hendrickson J, Tsai-Morris C, Marcotte P, Robinson C. Inactivation of aromatase *in vitro* by 4-hydroxy-4-androstene-3,17-dione and 4-acetoxy-4-androstene-3,17-dione and sustained effects *in vivo*. Steroids 1981;38:693–702.
7. Coombes C, Hall E, Gibson A. Randomized trial of exemestane after two to three years of tamoxifen therapy in postmenopausal women with primary breast cancer. N Engl J Med 2004;350:1081–92.
8. Buzdar A. Superior efficacy of letrozole versus tamoxifen as first-line therapy. J Clin Oncol 2002;20: 876–8.
9. Mouridsen H, Gershanovich M, Sun Y, et al. Superior efficacy of letrozole versus tamoxifen as first-line therapy for postmenopausal women with advanced breast cancer: results of a phase III study of the International Letrozole Breast Cancer Group. J Clin Oncol 2001;19:2596–606.
10. Goss P, Ingle J, Martino S, et al. A randomized trial of letrozole in postmenopausal women after five years of tamoxifen therapy. N Engl J Med 2003;349: 1793–802.
11. Baum M, Buzdar A, Cuzick J. Anastrozole alone or in combination with tamoxifen versus tamoxifen alone for adjuvant treatment of postmenopausal women with early-stage breast cancer: results of the ATAC (Arimidex, Tamoxifen Alone or in Combination) trial efficacy and safety update analyses. Cancer 2003;98:1802–10.
12. Wakeling A, Nicholson R, Gee J. Prospects for combining hormonal and nonhormonal growth factor inhibition. Clin Cancer Res 2001;7:4350–5.
13. Benz C, Scott G, Sarup J, et al. Estrogen-dependent, tamoxifen-resistant tumorigenic growth of MCF-7 cells transfected with HER2/neu. Breast Cancer Res Treat 1992;24:85–95.
14. Nicholson R, Gee J. Estrogen and growth factor cross-talk and endocrine insensitivity and acquired resistance in breast cancer. Br J Cancer 2000;82:501–13.
15. Lee A, Cui X, Oesterreich S. Cross-talk among estrogen receptor, epidermal growth factor, and insulin like growth factor signaling in breast cancer. Clin Cancer Res 2001;12:4429–35S; discussion 4411–4412S.
16. Shou J, Massarweh S, Osborne C, et al. Mechanisms of tamoxifen resistance: increased estrogen receptor-HER2/neu cross-talk in ER/HER2-positive breast cancer. J Natl Cancer Inst 2004;96:926–35.
17. Schiff R, Massarweh S, Shou J, Osborne C. Breast cancer endocrine resistance: how growth factor signaling and estrogen receptor coregulators modulate response. Clin Cancer Res 2003;9:447–54s.
18. Nicholson R, Gee J, Barrow D, Pamment J, Knowlden J, McClelland R. Endocrine resistance in breast cancer can involve a switch towards EGFR signaling pathways and a gain of sensitivity to an EGFR-selective tyrosine kinase inhibitor, ZD1839. AACR-NCI-EORTC International Conference on Molecular Targets and Cancer Therapeutics; 1999.
19. Sabnis G, Jelovac D, Long B, Brodie A. The role of growth factor receptor pathways in human breast cancer cells adapted to long-term estrogen deprivation. Cancer Res 2005;65:3903–10.
20. Brodie A, Jelovac D, Sabnis G, Long B, Macedo L, Goloubeva O. Model systems: mechanisms involved in the loss of sensitivity to letrozole. J Steroid Biochem Mol Biol 2005;95:41–8.
21. Jelovac D, Sabnis G, Long B, Macedo L, Goloubeva O, Brodie A. Activation of mitogen-activated protein kinase in xenografts and cells during prolonged treatment with aromatase inhibitor letrozole. Cancer Res 2005;65: 5380–9.
22. Dragnev K, Rigas J, Dmitrovsky E. The retinoids and cancer prevention mechanisms. Oncologist 2000;5: 361–8.
23. Goss P, Oza A, Goel R, et al. Liarozole fumarate (R85246): a novel imidazole in the treatment of receptor positive postmenopausal metastatic breast cancer. Breast Cancer Res Treat 2000;59:55–68.
24. Njar V. Cytochrome P450 retinoic acid 4-hydroxylase inhibitors: potential agents for cancer therapy. Mini Rev Med Chem 2002;2:261–9.
25. Patel J, Huynh C, Handratta V, et al. Novel retinoic acid metabolism blocking agents endowed with multiple biological activities are efficient growth inhibitors of human breast and prostate cancer cells *in vitro* and a human breast tumor xenograft in nude mice. J Med Chem 2004;47:6716–29.
26. Zhou D, Pompon D, Chen S. Stable expression of human aromatase complementary DNA in mammalian cells: a useful system for aromatase inhibitor screening. Cancer Res 1990;50:6949–54.
27. Zhou D, Wang J, Chen E, Murai J, Siiteri P, Chen S. Aromatase gene is amplified in MCF-7 human breast cancer cells. J Steroid Biochem Mol Biol 1993; 46:147–53.
28. Yue W, Zhou D, Chen S, Brodie A. A new nude mouse model for postmenopausal breast cancer using MCF-7 cells transfected with the human aromatase gene. Cancer Res 1994;54:5092–5.
29. Schwarzel W, Kruggel W, Brodie H. Studies on the mechanism of estrogen biosynthesis. The development of the enzyme system in the human placenta. Endocrinology 1973;92:866–80.
