Published in final edited form as:

Breast Cancer Res Treat. 2012 May ; 133(1): 137–144. doi:10.1007/s10549-011-1724-7.

Anti-tumor effects of a novel retinoic acid metabolism blocking agent VN/14-1 in the *N*-methyl-*N*-nitrosourea-induced rat mammary carcinoma model and its effects on the uterus

Paul E. Goss,

Breast Cancer Research, Massachusetts General Hospital Cancer Center, Breast Cancer Disease Program, Dana Farber/Harvard Cancer Center, Harvard Medical School, Boston, MA 02114, USA

Shangle Qi,

Breast Cancer Research, Massachusetts General Hospital Cancer Center, Breast Cancer Disease Program, Dana Farber/Harvard Cancer Center, Harvard Medical School, Boston, MA 02114, USA

Haiqing Hu,

Breast Cancer Research, Massachusetts General Hospital Cancer Center, Breast Cancer Disease Program, Dana Farber/Harvard Cancer Center, Harvard Medical School, Boston, MA 02114, USA

Lalji K. Gediya,

Department of Pharmaceutical Sciences, Jefferson School of Pharmacy and Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA

Puranik Purushottamachar,

Department of Pharmaceutical Sciences, Jefferson School of Pharmacy and Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA

Abhijit M. Godbole, and

Department of Pharmaceutical Sciences, Jefferson School of Pharmacy and Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA

Vincent C. O. Njar

Department of Pharmaceutical Sciences, Jefferson School of Pharmacy and Kimmel Cancer Center, Thomas Jefferson University, Philadelphia, PA 19107, USA

Paul E. Goss: pgoss@partners.org

Abstract

VN/14-1 [4-(±)-(1*H*-Imidazol-1-yl)-(E)-retinoic acid], a novel retinoic acid metabolism blocking agent (RAMBA), works by inhibiting the breakdown of all-*trans*-retinoic acid. The purpose of this study was to evaluate the anti-tumor effects of VN/14-1 on the *N*-methyl-*N*-nitrosourea (MNU)-induced rat mammary carcinoma model, and peripheral organ effects on the uteri of immature

ovariectomized (OVX) rats. In tumor burden experiments, after 56 days of administration of VN/14-1 5, 10, and 20 mg/kg/day, significant tumor reductions in mean tumor weight of 19.1, 34.4, and 44.3%, compared to tumors in control animals occurred. Cumulative tumor growth was also significantly slower in a dose-dependent manner in groups receiving 5, 10, and 20 mg/kg/day of VN/14-1 compared to growth rates in the control group. Tumor apoptosis was significant increases in animals treated with 5, 10, and 20 mg/kg/day of VN/14-1. In uterotrophic experiments, immature OVX rats given VN/14-1 significantly reduced uterine weight and blocked endometrial stimulation induced by unopposed β -estradiol (E2). In both rat models, adverse toxicities included weakness, anorexia, and reduction in body weight in the groups given the highest dose of 20 mg/kg/day. In summary, VN/14-1 inhibited tumor growth in the MNU-induced estrogen receptor (ER)-positive rat mammary tumor model, and antagonized the stimulatory effect of estrogens on the uterus. The studies suggest that VN/14-1 may be a useful novel therapy for ER-positive breast cancer.

Keywords

Breast cancer; Retinoic acid metabolism blocking agent (RAMBA); VN/14-1; Anti-tumor efficacy; Blockage of estrogenic stimulation on uterus

Introduction

All-*trans*-retinoic acid (ATRA), the biologically most active metabolite of vitamin A, as well as other natural and synthetic retinoids, play key roles in many biological functions such as cellular differentiation, proliferation, apoptosis, and regulation of gene expression, as well as in prevention and therapy of many proliferative diseases including dermatologic disorders and cancers [1-7].

Retinoic acid metabolism blocking agents (RAMBAs) increase endogenous levels of ATRA by inhibiting cytochrome p450-dependent CYP26s enzymes responsible for ATRA metabolism [8]. Thus, they are believed to mimic the effects of retinoid treatment. We have previously published on the significant efficacy of liarozole, a dual RAMBA and aromatase (estrogen synthetase) inhibitor in both preclinical models and in patients with advanced breast cancer [9-11].

