Bexarotene Induces Cellular Senescence in MMTV-Neu Mouse Model of Mammary Carcinogenesis

Anne Shilkaitis, Laura Bratescu, Albert Green, Tohru Yamada, and Konstantin Christov*

Department of Surgery, Division of Surgical Oncology, University of Illinois at Chicago, Chicago, IL 60612

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E-Mails: anneshil@uic.edu, g3607273@uic.edu, bratescu1462@comcast.net, tohru@uic.edu, christov@uic.edu

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*To whom correspondence should be addressed:
Konstantin Christov, M.D., Ph.D.
Department of Surgical Oncology
University of Illinois at Chicago
840 South Wood Street (M/C 820)
Chicago, IL 60612
Phone: 312-996-5347, Fax: 312-996-9365
E-mail: christov@uic.edu
Abstract

Previous studies have shown that retinoids and rexinoids can prevent breast cancer in animal models and in women with increased risk of developing the disease. The cellular effects of these Vitamin A analogs have been primarily associated with induction of differentiation and inhibition of proliferation. In this study, we tested the hypothesis that bexarotene (LGD1069, Targretin), a rexinoid, can not only inhibit cell proliferation but also induce cellular senescence in mammary epithelial cells, premalignant lesions, and tumors of the MMTV-Neu model of mammary carcinogenesis, which develops estrogen receptor-negative tumors. Mice with palpable mammary tumors were treated for 4 weeks with bexarotene at 80 or 40 mg/kg bw, and senescent cells were determined by SA-β-Gal assay. Bexarotene decreased in a dose-dependent manner the multiplicity of premalignant lesions and tumors, and this was associated with inhibition of cell proliferation and induction of cellular senescence and apoptosis. By double labeling of senescent cells, first by SA-β-Gal and then by antibodies against genes related to cellular senescence, we found that p21, p16, and RARβ, but not p53, were up-regulated by bexarotene in mammary tumors and in breast cancer cell lines, suggesting involvement of multiple signaling pathways in mediating the senescence program of rexinoids. These findings indicate that, in addition to cell proliferation and apoptosis, cellular senescence could be used as a potential biomarker of response in breast cancer prevention and therapy studies with rexinoids and possibly with other antitumor agents.
Introduction

Over the last 20 years, significant progress has been made in preventing and treating estrogen receptor-positive (ER\(^+\)) breast cancer by tamoxifen and aromatase inhibitors (1, 2). Little has been achieved for estrogen receptor-negative (ER\(^-\)) breast cancer, which comprises about 30% of all breast carcinomas, most of them with unfavorable prognosis (3). For ER\(^-\) breast cancer, retinoids and rexinoids have been considered potential alternatives because they suppress the growth of both ER\(^+\) and ER\(^-\) breast cancer cell lines, relevant animal models of mammary carcinogenesis, as well as breast cancer development in women (4-7). Rexinoids appear less toxic than retinoids; therefore, they have advantage in clinical studies (8, 9). Rexinoids are ligands of retinoid X receptors \(\alpha, \beta, \gamma\) (RXRs \(\alpha, \beta, \gamma\)) and exert their biological effects by receptor-dependent and -independent mechanisms (10-12). The cellular effects of retinoids and rexinoids are primarily associated with inhibition of proliferation and induction of differentiation (13-16). To the best of our knowledge, no data are available about whether rexinoids, particularly bexarotene, can induce cellular senescence (CS) in ER\(^-\) models of mammary carcinogenesis. Most previous and recent studies on CS have been performed \textit{in vitro} on mouse embryo fibroblasts and on various normal and tumor cell lines treated with cytotoxic agents or irradiation that induce DNA damage and gene instability, both leading to p53 and/or p21 up-regulation (17-19). RAS, MYC, and BRAF oncogenes, when activated, can also induce CS, which appears also associated with DNA alterations (20, 21). Previously, we found that all-\textit{trans} retinoic acid (atRA) is an efficacious inducer of CS in ER\(^+\) MCF-7 breast cancer cells, and we developed an \textit{in vitro} assay to discriminate quiescent cells from senescent cells (SC) (22). By employing the SA-\(\beta\)-Gal assay, we showed in animal carcinogenesis models that two other retinoids, 9-\textit{cis}-retinoic acid (9cRA) and 4-Hydroxyphenilretinamide (4-HPR, Fenretinide), can also induce CS in mammary premalignant lesions and tumors (23). Recently, we found that 9cRA suppressed prostate carcinogenesis in p27-deficient mice, and this was associated with inhibition of cell proliferation and induction of CS (24).
MCF-7 cells treated with atRA (25) and on normal human breast epithelial cells treated with bexarotene (26) revealed up- and down-regulation of hundred of genes, most of them associated with cell cycle regulation, intercellular matrix proteins, cell differentiation, retinoid metabolism, various transcription factors, and secretion of cytokines that may affect surrounding and distant cells and tissues. However, the affected genes in both studies, except for RARβ transcription, were not directly associated with CS (26).