30. Inkster S, Brodie A. Immunocytochemical studies of aromatase in early and full-term human placental tissues: comparison with biochemical assays. Biol Reprod 1989;41:889–98.
31. Brodie A, Schwarzel W, Shaikh A, Brodie H. The effect of an aromatase inhibitor, 4-hydroxy-4-androstene-3,17-dione, on estrogen-dependent processes in reproduction and breast cancer. Endocrinology 1977; 100:1684–95.
32. Brodie H, Kripliani K, Possanza G. Studies on the mechanisms of estrogen biosynthesis. VI. The stereochemistry of hydrogen elimination of C-2 during aromatization. J Am Chem Soc 1969;91:1241–3.
33. Long B, Jelovac D, Thaintanawat A, Brodie A. The effect of second-line antiestrogen therapy on breast tumor growth after first-line treatment with the aromatase inhibitor letrozole. Long-term studies using the intratumoral aromatase postmenopausal breast cancer model. Clin Cancer Res 2002;8:2378–88.
34. Long B, Tilghman S, Yue W, Thaintanawat A, Grigoryev D, Brodie A. The steroidal antiestrogen ICI 182,780 is an inhibitor of cellular aromatase activity. J Steroid Biochem Mol Biol 1998;67:293–304.
35. Long B, Jelovac D, Haradatta V, et al. Therapeutic strategies using the aromatase inhibitor letrozole and tamoxifen in a breast cancer model. J Natl Cancer Inst 2004;96:456–65.
36. Katzenellenbogen B, Kendra K, Norman M, Berthois Y. Proliferation, hormonal responsiveness, and estrogen receptor content of MCF-7 human breast cancer cells grown in the short-term and long-term absence of estrogens. Cancer Res 1987;47:4355–60.
37. Sjogren S, Inganas M, Lindgren A, Holmberg L, Bergh J. Prognostic and predictive value of c-erbB-2 overexpression in primary breast cancer, alone and in combination with other prognostic markers. J Clin Oncol 1998;16:462–9.
38. Zidan J, Dashkovsky I, Stayerman C, Basher W, Cozacov C, Hadary A. Comparison of HER-2 overexpression in primary breast cancer and metastatic sites and its effect on biological targeting therapy of metastatic disease. Br J Cancer 2005;93:552–6.
39. Yue W, Wang J, Conaway M, Li Y, Santen R. Adaptive hypersensitivity following long-term estrogen deprivation: involvement of multiple signal pathways. J Steroid Biochem Mol Biol 2003;86:265–74.
40. Kurokawa H, Arteaga C. ErbB (HER) receptors can abrogate antiestrogen action in human breast cancer by multiple signaling mechanisms. Clin Cancer Res 2003;9: 511–5S.
41. Ciolino H, Wang T, Sathyamoorthy N. Inhibition of aromatase activity and expression in MCF-7 cells by the chemopreventive retinoid *N*-(4-hydroxy-phenyl)-retinamide. Br J Cancer 2000;83:333–7.
42. Bhatnager A, Brodie A, Long B, Evans D, Miller W. Intracellular aromatase and its relevance to the pharmacological efficacy of aromatase inhibitors. J Steroid Biochem Mol Biol 2001;76:199–202.
43. Simeone A, Tari A. How retinoids regulate breast cancer cell proliferation and apoptosis. Cell Mol Life Sci 2004;61:1475–84.
44. Semeniuk E, Anchim T, Dziêcio J, Dbrowska M, Sawomir Wo czyñski. Can transforming growth factor-1 and retinoids modify the activity of estradiol and antiestrogens in MCF-7 breast cancer cells? Acta Biochimica Polonica 2004;51:733–45.
45. Teixeira C, Pratt C. CDK2 is a target for retinoic acid-mediated growth inhibition in MCF-7 human breast cancer cells. Mol Endocrinol 1997;11:1191–202.
46. Pratt C, Niu M, White D. Differential regulation of protein expression, growth, and apoptosis by natural and synthetic retinoids. Cell Biochem 2003;90:692–708.
47. Zhou Q, Stetler-Stevenson M, Steeg P. Inhibition of cyclin D expression in human breast carcinoma cells by retinoids *in vitro*. Oncogene 1997;15:107–15.
48. Woelfle U, Sauter G, Santjer S, Brakenhoff R, Pantel K. Down-regulated expression of cytokeratin 18 promotes progression of human breast cancer. Clin Cancer Res 2004;10:2670–4.
49. Jing Y, Zhang J, Waxman S, Mira-y-Lopez R. Upregulation of cytokeratins 8 and 18 in human breast cancer T47D cells is retinoid-specific and retinoic acid receptor-dependent. Differentiation 1996;60:109–17.
50. Korsching E, Packeisen J, Agelopoulos K, et al. Cytogenetic alterations and cytokeratin expression patterns in breast cancer: integrating a new model of breast differentiation into cytogenetic pathways of breast carcinogenesis. Lab Invest 2002;82:1525–33.
51. Rubin M, Fenig E, Rosenauer A, et al. 9-*Cis* retinoic acid inhibits growth of breast cancer cells and down-regulates estrogen receptor RNA and protein. Cancer Res 1994;54:6549–56.
52. Lu Q, Liu Y, Long B, Grigoryev D, Gimbel M, Brodie A. The effect of combining aromatase inhibitors with antiestrogens on tumor growth in a nude mouse model for breast cancer. Breast Cancer Res Treat 1999;57:183–92.
53. Brodie A, Jelovac D, Long B. Predictions from a preclinical model: studies of aromatase inhibitors and antiestrogens. Clin Cancer Res 2000;9:455–9S.

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