We designed and synthesized VN/14-1 [4-($\pm$)-(1*H*-Imidazol-1-yl)-(*E*)-retinoic acid], a structural analog of ATRA [12, 13]. VN/14-1 is a new generation and novel RAMBA that inhibits the metabolism of ATRA and keeps more endogenous retinoic acid (RA) available within cancer cells which redirects the cells back into their normal growth patterns, including programmed cell death. VN/14-1 was the most potent and effective RAMBA, inducing cell differentiation, apoptosis, and cell cycle arrest in cell culture and showing efficacy in breast tumor xenograft models [13-16]. VN-14 also inhibits aromatase in preclinical models [14]. In this study, we used the *N*-methyl-*N*-nitrosourea (MNU)-induced mammary carcinoma model that is more estrogen dependent than DMBA-induced tumors and widely used as a model of human breast cancer [17]. For the evaluation of the uterotrophic effects of VN/14-1, the immature ovariectomized (OVX) Sprague–Dawley rat is a suitable model because no interfering estrogens mask any possible inherent uterotrophic

effects of the compound [18]. Thus, the purpose of this study was to evaluate the anti-tumor effects of VN/14-1 on the MNU-induced estrogen-dependent mammary carcinomas in rats, a model which has normal immunological function. In addition, we investigated potential biological effects of VN/14-1 on key endocrine sensitive end-organs such as bone, lipids in MNU-induced estrogen-dependent mammary carcinoma rat model, as well as its effects alone and in combination with β -estradiol (E2) on the uterus of the immature OVX rat.

Materials and methods

Chemicals

RAMBA VN/14-1 was designed and synthesized in the laboratory of VN (12). For these experiments, the compound was dissolved in 20% hydroxypropyl- β -cyclodextrin (in saline) for oral gavage. β -estradiol (Sigma-Aldrich, St. Louis, MO, USA) was dissolved in 5% ethanol (in saline) for subcutaneous injection. MNU was purchased from Ash Stevens, Detroit, MI, USA.

Tumor burden experiments

Animals and experimental design

All animals received care according to guidelines established by the Massachusetts General Hospital Subcommittee on Care of Research Animals. Forty-two-day-old pathogen-free female Sprague–Dawley rats (Indianapolis, IN, USA) were housed in a pathogen-free environment under controlled conditions of light, humidity, and room temperature and provided food and tap water. The animals were acclimatized for 7 days before the start of the experiment. At 50 days of age, the animals received an intraperitoneal injection of 50 mg/kg MNU, dissolved in 0.9% NaCl solution and acidified to pH 4 with acetic acid within 20 min of preparation [19, 20]. The mammary glands were palpated at weekly intervals starting 4 weeks after MNU administration. Animals bearing one or more tumors greater than 10 mm in diameter were matched according to mean tumor volume and assigned to 4 experimental groups of 12 animals per group: group 1, a control group, received 20% hydroxypropyl- β -cyclodextrin once daily by oral gavage; group 2, received VN/14-1 5 mg/kg/day; group 3, received VN/14-1 10 mg/kg/day; and group 4, received VN/14-1 20 mg/kg/day. VN/14-1 was given to all animals once daily by oral gavage, and continued for 8 consecutive weeks (5 days per week). Tumors were measured with an electronic digital caliper, and tumor volumes were calculated according to the formula $4/3\pi r_1^2 r_2$, where r_1 is the smaller radius and r_2 is the large radius. The total cumulative tumor volume was calculated for each animal and recorded weekly. After 8 weeks of treatment, the animals were euthanized by cardiac puncture under ketamine anesthesia. All animals were fasted overnight before blood collection for serum lipid assay and plasma RA analysis. The whole lumbar spine and femora from each animal were excised for measurements of bone mineral density (BMD) and biomechanical testing. Tumor sections were used for apoptosis assays.

In situ apoptosis staining

Tumors were harvested and placed in 10% formalin, embedded in paraffin, and cut into sections at 5- μ m thickness. To evaluate apoptosis, the sections were subjected to terminal

deoxynucleotidyl transferase (TdT)-mediated deoxyuridine triphosphate nick end-labeling (TUNEL) analysis using ApopTag[®] Peroxidase In Situ Apoptosis Detection kits (Millipore, Bedford, MA, USA) according to the manufacturer's directions.

In brief, 5- μ m sections of formalin-fixed paraffin embedded tumor tissue were deparaffinized and rehydrated. After permeabilization with proteinase K (20 μ g/ml) for 15 min at room temperature, sections were quenched with endogenous peroxidase in aqueous 3% H₂O₂ for 5 min at room temperature.