Here, we employed the MMTV-Neu mammary carcinogenesis model in mice and breast cancer cell lines to determine potential target genes associated with the bexarotene-induced CS. Previous studies from Powel Brown’s group have shown that bexarotene suppressed mammary carcinogenesis in MMTV-Neu mice by a dose-dependent manner, and this was associated with no changes in ErbB2 expression, but with inhibition of cell proliferation (12, 16, 27). The combination of bexarotene with the Cox-2 inhibitor celecoxib further increased inhibitory effects on mammary carcinogenesis (28). Those authors also showed that bexarotene is superior in p53-negative mammary tumor cell lines, as compared to the tyrosine kinase inhibitor gefitinib (29). The combination of another rexinoid (LG100268) with tri-terpenoids or selective estrogen receptor modulators (SERMs) was also more efficacious in inhibiting mammary carcinogenesis than both agents given separately (13, 15, 30). In the above in vivo studies, the decreased cell proliferation in mammary epithelial cells (MEC) and tumors was the principal biomarker of response to rexinoids. Here, we showed that bexarotene, in addition to inhibiting cell proliferation also induces CS in normal MEC, premalignant lesions, and tumors of MMTV-Neu mice, and that p21, p16, and RARβ are over-expressed in SC.

Material and Methods

Mice and tumors: MMTV-Neu female mice at the age of 4 weeks were purchased from the Jackson Laboratory, Bar Harbor, ME. Animals were fed a controlled MIN-76 purified mouse diet from Harlan Teklad, Madison, WI. When palpable tumors (about 2-3 mm/diameter)
occurred, usually at the age of 5-6 months, mice were randomized in placebo (sesame oil) and bexarotene-treated groups. A separate group of animals with mammary tumors were also treated for 7 days with doxorubicin at 3.0 mg/kg body weight (bw) to induce CS and serve as positive controls. Animal's weight and tumor growth were monitored twice weekly, according the requirements of the animal care committee at the University of Illinois at Chicago. At sacrifice of animals, tumors were dissected and cut in two halves; one was frozen in liquid nitrogen and kept at -80°C until examination, and the other was fixed in 10% neutral formalin, embedded in paraffin blocks, and used for histology and immunocytochemistry (ICH). Tissues free of tumors were also collected from abdominal and thoracic mammary glands and fixed in formalin or frozen in liquid nitrogen. These were used for identification of hyperplastic (atypical hyperplasia-AH) and premalignant (minimal intraepithelial neoplasia-MIN) lesions, as described by Powel Brown’s group (12, 16).

**Rexinoids and retinoids:** Bexarotene (LGD1069, Targretin) was provided from the NCI repository. Bexarotene at 80 or 40 mg/kg bw was mixed in sesame oil (0.1 ml) and given by gavage 6 days/week for 4 weeks. atRA was purchased from Sigma, Inc. (St. Louis, MO), and 4-HPR was obtained from the NIH repository. Doxorubicin was purchased from Ben Venue Laboratories, Inc., Bedford, OH.

**Histology and ICH:** For ICH, 4-µm paraffin sections were deparaffinized, endogenous peroxidase was blocked by 3.0% hydrogen peroxide; and sections were transferred to a pressure cooker for 6 min with 0.01 M citric buffer. Then slides were treated for 20 min with corresponding blocking serum, followed by specific antibodies against: p21 (AB-5, 1:100, Lab Vision, Kalamazoo, MI), p16 (M-156, 1:100, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), phospho-pRb (AB-7, 1:40, Lab Vision, Kalamazoo, MI), p53 ( DO-7, sc-47698, 1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA), RXRα (D-20, sc-553, 1:50, Santa Cruz Biotechnology, Inc., Santa Cruz, CA) and RARβ (Abcam, Cambridge, MA). ABC kit and 3, 3'- diaminobenzidine (DBA) from Vector Laboratories (Burlingame, CA) were used to identify the above antigens.
**Cell proliferation:** Proliferating cells in mammary tumors and tissues were determined by BrdU-labeling, as we described previously (31).

**Apoptosis:** Cells in apoptosis were evaluated by TUNEL assay, as recommended by the ApopTag *in situ* hybridization detection kit (Oncor, Co., Gaithersburg, MD) (31).

