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

Aashvini Belosay, Angela M.H. Brodie, and Vincent C.O. Njar

Department of Pharmacology and Experimental Therapeutics, School of Medicine and the Greenebaum Cancer Center, University of Maryland, Baltimore, Maryland

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 ER<sup>a</sup> 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<sub>50</sub> of 8.5 and 10.5 nmol/L, respectively. In human placental microsomes, aromatase activity was inhibited with IC<sub>50</sub> of 8.0 pmol/L. The IC<sub>50</sub> in LTLC cells was 0.83 nmol/L, similar to letrozole (IC<sub>50</sub> 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 ER<sup>a</sup>, 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; <i>P </i>&lt; 0.002). Decreases in ER<sup>a</sup>, 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 ER<sup>a</sup>. The binding of estrogen to ER<sup>a</sup> 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 steroid 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 lose 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
studies have been carried out in our laboratory to investigate the effects of resistance to aromatase inhibitor letrozole \textit{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 \textit{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 \textit{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-rietinoic acid (ATRA) and its isomers as well as other retinoids, such as fenretinide \cite{23} are retinoids. All-chemotherapeutic agents used in the treatment of breast cancer \textit{in vitro} to determine loss of sensitivity to aromatase inhibitor letrozole.

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 hormone therapies (23).

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 placenta and stored in 0.1 mol/L phosphate buffer (pH 7.4) in –70 °C until required as described elsewhere (30).

Radiometric $^3$H$_2$O Release Assay for Measuring the Aromatase Activity

Microsomal and cellular assay. This assay was done as reported earlier (31, 32). Tritiated water ($^3$H$_2$O) formed during the aromatization of [1$^\text{H}$]-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
Western Immunoblotting

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 × 10⁶ 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: $V = \frac{4}{3} \pi r_1 r_2$, where $r_1$ is the smaller radius and $r_2$ is the larger radius.

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. 2A). 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. 2A). 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. 2A). 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 (E2; ref. 33). MCF-7Ca cells show maximum growth stimulation in response to 1 nmol/L E2 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 E2 or androstenedione, indicating that their growth was no longer dependent on estrogen (Fig. 2B). 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. 2C). 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 ERβ 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. 2C). In addition, evidence of HER-2/MAPK growth factor signaling pathways driving the growth of LTLC 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 LTLC-Ca cells, LTLC cells also showed up-regulation of HER-2 as well as overexpression of Grb2, pMEK1/2, and pMAPK1/2 proteins (Fig. 2C) compared with the parental MCF-7Ca cells (21).

**Effect of V/N14-1 on growth of MCF-7Ca and LTLC cells.** The effect of V/N14-1 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 IC₅₀ of 10.5 nmol/L, whereas letrozole inhibited growth with an IC₅₀ of 0.4 nmol/L (Fig. 3A). Interestingly, LTLC cells were notoriously sensitive to VN/14-1 and growth was inhibited with an IC₅₀ of 0.83 nmol/L (Fig. 3B), whereas letrozole was essentially ineffective with an IC₅₀ of ~100 μmol/L. Thus, LTLC cells were significantly more...
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. 4A), 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. 4B), it is a potent aromatase inhibitor in addition to being a potent RAMBA (25).

![Figure 2 A](https://example.com/figure2a.png)
![Figure 2 B](https://example.com/figure2b.png)
![Figure 2 C](https://example.com/figure2c.png)

**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, ER$_{a}$, and AIB1 proteins. Experimental protocol was as described in Materials and Methods. Blots were stripped and probed for $\beta$-actin to verify equal amount of protein loaded in each lane. Representative of three independent experiments.
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 10.5 nM (1.2-fold) and HER-2 at 10 nM (3.35-fold; 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 G1 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

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).
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 G1 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 cytokeratin 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 cytokeratin 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 cytokeratin 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 malignantities, 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. 6A). After a period of 6 weeks, when the tumors had reached a size of 100 mm³, 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.
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. 6B). 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...
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} \text{ to } 10^{-4} \text{ 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 \text{ mmol/L letrozole}, although they were marginally inhibited by 10 and 100 \text{ mmol/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. 2A; 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-I, 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 ER\alpha and steroid coactivator AIB1. In LTLC cells, estrogen signaling was significantly increased. Figure 5 shows increased ER\alpha, its coactivator AIB1, cyclin D1 (estrogen-responsive gene), and CDK4. Treatment with VN/14-1 caused marked down-regulation of ER\alpha, 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 \mu g/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, ER\alpha, and AIB1) further strengthen the effectiveness of this compound. Down-regulation of HER-2 at 10 \mu mol/L and pMAPK at 1 \mu mol/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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