After a TdT enzyme reaction in a humidified chamber for 1 h at 37°C, anti-digoxigenin conjugate was applied to sections in a humidified chamber for 30 min at room temperature. Final visualization was achieved using Vectastain ABC Kit (Vector Laboratories, Burlingame, CA, USA). Counter-staining with 0.5% methyl green (MP Biomedicals, LLC, OH, USA) was carried out before the slides were mounted.

For quantitative apoptotic evaluation, the number of TUNEL-positive apoptotic cells and the malignant cells in three different fields at random were counted under Olympus BX60 microscope (Olympus, Japan), and the percentage of TUNEL-stained apoptotic cells was calculated against the total number of mammary carcinoma cells.

Bone densitometry

The cleaned, excised lumbar spine and left femur of individual rats were scanned by dual energy X-ray absorptiometry using a Lunar PIXImus2 densitometer (GE Medical System Lunar, Madison, WI, USA) with a scan resolution of 0.1 $\times$ 0.1 mm. Whole left femur and lumbar vertebrae (first through sixth) were placed on a polystyrene tray with water to mimic soft tissue. The bone mineral content (BMC) and area were measured, and BMD was calculated automatically as BMC/area (g/cm²).

Biomechanical tests

The biomechanical failure properties of the femora and vertebrae were evaluated using an Instron 8501 material testing system (Instron Corp, Canton, MA, USA). Force and deformation data were collected at a rate of 25 Hz using a 12-bit data acquisition card (National Instruments, Austin, TX, USA), Labview 5.0 data acquisition software (National Instruments, USA).

The diaphysis of the right femur was tested for mechanical failure using three-point bending according to a procedure previously described [21]. In brief, samples were subjected to a pre-load of 1 N and then deformed at a rate of 1 mm/min until failure. The point of failure was defined as a successive drop in load greater than 10%. The body of the fifth lumbar vertebra was tested to failure by unconfined compression using a similar procedure as previously described [22]. A pre-load of 2 N was applied to the sample and then deformed at a rate of 2 mm/min until failure occurred. The point of failure was defined as a successive drop in load of greater than 5%.

Serum lipid assays

Blood samples were allowed to clot at 4°C for 2 h, and then centrifuged at $2000 \times g$ for 10 min. The serum was transferred to new tubes for lipid assays. Serum total cholesterol (CH), high-density lipoprotein (HDL) cholesterol, low-density lipoprotein (LDL) cholesterol, and tri-glyceride (TG) levels were measured using the Roche Diagnostics' reagents and assayed on a Hitachi 917 Automatic Analyzer (Hitachi, Tokyo, Japan).

Quantification of RA in blood plasma

RA was extracted from plasma and quantified. The analysis was performed on an HPLC Waters Alliance System coupled with 2695 Separation Module and Waters 2998 PDA detector operated at 350 nm. HPLC analysis was conducted on a 5 μ m Waters Symmetry® C18 Column (4.6 $\times$ 75 mm). The HPLC analysis was performed at ambient temperature, and data acquisition and integration were achieved with a Waters millennium chromatography manager as we and others have previously described [23, 24].

Uterotrophic experiments

Animals and experimental design

Immature (21-day old, 38–45 g) female Sprague–Dawley rats (Indianapolis, IN, USA) were OVX 7 days before the start of the experiment. The animals were housed with their mothers as described above. The immature animals were acclimatized for 2 days before being dosed. At 21 days of age, the animals were randomized to 6 experimental groups (6 rats per group), as follows: group 1, controls received 20% hydroxypropyl- β -cyclodextrin once daily by oral gavage; group 2, β -estradiol (E2) 10 μ g/kg/day; group 3, VN/14-1 10 mg/kg/day; group 4, VN/14-1 10 mg/kg/day + E2 10 μ g/kg/day; group 5, VN/14-1 20 mg/kg/day; and group 6, VN/14-1 20 mg/kg/day + E2 10 μ g/kg/day. After being allocated to the respective groups, animals were then treated with VN/14-1, E2 either alone or in combination for three consecutive days. VN/14-1 was given once daily by oral gavage, and E2 was given once daily by subcutaneous injection. After 3 days of treatment, the animals were sacrificed on day 4. The uteri were removed for measurement of wet weight and for the uterotrophic assay.

Uterine weight and histology

The uteri were excised, trimmed free of fat, pierced, and blotted to remove excess fluid. The body of the uterus was cut just above its junction with the cervix and at the junction of the uterine horns with the ovaries. The wet weight of the uterus was then determined [25].