**Senescent cells (SC):** SA-β-Gal activity assay (Cell Signaling Technology, Inc., Danvers, MA) was employed to identify SC in mammary tissues and tumors and in breast cancer cell lines (32). In brief, frozen section (4- to 6-μm thick) from control and bexarotene-treated animals were fixed in 3.0% formaldehyde for 5 min., washed in PBS, and stained with X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactosidase) kit at pH 5.5-6.0 for 24 hrs at 37°C. To determine SC *in vitro*, cells grown on cover slips were treated for 7 days with bexarotene at 1.0 or 5.0 μM, and the percentage of CS was determined by SA-β-Gal staining. To confirm whether SC over-express p53, p21, p16, pRb, and RARβ, double staining protocols were developed in which frozen sections were first stained by SA-β-Gal overnight to detect SC and then the same slides were treated for 2 hrs by one of the above antibodies. Because SA-β-Gal stains the cytoplasm, whereas the above biomarkers are expressed in the nucleus, it was possible with relative high accuracy to determine the level of their nuclear expression in SC and non-SC. A progressive dilution (from 1:40 to 1:500) of the above antibodies was used to discriminate cells with high and low levels of gene/protein expression. A four-point grading system (0-3) was introduced to evaluate semi-quantitatively the expression level of the above biomarkers in SC and non-SC with: 0 = no expression (similar to the background level); 1 = weak expression, 2 = moderate expression and 3 = strong expression). The percentage of SC with no (0, score) or low levels of expression (scores of 1 and 2) of the above biomarkers were compared with those with high expression (score 3) in control and bexarotene-treated animals. Two pathologists (AS and KC) independently evaluated the slides in a blind fashion, and the scores were in the range of 15-20% difference.
Cell lines and western blotting: To determine the effects of bexarotene on CS in vitro, ER+, MCF-7, T47D, and BT474 and ER-, MDA-MB-231 breast cancer cell lines were employed. The above cell lines were obtained from ATCC. For western blotting, cells were cultured in 75 cm² flasks, treated for 24 or 72 hrs with 0.0, 1.0, or 5.0 uM bexarotene, atRA or 4-HPR; the total protein was isolated by lysis buffer (Roche, Indianapolis, IN), subjected to electrophoreses, and the gels were transblotted to Immobilon-P membranes (Millipore, Bedford, MA). Blots were probed with primary antibody and correspondent secondary antibody.

Statistical analysis: Data for multiplicity of premalignant lesions and tumors, as well as for CS, cell proliferation, apoptosis, RARβ, and RXRα, in control and bexarotene-treated animals and cells were compared using Student’s t test.

Results

Bexarotene Inhibits Multiplicity of Mammary Premalignant Lesions and Tumors

To assess the effect of bexarotene on the development and progression of mammary premalignant lesions and tumors, animals with the first palpable mammary tumor detected were treated for 4 weeks with placebo (sesame oil) or bexarotene given at two doses: 40mg/kg bw (low dose-L) or 80 mg/kg bw (high dose-H). At the beginning of the experiment, we used 100 mg/kg bw bexarotene as a high dose, but some animals lost weight and developed signs of toxicity; therefore, we decreased the high dose to 80 mg/kg bw. In addition to tumors, lesions with characteristics of AH and MIN were also identified (Fig. 1Aa and 1Ab). As shown in Fig. 1B, 13 control animals (placebo treated) with 13 palpable tumors at the start of experiment four weeks later developed 3.1 tumors per animal, as determined by histological examination of all mammary glands. Bexarotene decreased tumor multiplicity in a dose-dependent manner, from 3.1 ± 1.1 per animal in the placebo group to 2.2 ± 1.0 (*, p < 0.05) in the low-dose group and further to 1.7 ± 0.8 (**, p < 0.001) in the high-dose group. In one animal treated with high-dose bexarotene, the palpable tumor totally regressed; in two other animals, tumors were almost
totally replaced by inflammatory and death cells. As shown in Fig. 1Ad, bexarotene at 80 mg/kg bw induced disintegration of tumor cells in peripheral areas (arrows) where proliferating cells predominate. In addition to mammary tumors, three animals developed salivary gland tumors and two developed uterine tumors. MIN was detected in 8 of 13 placebo-treated animals, and its number progressively decreased, from 1.2 ± 0.4 per animal in the control group to 0.9 ± 0.6 in low-dose and to 0.6 ± 0.4 (*, p < 0.05) in high-dose (Fig. 1B). Lesions with characteristics of AH were identified in only a small number of animals, and their number decreased from 1.5 ± 0.9 in placebo animals (n = 7) to 0.8 ± 0.4 low-dose bexarotene-treated animals (n = 4, p < 0.1) and further to 0.6 ± 0.3 in high-dose animals (n = 5, *, p < 0.05) (Fig. 1B).