10% phosphate buffered formalin-fixed uteri were processed for conventional paraffin embedding. Cross sections at 4- μ m thickness were prepared from both horns of each uterus and stained with hematoxylin and eosin. The epithelial lining cell height was measured using a Quantimet 500 MC automated image analysis system (Leica, Germany). The image analysis system is attached to an Orthoplan microscope and a JVC color camera.

Statistical analysis

Data are expressed as the mean $\pm$ standard error of the mean (SEM) of each group. Data were analyzed using Student's *t* test or one-way analysis of variance with SAS statistical software (SAS Institute Inc, Cary, NC, USA). Pair-wise comparisons between various groups were performed using a Tukey–Kramer adjustment. Statistical significance was considered at $P < 0.05$.

Results

Tumor burden experiments

Effects of VN 14-1 on tumor growth and tumor apoptosis—The effects of VN/14-1 on the mean tumor weight of the MNU-induced rat mammary tumors are shown in Fig. 1a. Treatment with VN/14-1 produced a dose-dependent inhibition of tumor growth. At the planned end of the treatment period, the administration at doses of 5, 10, and 20 mg/kg/day of VN/14-1 caused significant reductions of 19.1, 34.4, and 44.3%, respectively, in mean tumor weight compared to weights in control animals (all $P < 0.05$). As shown in Fig. 1b, the cumulative tumor growth in groups receiving 5, 10, and 20 mg/kg/day of VN/14-1 was significantly inhibited in a dose-dependent manner compared to growth in the control group (all $P < 0.05$).

Treatment with VN/14-1 induced the apoptosis of mammary carcinoma cells as revealed by in situ end labeling of DNA strand breaks (TUNEL assay). Quantifications of apoptosis caused by various doses of VN/14-1 are presented in Fig. 2. The mean percentages of TUNEL-positive staining cells in the tumors were significant increases in the animals treated with VN/14-1. At the endpoint of the treatment period, administration of doses of 5, 10, and 20 mg/kg/day of VN/14-1-induced apoptosis was 5.31, 8.26, and 10.22% (all $P < 0.05$ vs. controls), respectively, as compared with controls which had 1.19% apoptosis.

Changes in body weight—The effect of vehicle and VN/14-1 on the animals overall body weight is shown in Table 1. Eight weeks of treatment with 10 and 20 mg/kg/day of VN/14-1 caused 5 and 9% decrease, respectively, in body weight gain compared to controls (all $P < 0.05$).

Effects of VN 14-1 on lipid metabolism—No significant differences were observed in serum total CH, LDL, TG, and HDL between rats treated with all doses of VN/14-1 and controls (Table 1).

BMD and biomechanical properties—The effects of an 8-week treatment with VN/14-1 on lumbar vertebral and fifth lumbar vertebral BMDs are shown in Table 2. After 8 weeks of treatment, lumbar vertebral BMDs were 7.0 and 9.1% lower in animal given 10 and 20 mg/kg/day of VN/14-1, respectively, than in controls (all $P < 0.05$). Similar reductions were also observed on femoral BMD measurements. Administration with 10 and 20 mg/kg/day of VN/14-1 also significantly decreased the mechanical stress “failure properties” of the rat femora as evaluated from the three-point bending strength, as well as of the lumbar vertebrae as measured by compressive strength (Table 2).

RA parameter—As shown in Table 1, eight weeks of oral treatment of 5, 10, and 20 mg/kg/day of VN/14-1 caused significant increases of 43, 91, and 618%, respectively, in plasma RA levels compared to control animals (all $P < 0.05$).

Uterotrophic experiments

Uterine wet weight and epithelial cell height—The effects of VN/14-1 alone or in combination with β -estradiol (E2), on uterine weight in the OVX immature rats, are shown in Fig. 3. After 3 days of administration with E2 10 μ g/kg/day, OVX rats had a 74% increase in uterine wet weight ($P < 0.001$ vs. OVX controls). OVX rats given VN/14-1 at doses of 10 and 20 mg/kg/day had reductions in uterine wet weight of 52 and 56%, respectively, compared to OVX controls ($P < 0.001$). VN/14-1 10 and 20 mg/kg/day in combination with E2 resulted in a reduction in uterine wet weight of 42 and 58%, respectively, compared to the OVX rats given E2 as controls ($P < 0.001$).