**Bexarotene Decreased Cell Proliferation and Induced Apoptosis in MEC, MIN, and Tumors**

As shown in Table 1, BrdU-labeled (proliferating) cells progressively increased in the course of mammary carcinogenesis: from 2.6 ± 0.8% in MECs to 4.4 ± 1.0% (*, p < 0.02) in AH, to 8.3 ± 1.5% (**, p < 0.001) in MIN, and to 9.6 ± 2.6% (**, p < 0.001) in tumors. Bexarotene decreased proliferating cells in MECs, AH, MIN, and tumors in a dose-dependent manner from 2.6 ± 0.8% in placebo, to 1.8 ± 0.7% in low-dose (*, p < 0.02); and 1.5 ± 0.5% in high-dose-treated animals (*, p < 0.02). Similar decreases in cell proliferation occurred in AH (a2, p < 0.02) for high-dose group; in MIN for low- (b1, p < 0.05) and high-dose (b2, p < 0.001) groups; and in tumors for low- (c1, p < 0.05) and high-dose (c2, p < 0.005) groups (Table 1). In addition to a decrease in cell proliferation, bexarotene induced apoptosis in MEC, MIN, and tumors (Fig. 2A and 2B). Apoptosis was rare in MEC, MIN, and tumors of placebo-treated animals (Fig. 2Aa, c brown-stained cells) and increased in bexarotene-treated animals (Fig. 2Ab, d and Fig. 2B): for MEC (*, p < 0.02 and **, p < 0.005), MIN (*, p < 0.01 and **, p < 0.02) and tumors (*, p < 0.001 and **, p < 0.001) for low- and high-dose groups, respectively. Note the relatively high
number of cells in apoptosis among MEC of lobules (Fig. 2Ab) and in tumors (Fig. 2Ad) of bexarotene-treated animals.

**Bexarotene Induced Cellular Senescence (CS) in MEC, MIN, and Tumors**

To determine CS, frozen tissue and tumor sections were stained overnight by SA-β-Gal reaction, which has been consistently used for identification of SC, both in vitro and in vivo (32). As shown in Fig. 3Aa, single SC (blue-stained) were identified among MECs of lobules (arrows) and in AH (arrow) of a control animal. Single SC were also detected in tumors (T) and in surrounding stromal cells (Fig. 3Ab, arrows) of control animals. Treatment of animals with bexarotene significantly increased SC in ducts (D), lobules (L, arrows), and terminal end buds (TEB, arrow), which are considered places of origin of mammary tumors (Fig. 3Ac, arrows). Note that, in some lobules, almost all epithelial cells are SA-β-Gal-positive (L-arrows). SC also increased in tumors (Fig. 3Ad, blue-stained cells). In animals treated with bexarotene, SC usually formed clusters (Fig. 3Ad) or were preferentially localized in differentiated structures (acinar or papillary formations; see Fig. 4Aa, b, c also). As shown in Fig. 3B, in placebo-treated animals, 1.5 ± 0.5% SC were detected among MEC of lobules and ducts, and their number progressively increased to 4.0 ± 1.1% (*, p < 0.05) in low-dose and further to 12.8 ± 2.9% (**, p < 0.001) in high-dose-treated animals. A similar increase of SC was also found in MIN (*, p < 0.02 and **, p < 0.02) and in tumors (*, p < 0.01 and **, p < 0.001) for low- and high-dose treated animals, respectively. The values of SC in mammary tumors of doxorubicin-treated animals were much higher, as compared to those of bexarotene-treated animals (***, p < 0.001, high-dose group) (Fig. 3B). Doxorubicin is a cytotoxic agent that induces DNA breaks and CS; thus, it served as a positive control to bexarotene-induced CS. No difference was found in localization and cytological appearance of SC between bexarotene- and doxorubicin-treated animals.

**Bexarotene-induced CS is Associated with Increased p21 and p16 Expression**
Most studies on CS have been performed in vitro with genotoxic agents, which up-regulate p53-p21 expression (17-19, 33, 34). Here, we asked whether bexarotene, a cell differentiation agent, can also induce CS by affecting the above biomarkers in mammary tumors. To answer this question, we first examined by ICH p53, p21, p16, and pRb expression in tumor cells of control and bexarotene-treated animals. In formalin-fixed paraffin-embedded tissue sections, all the above biomarkers were detected in the nucleus of almost all (>90%) tumor cells, and it was difficult to find any difference in the level of their expression between placebo and bexarotene-treated animals. To determine whether some of the above biomarkers are over-expressed in SC, double labeling of SC was conducted, as indicated in Materials and Methods. In frozen sections, p53 was detected at low level in all tumor cells, and its expression did not increase in SC of control and bexarotene-treated animals. As shown in Fig. 4Aa, a weak p53 nuclear expression in tumor cells of animals treated with bexarotene is evident, but it did not increase in SC (blue stained). However, a parallel frozen section from the same tumor showed clear p21 over-expression in SC (Fig. 4Ab, arrows). Because p21 was over-expressed in single SC of control animals as well, we compared SC with p21 over-expression in control (C) and bexarotene-treated (high-dose) animals. As shown in Fig. 4Ba, bexarotene significantly increased the percentage of SC that over-express p21 in mammary tumors: 36.2 ± 6.5% in bexarotene-treated (high-dose) vs. 12.3 ± 3.3% (*, p < 0.001) in placebo-treated animals. The same approach was also used for p16 and pRb expression in SC (Fig. 4Ac, d). Bexarotene induced p16 expression in SC of tumors, as evident in Fig. 4Ac (arrows) and in Fig. 4Ba, with 21.2 ± 5.8% in bexarotene-treated vs. 40.1 ± 12.2% (H, **, p < 0.01) in placebo-treated animals. pRb protein (Fig. 4Ad, arrows) was also over-expressed in the nucleus of some SC, but no difference was found in the values between control and bexarotene-treated animals (Fig. 4Ba).

**Bexarotene Induces Differentiation and p21 Expression in Breast Cancer Cell Lines**

To make sure that p21 is one of the bexarotene targets, as was shown above in mammary tumors, MDA-MB-231, BT474, MCF-7, and T47D cells, which differentially express
p21 and differ in ER, PR, and Her2/Neu status, were treated for 24 hrs with 1.0 umol/L bexarotene, and p21 was examined by western blotting. As shown in Fig. 4Bb, bexarotene induced p21 expression in MDA-MB-231 and BT-474 cells, had no effect in T47D cells, and decreased its expression in MCF-7 cells. To confirm that bexarotene induces cell differentiation, MDA-MB-231 and BT474 cells were treated for 24 and 72 hrs with 1.0 or 5.0 umol/L, and cytokeratin (pan-cytokeratin) expression was determined (Fig. 4Bc). Bexarotene increased pan-cytokeratin expression in MDA-MB-231 cells within the first 24 hrs with similar effect after 3 days of treatment. The cytokeratin induction in the above cell lines by bexarotene correlated with a p21 increase, suggesting that the latter is a consequence of cell differentiation. Cytokeratin was also up-regulated in BT474 cells treated for 3 days with bexarotene (Fig. 4Bc).

**Bexarotene Differentially Affect RARβ and RXRα Expression in MEC and Tumors**

RARβ expression was detected by ICH in MEC of ducts and lobules and in tumors of control and bexarotene-treated animals. As shown in Fig. 5Aa, only single RARβ positive cells were found in tumors (T). Bexarotene induced RARβ in MEC: from 7.2 ± 1.6% in placebo to 15.3 ± 3.8% (*, p < 0.05) in bexarotene-treated animals; and in 3 of 8 tumors examined, with variability in the values in individual tumors between 1.2% and 15.4% positive cells (Fig. 5Ab). To determine whether RARβ is over-expressed in SC, frozen sections were first treated by SA-β-Gal agents and then with anti-RARβ antibody from Abcam (Cambridge, MA). No RARβ+SA-β-Gal cells were identified among placebo and bexarotene-treated animals. We also found that RXRα was expressed in 82.2 ± 18.8% of MECs and in 75.5 ± 10.3% of tumor cells of control animals, with no significant difference between the values (Fig. 5Ac). Treatment of animals with high doses of bexarotene did not affect RXRα-positive MEC, whereas the high dose decreased receptor expression in tumor cells (Fig. 5Ad) from 75.5 ± 10.3% in placebo to 60.2 ± 12.8% (Fig. 5Bb, *, p < 0.02). To assess in vitro the effect of bexarotene on RARβ and RXRα expression, MDA-MB-231 cells were treated for 24 hrs with 1.0 and 5.0 umol/L of 4-HPR, bexarotene, and atRA (Fig. 5C); the nuclear protein was isolated, and western blotting was performed. As shown
in Fig. 5C, 4-HPR, a ligand of RARγ, did not affect RARβ2 expression; whereas, both bexarotene at 1.0 umol/L and atRA at 1.0 and 5.0 umol/L induced RARβ2 expression. Bexarotene also decreased RXRα expression in MDA-MB-231 cells, but had no effect in BT474 cells (Fig. 5C).