As shown in Fig. 4, OVX rats given VN/14-1 at doses of 10 and 20 mg/kg/day had reductions in uterine epithelial cell height of 59 and 64%, respectively, compared to OVX controls ($P < 0.001$). VN/14-1 10 and 20 mg/kg/day in combination with E2 resulted in reductions in uterine epithelial cell height of 47 and 62%, respectively, compared to the OVX rats given E2 as control ($P < 0.005$).

Discussion

VN/14-1 is a novel retinoid-mimetic agent being investigated as a novel therapy for breast cancer. RA belongs to a class of compounds known as retinoids that are known to be critical signaling molecules that regulate gene transcription and the cell cycle and that play key roles in regulating gene transcription. As such they govern multiple functions in the body, such as cell division and differentiation, immune response, and embryonic development. They also control the development and spread of cancer cells, and some retinoids, including RA, can inhibit tumor growth by preventing cancer cell proliferation. Retinoids themselves are now in clinical trials for treatment of head, neck, and breast cancers [15, 26-28] and also leukemia and neuroblastoma [29].

The putative advantage of VN/14-1 over exogenously administered retinoids such as RA is that it is a relatively weak retinoid but importantly allows accumulation (via inhibition of metabolic CYP 26) of natural endogenous retinoids in tumor and other tissues.

As such is it not vulnerable to the short half-life and self-induced metabolism that is characteristic of exogenously administered retinoids. The latter in part explains why cancer patients frequently become resistant to RA therapy over time [26].

In this set of experiments, we set out to demonstrate anti-tumor activity in a standard preclinical breast cancer rat model and to confirm that VN/14-1 caused accumulation of RA in the animal's plasma and tissues. Other hormonal agents and the retinoid-mimetic liarozole which we previously studied in animals and breast cancer patients demonstrated a degree of retinoid-mimetic effects as well as aromatase (estrogen synthetase) inhibition [9-11]. We showed that liarozole caused bone loss and atrophic attenuation of the uterine endometrium.

In the clinic liarozole proved to have significant toxicities particular in skin and mucus membranes leading to it being withdrawn from clinical development. Thus, the experiments designed here are important stage setting evaluations of toxicities in preparation for clinical development of VN/14-1. In terms of anti-tumoral effects, our results confirm the anti-tumor properties of VN/14-1 in the MNU-induced mammary carcinoma rat model. This rat model differs from the nude mouse model in that it is not in immune deficient animals and that the rat model is an estrogen-dependent breast cancer model. However, the anti-tumor results are consistent with the MCF-7 tumor xenograft model in OVX female athymic nude mice which is also hormone dependent [16].

Figure 1b shows weekly changes in mean tumor volume for treatment with three doses of 5, 10, and 20 mg/kg/day of VN/14-1 compared to controls. At all time points during the 8-week treatment period, the tumor growth in groups receiving the three doses was significantly suppressed in a dose-dependent manner compared to the control (vehicle) group (all $P < 0.05$). In the first 2-3 weeks following VN/14-1 administration, tumor growth appears to be suppressed, and thereafter, the slope of the growth curves for all three doses appear similar and follow parallel slopes, meaning that growth suppression due to VN/14-1 became modest after the initial weeks remained stable but sustained with ongoing treatment. This may be explained by exponential growth of the tumors, as is typical for a variety of cancers. Molecular analyses of the tumors in these two growth phases may improve our understanding of the effects of VN/14-1.

In terms of confirming the retinoid-mimetic effects of VN/14-1, at the end of the treatment period in our study, significant increases in RA levels were observed in the VN/14-1-treated groups compared to controls. In the 20 mg/kg/day-treated animals, RA levels reached as high as six times those in the control animals. The results confirm the known mechanism of action of VN/14-1, namely, increasing the levels of intra-tumoral RA. With respect to key end-organ effects, we first looked at effects on the uterine endometrium of VN/14-1. We observed that treatment with VN/14-1 causes significant reduction in uterine wet weight and epithelial lining height in the OVX immature rat, which indicates that VN/14-1 antagonizes the stimulatory effect of β -estradiol on the uterus. Compatible with this is that our results also show that VN/14-1 can effectively block the uterotrophic activity of estrogens produced by peripheral aromatization of androstenedione [9, 30]. Retinoids are known to abrogate the stimulatory effects of β -estradiol on the uterus.

In terms of effects on bone metabolism and BMD, we showed that treatment with 10 and 20 mg/kg/day of VN/14-1 causes significant reductions in BMD and mechanical properties bone in the MNU-induced estrogen-dependent mammary carcinoma rat model. The mechanism of this is unclear but may represent an anti-estrogen effect. These effects on key endocrine sensitive end-organs need to be further investigated in an adult OVX female rat model and eventually in patients.