**Discussion**

In this study, we found that bexarotene not only inhibited cell proliferation but also induced CS and apoptosis in MEC, MIN, and tumors of the MMTV-Neu model of mammary carcinogenesis. Our data confirm previous studies of Powel Brown's group, which found that bexarotene suppressed mammary carcinogenesis and tumor growth in MMTV-Neu mice and that this was associated with inhibition of cell proliferation (12, 16). For assessment of apoptosis, we used the TUNEL assay, whereas Brown’s group employed that caspase-3 assay; that may explain the differences in the percentage of cells in apoptosis between their and our study. It is generally believed that apoptosis induced by retinoids is a consequence of oxidative species, which, depending on the cell system and the dose used, may also cause CS (35). Here, we tried to distinguish the effect of bexarotene on cell proliferation and CS from apoptosis by employing two doses of bexarotene (80 and 40 mg/kg bw), assuming that the high dose will preferentially induce apoptosis whereas the low dose will suppress cell proliferation and eventually induce CS. We found a dose-dependent increase in SC concomitantly with increase in apoptosis and decrease in proliferation of MEC, MIN, and tumors. Potential implementation of much lower doses of bexarotene (20, 10 mg/kg bw) may distinguish CS from apoptosis and cell proliferation, as has been reported for *in vitro* studies. Bexarotene was highly efficacious in inducing CS in TEBs, which have high proliferation activity and are considered places of origin of most mammary tumors, thus supporting our data and those of others that retinoids and rexinoids are more efficacious in inhibiting early than late (MIN, tumors) stages of mammary carcinogenesis (16, 23).
Increased CS and apoptosis in MEC of lobules and ducts suggest that bexarotene, in addition to inhibition of carcinogenesis, can also alter mammary gland architecture, as has been reported for 4-HPR in earlier studies (6). Bexarotene, like other rexinoids and retinoids, is a cell differentiation agent (4, 9, 10, 36), as we also confirmed in this study by cytokeratin expression in MDA-MB-231 and BT474 tumor cells (4Bc), suggesting involvement of distinctive signaling pathways different from those induced by cytotoxic agents. For instance, we found that p21 and p16, but not p53, were over-expressed in SC of mammary tumors of bexarotene-treated animals (Fig. 4Ab,c), contrasting the data from cytotoxic agents which primarily induce DNA damage leading to p53 transcription and overexpression (25, 34). Collateral in vitro data provided in this study support the in vivo data and showed increased p21 expression in MDA-MB-231 and BT474 cells treated with 1.0 umol/L bexarotene (Fig.4Bb). Surprisingly, in ER+ MCF-7 and T47D cells, bexarotene had the opposite effect; it suppressed p21 expression, suggesting involvement of different molecular mechanisms in cell and tumor growth inhibition by rexinoids in ER+ and ER- breast carcinomas (37, 38). In addition to p21, p16 was also over-expressed in SC of control and bexarotene-treated animals (Fig. 4Ac). p16ink4a is considered a tumor suppressor gene, and its activation by bexarotene or by other antitumor agents is associated with inhibition of cell proliferation and induction of CS (33, 39, 40). Because, p16 cooperates with pRb, the latter was also evaluated in mammary tumors of control and bexarotene-treated animals. Although in some SC a clear pRb overexpression was found, no significant difference was established when the values were compared with those in non-SC. Another alternative for bexarotene-induced CS is through up-regulation of RARβ. Previous studies have shown that RARβ and particularly its RARβ2 isoform are increased in human breast epithelial cells, which after continuous (>20 passages) in vitro culturing develop senescent phenotype (41). In breast premalignant lesions and tumors, RARβ2 is lost, suggesting its tumor suppressor role (42, 43). Transfection of RARβ2 in tumor cells lacking the receptor was associated with cell growth inhibition and induction of apoptosis (44). Our previous
data obtained from the promoter luciferase activity assay (45) and those of others obtained by
gene analysis of human breast epithelial cells (26) revealed that bexarotene, in addition to
RXRs, can also induce RARβ transcription and thus promotes CS. Here we showed that atRA is
a more powerful inducer of RARβ than bexarotene, which correlates well with differences in
their binding affinity to RARs (38, 46). RXRα expression in mammary tumors was found lower
as compared to normal MEC, most probably as a consequence of overall RARs and RXRs
decrease in tumor development and progression. Bexarotene also decreased RXRα expression
in MDA-MB-231 and BT474 cells, suggesting receptor protein degradation, as has been
reported for RARα in MCF-7 cells treated with atRA (47). The role of RARβ and RXRα in
mediating antitumor potential of retinoids and rexinoids seems to be overestimated, as reported
by gene profiling as well (25, 26). Previous studies (15, 22, 25) and our data in this study
showed that retinoids and rexinoids can, in addition to modulating RARs and RXRs activity, also
affect expression of p21, cyclins-D-A, cdk2/4, pRb, E2F, and other genes/proteins and thus
facilitate retinoid receptor-independent cell and tumor growth inhibition as well as development
of CS. In support of this data, we recently found that about 30% of early passages (< 12
passages) of breast cancer cells did not respond to atRA, 9cRA, and 4-HPR, and that the
expression of both RARβ isoforms (β2 and β5) did not predict cell sensitivity to retinoids (48,
49). In addition, SC induced by bexarotene may secrete cytokines, which may stimulate
proliferation of fibroblasts or activate microphages and tumor suppressor lymphocytes and thus
by collateral mechanisms inhibit mammary carcinogenesis (50). Thus, our data and those of
others suggest that the modulation of one or more of the above genes may prevent tumor cells
to senesce and thus increase their resistance to rexinoids and other antitumor agents. However,
bexarotene is not a pure RXR agonist, but also induces RAR expression; that increases its
cytotoxicity, and thus its long-term clinical implication in cancer prevention and treatment trials
remains questionable (8, 12). SC with their permanent growth arrest can give additional
information about the long-term efficacy and cellular mechanisms of cancer prevention agents.
CS also opens new avenues in developing drugs that selectively induce CS and thus suppress tumor development and progression. This study strongly supports the notion that CS, in addition to inhibition of cell proliferation, can bring additional information about the efficacy and molecular mechanisms of rexinoids in breast cancer prevention and therapy studies. Findings also showed that bexarotene-induced CS is a consequence of multiple signaling pathways involving p21, p16, and RARβ expression. Clinical studies need to confirm whether CS, alone or in combination with other biomarkers, could be used in assessing the efficacy of bexarotene and other agents in preventing and treating breast and other types of cancer.

**Disclosure of potential conflict of interest:**

No potential conflict of interest were disclosed

**Author’s Contributions:**

Conception and design: K. Christov, designed and coordinated the study, evaluated animal and molecular data

Development of methodology: K. Christov and A. Shilkaitis

Acquisition of data: K. Christov, A. Shilkaitis, A. Green, L. Bratescu, T. Yamada

Analysis and interpretation of data: K. Christov, T. Yamada, A. Shilkaitis

Writing, review and revision of manuscript: K. Christov

Administrative, technical and material support: K. Christov, A. Shilkaitis, A. Green

Study supervision: K. Christov

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Table 1: Bexarotene inhibits cell proliferation in MEC, AH, MIN and tumors

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>MECs</th>
<th>AH</th>
<th>MIN</th>
<th>Tumors</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg bw</td>
<td>No X ± SD</td>
<td>No X ± SD</td>
<td>No X ± SD</td>
<td>No X ± SD</td>
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<tr>
<td>Placebo</td>
<td>0</td>
<td>10 2.6 ± 0.8</td>
<td>5 4.4 ± 1.0*</td>
<td>8 8.3 ± 1.5**</td>
<td>26 9.6 ± 2.6**</td>
</tr>
<tr>
<td>Bexarotene</td>
<td>40</td>
<td>11 1.8 ± 0.7a₁</td>
<td>6 4.0 ± 1.2</td>
<td>6 6.4 ± 1.4b₁</td>
<td>18 8.0 ± 2.5c₁</td>
</tr>
<tr>
<td>Bexarotene</td>
<td>80</td>
<td>5 1.5 ± 0.5a₂</td>
<td>5 2.8 ± 0.8b₂</td>
<td>5 3.7 ± 0.9b₂</td>
<td>15 6.6 ± 2.8b₂</td>
</tr>
</tbody>
</table>

a₁, p < 0.02; a₂, p < 0.02; b₁, p < 0.05; b₂, p < 0.001; c₁, p < 0.05; c₂, p < 0.005;
**Figure Legends:**

**Fig. 1A: Bexarotene induces alteration in mammary gland tumors**

Aa: Histomorphology of AH in mammary gland of 5 months old control MMTV-Neu mice. Note the papillary and alveolar structures within AH. The slide is stained by hematoxylin-eosin (H&E) x 200.