General adverse toxicities in the animals were important to study as well, particularly in light of the significant side effects from liarozole. Adverse toxic effects such as weakness, anorexia, and reduction of body weight occurred in the groups given 20 mg/kg/day of VN14-1 in both the OVX immature and MNU-induced mammary carcinoma rat model were

observed. We tested a higher dose of 40 mg/kg/day in both models, and toxicity was higher yet, resulting in death in the animals. These non-specific toxicities of weakness, anorexia, and reduction of body weight are seen with high doses of exogenous retinoids as well. We strove to reduce these toxicities by introducing a 2 day break in the weekly treatment regimen, giving the animals 5 day per week treatments for 8 consecutive weeks. VN/14-1 and the structural analogs of ATRA need to be further improved for reduction of these generalized side effects.

In conclusion, in this study, we demonstrated VN/14-1 significantly inhibits the growth of tumors, and induces cell apoptosis in the MNU-induced estrogen receptor (ER)-positive rat mammary tumor model, as well as effectively antagonized the stimulatory effect of estrogens on the uterus indicating an absence of an intrinsic estrogenic signal. The loss of BMD and diminution of bone strength we observed in our experiment needs to be further evaluated. With the anti-tumor effects noted VN/14-1 merits further investigation as a novel therapy for ER-positive breast cancer. Further experiments are also needed to evaluate VN/14-1 in ER-negative breast cancer models.

Acknowledgments

We thank Endocrine Unit and Dr. Ernestina Schipani laboratory, Massachusetts General Hospital and Harvard Medical School for friendly providing the equipment for the bone densitometry. The study was supported in part by grants from the US Department of Defense under the Peer Reviewed Medical Research Program (PRMRP, W81XW-04-1-0101, Njar, VCO), and US National Institutes of Health and National Cancer Institute (NIH/NCI, 1R01CA12379-01A2, Njar VCO) and by Golfers against Cancer.

References

1. Mangelsdorf, DJ.; Umesono, K.; Evans, RM. The retinoid receptors. In: Sporn, MB.; Roberts, AB.; Goodman, DS., editors. The retinoids. 2. Raven Press; New York: 1994. p. 319-349.
2. Moon RC, Mehta RG, McCormick DL. Retinoids and mammary gland differentiation. Ciba Found Symp. 1985; 113:156–167. [PubMed: 3896699]
3. Chambon P. A decade of molecular biology of retinoic acid receptors. FASEB J. 1996; 10:940–954. [PubMed: 8801176]
4. Dragnev KH, Rigas JR, Dmitrovsky E. The retinoids and cancer prevention mechanisms. Oncologist. 2000; 5:361–368. [PubMed: 11040271]
5. Altucci L, Gronemeyer H. The promise of retinoids to fight against cancer. Nat Rev Cancer. 2001; 1:181–193. [PubMed: 11902573]
6. Boyle JO. Retinoids mechanisms and cyclins. Curr Oncol Rep. 2001; 3:301–305. [PubMed: 11389813]
7. Fontana JA, Rishi AK. Classical and novel retinoids: their targets in cancer therapy. Leukemia. 2002; 16:463–472. [PubMed: 11960323]
8. Njar VC. Cytochrome p450 retinoic acid 4-hydroxylase inhibitors: potential agents for cancer therapy. Mini Rev Med Chem. 2002; 2:261–269. [PubMed: 12370067]
9. Goss PE, Strasser K, Marques R, et al. Liarozole fumarate (R85246): in the treatment of ER negative, tamoxifen refractory or chemotherapy resistant postmenopausal metastatic breast cancer. Breast Cancer Res Treat. 2000; 64:177–188. [PubMed: 11194453]
10. Goss PE, 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. [PubMed: 10752680]

11. Goss PE, Strasser-Weippl K, Qi S, et al. Effects of liarozole fumarate (R85246 in combination with tamoxifen on *N*-methyl-*N*-nitrosourea MNU-induced mammary carcinoma and uterus in the rat model. *BMC Cancer*. 2007; 7:26–32. [PubMed: 17266767]
12. Njar VCO, Nnane IP, Brodie AM. Potent inhibition of retinoic acid metabolism enzyme(s) by novel azolyl retinoids. *Bioorg Med Chem Lett*. 2000; 10:1905–1908. [PubMed: 10987414]
13. Patel JB, Huynh CK, Handratta VD, 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; 47:6716–6729. [PubMed: 15615521]
14. Belosay A, Brodie AM, Njar VC. Effects of novel retinoic acid metabolism blocking agent (VN/14-1) on letrozole-insensitive breast cancer cells. *Cancer Res*. 2006; 66:11485–11493. [PubMed: 17145897]
15. Njar VC, Gediya L, Purushottamachar P, et al. Retinoic acid metabolism blocking agents (RAMBAs) for treatment of cancer and dermatological diseases. *Bioorg Med Chem*. 2006; 14:4323–4340. [PubMed: 16530416]
16. Patel JB, Mehta J, Belosay A, et al. Novel retinoic acid metabolism blocking agents have potent inhibitory activities on human breast cancer cells and tumour growth. *Br J Cancer*. 2007; 8:1204–1215. [PubMed: 17387344]
17. Rose DP, Pruitt B, Stauber P, et al. Influence of dosage schedule on the biological characteristics of *N*-nitrosomethylurea-induced rat mammary tumors. *Cancer Res*. 1980; 40:235–239. [PubMed: 7356506]
18. Kang K-S, Kim HS, Ryu DY, et al. Immature uterotrophic assay is more sensitive than ovariectomized uterotrophic assay for the detection of estrogenicity of *p*-nonylphenol in Sprague-Dawley rats. *Toxicol Lett*. 2000; 118:109–115. [PubMed: 11137316]
19. Turcot-Lemay L, Kelly PA. Characterization of estradiol, progesterone, and prolactin receptors in Nitrosomethylurea-induced mammary tumors and effect of antiestrogen treatment on the development and growth of these tumors. *Cancer Res*. 1980; 40:3232–3240. [PubMed: 6253058]
20. Thompson HJ, Adlakha H. Dose-responsive induction of mammary gland carcinoma by the intraperitoneal injection of 1-Methyl-1-nitrosourea. *Cancer Res*. 1991; 51:3411–3415. [PubMed: 2054781]
21. Kasra M, Vanin CM, MacLusky NJ, et al. Effect of different estrogen and progestin regimens on the mechanical properties of rat femur. *J Orthop Res*. 1997; 15:118–123. [PubMed: 9066535]
22. Chachra D, Vanin CM, MacLusky NJ, et al. The effect of different hormone replacement therapy regimens on the mechanical properties of rat vertebrae. *Calcif Tissue Int*. 1995; 56:130–134. [PubMed: 7736321]
23. Wu C, Njar V, Brodie A, et al. Quantification of a novel retinoic acid metabolism inhibitor, 4-(1*H*-imidazol-1-yl)retinoic acid (VN14-1 RA) and other retinoids in rat plasma by liquid chromatography with diode-array detection. *J Chromatogr B*. 2004; 810:203–208.
24. Stoppie P, Borgers M, Borghgraef P, et al. R115866 inhibits all-trans-retinoic acid metabolism and exerts retinoidal effects in rodents. *J Pharmacol Exp Ther*. 2000; 293:304–312. [PubMed: 10734183]
25. Odum J, Lefevre PA, Tittensor S, et al. The rodent uterotrophic assay: critical protocol features, studies with nonyl phenols, and comparison with a yeast estrogenicity assay. *Regul Toxicol Pharmacol*. 1997; 25:176–188. [PubMed: 9185893]
26. Budhu AS, Noy N. Direct channeling of retinoic acid between cellular retinoic acid-binding protein II and retinoic acid receptor sensitizes mammary carcinoma cells to retinoic acid-induced growth arrest. *Mol Cell Biol*. 2002; 8:2632–2641. [PubMed: 11909957]
27. Thatcher JE, Isoherranen N. The role of CYP26 enzymes in retinoic acid clearance. *Expert Opin Drug Metab Toxicol*. 2009; 5:875–886. [PubMed: 19519282]
28. Hua S, Kittler R, White KP. Genomic antagonism between retinoic acid and estrogen signaling in breast cancer. *Cell*. 2009; 137:1259–1271. [PubMed: 19563758]
29. Altucci L, Leibowitz MD, Ogilvie KM, et al. RAR and RXR modulation in cancer and metabolic disease. *Nat Rev Drug Discov*. 2007; 6:793–810. [PubMed: 17906642]

30. Jelovac D, Macedo L, Goloubeva OG, et al. Additive antitumor effect of aromatase inhibitor letrozole and antiestrogen fulvestrant in postmenopausal breast cancer model. *Cancer Res.* 2005; 65:5439–5444. [PubMed: 15958593]

Fig. 1.

Effect of VN/14-1 on the MNU-induced rat mammary carcinoma model. **a** Mean tumor weight at the endpoint of the treatment. **b** Changes in mean tumor volume in the 8-week period of the treatment

Fig. 2. Effects of VN/14-1 on tumor apoptosis. Graph showing apoptosis induced by VN/14-1. Groups: controls, VN/14-1 5 mg/kg/day, VN/14-1 10 mg/kg/day, and VN/14-1 20 mg/kg/day. Scale bars represent the mean $\pm$ SEM ($n = 12$). * $P < 0.05$ versus controls

Fig. 3.

Effects of VN/14-1 on uterine wet weight in the immature OVX rat model. Groups: OVX controls (OVX), OVX + β -estradiol 10 μ g/kg/day (OVX + E2), OVX + VN/14-1 10 mg/kg/day (OVX + VN 10 mg), OVX + VN/14-1 10 mg/kg/day + β -estradiol 10 μ g/kg/day (OVX + VN 10 mg + E2), OVX + VN/14-1 20 mg/kg/day (OVX + VN 20 mg), and OVX + VN/14-1 20 mg/kg/day + β -estradiol 10 μ g/kg/day (OVX + VN 20 mg + E2). Scale bars represent the mean $\pm$ SEM ($n = 6$). * $P < 0.001$ versus OVX, + $P < 0.001$ versus OVX + E2

Fig. 4.

Effects of VN/14-1 on uterine epithelial cell height in the immature OVX rat model. Groups: OVX controls (OVX), OVX + β -estradiol 10 μ g/kg/day (OVX + E2), OVX + VN/14-1 10 mg/kg/day (OVX + VN 10 mg), OVX + VN/14-1 10 mg/kg/day + β -estradiol 10 μ g/kg/day (OVX + VN 10 mg + E2), OVX + VN/14-1 20 mg/kg/day (OVX + VN 20 mg), and OVX + VN/14-1 20 mg/kg/day + β -estradiol 10 μ g/kg/day (OVX + VN 20 mg + E2). Scale bars represent the mean $\pm$ SEM ($n = 6$). * $P < 0.001$ versus OVX, + $P < 0.005$ versus OVX + E2

Table 1

Retinoic acid (RA) parameter, body weight gain, and serum lipid levels

Group	RA levels (ng/ml)	Body weight gain (g)	CH (mg/dl)	LDL (mg/dl)	TG (mg/dl)	HDL (mg/dl)
Controls	0.7183 ± 0.2660	2.9 ± 1.9	100.0 ± 4.9	15.1 ± 2.3	81.3 ± 3.6	78.7 ± 2.9
VN/14-1 (5 mg/kg/day)	1.0262 ± 0.4434 ^a	-0.4 ± 1.9	98.5 ± 4.1	15.5 ± 2.7	84.8 ± 4.3	65.9 ± 4.2
VN/14-1 (10 mg/kg/day)	1.3708 ± 0.3783 ^a	-6.3 ± 2.1 ^a	104.4 ± 3.6	14.4 ± 2.9	86.9 ± 3.2	72.6 ± 3.2
VN/14-1 (20 mg/kg/day)	5.1557 ± 1.5073 ^a	-24.2 ± 2.7 ^a	105.1 ± 4.8	15.2 ± 3.1	82.1 ± 3.1	73.4 ± 3.7

Data are mean ± SEM, 12 rats per group

^a $P < 0.05$ versus controls

Table 2

BMD values and mechanical properties

Group	Lumbar vertebral BMD (g/cm ²)	Femoral BMD (g/cm ²)	Bending strength (MPa)	Compressive strength (MPa)
Controls	0.1436 ± 0.002	0.1691 ± 0.002	161.3 ± 2.3	7.19 ± 0.17
VN/14-1 (5 mg/kg/day)	0.1433 ± 0.002	0.1693 ± 0.003	160.4 ± 2.8	7.16 ± 0.20
VN/14-1 (10 mg/kg/day)	0.1335 ± 0.002 ^a	0.1596 ± 0.003 ^a	150.0 ± 2.3 ^a	5.87 ± 0.09 ^a
VN/14-1 (20 mg/kg/day)	0.1306 ± 0.002 ^a	0.1569 ± 0.002 ^a	145.4 ± 3.4 ^a	5.65 ± 0.11 ^a

Data are mean ± SEM, 12 rats per group

^a*P* < 0.05 versus controls