Ab: Morphological characteristics of MIN. Cells are similar in size and occupy the entire duct (arrow). Two normal ducts cover with single layer of epithelial cells are also visible. H&E x 200.

Ac: Representative picture of mammary tumor in a control 7 months old mice. H&E x 200.

Ad: Representative picture of a mammary tumor from an animal treated for 4 weeks with bexarotene. Note disintegration of tumor parenchyma (arrows) in peripheral tumor areas. H&E x 200.

**1B: Bexarotene inhibits multiplicity of mammary premalignant lesions and tumors.**

After 4 weeks of treatment with bexarotene a significant decrease in multiplicity of mammary tumors (T), ($p < 0.05^*$ and $p < 0.01^{**}$ for low and high dose groups); MIN ($p < 0.05^*$, for high dose) and AH ($p < 0.05^*$, for high dose) was found. Inside columns are given numbers of animals examined. C: control, placebo treated animals; L, low dose (40 mg/kg bw) and H, high dose (80 mg/kg bw) bexarotene-treated animals.

**Fig. 2: Bexarotene induces apoptosis in MEC, MIN and tumors**

2Aa: Cells in apoptosis were identified by TUNEL assay. Note, single cells in apoptosis (brown stained, arrows) among ductal cells of a control animal. The slide is counterstained by methyl green. x 200;

Ab: Increased cells in apoptosis (arrows) are presented among lobular cells of animal treated for 4 weeks with high dose of bexarotene. x 200

Ac: Several cells in apoptosis (arrows) in mammary tumor of a control animal. x 200;
Ad: Sharp increase in apoptotic cells (brown stained) in mammary tumor of animal treated with high dose of bexarotene. x 200

2B: Percentage of cells in apoptosis in MEC, MIN and tumors. A dose-dependent increase in apoptosis was found in MEC ($p < 0.02^*$ and $p < 0.005^{**}$), MIN ($p < 0.01^*$ and $p < 0.02^{**}$) and tumors ($P < 0.001^* $ and $p < 0.001^{**}$) respectively for low and high doses. The number of animals examines is given inside the columns.

Fig. 3: Bexarotene induces CS in mammary tissues and tumors.

3Aa: SA-β-Gal positive cells (blue-stained) were identified in normal lobules (arrows) and in AH of a control 6 months old mice. The slide is counterstained by neutral fast red. x 200.

Ab: Single SA-β-Gal positive cells were detected among tumor cells (arrow) and among fibroblast surrounding tumor (arrows) of a control animal. x 400.

Ac: Plenty of SA-β-Gal positive cells (blue-stained) among epithelial cells of ducts (D), lobules (L) and terminal end buds (TEB) of a 5 months old mice treated with bexarotene, 80 mg/kg bw. x 200.

Ad: High number of SA-β-Gal positive cells (blue-stained) among tumor parenchyma of animal treated with bexarotene for 4 weeks. x 400.

3B: Note, dose-dependent increase of SC among MEC ($p < 0.05^*$ and $p < 0.001^{**}$, low and high doses respectively), MIN ($p < 0.02$ and $0.02$) and tumors ($p < 0.001$ and $p < 0.001$) bexarotene-treated animals. C, control animals; L, treated with low dose bexarotene; H, treated with high dose bexarotene and D, treated with doxorubicin. Most significant increase in SC was observed in doxorubicin treated animals ($p < 0001^{***}$).

Fig. 4: Senescent cells in mammary tumors over-expressed p21 and p16
Aa: Representative picture of mammary tumor from an animal treated with bexarotene; p53 is expressed at low level in almost all tumor cells. Note, the lack of p53 over-expression in SA-β-Gal positive cells. x 200.

Ab: Parallel section from the same tumor as in Aa, the slide is double stained by SA-β-Gal and p21 antibody. In most SC p21 is over-expressed (dark stained nuclei). x 200.

Ac: Double staining of tumor tissue section by SA-β-Gal and p16 antibody from animal treated with high dose of bexarotene. Most tumor cells expressed p16 (brown stained). However, p16 was over-expressed in most SC (arrows). x 400.

Ad: Representative picture of pRB overexpression in the nucleus of SC (arrows). x 400.

4Ba: The values of SC that overexpress p53, p21, p16, and pRb are presented. C, control animals; H, animals treated with high dose of bexarotene. There is no difference in p53 expression between SC and non-SC (p > 0.5). However, p21 (p < 0.001**) and p16 (p < 0.01*) are over-expressed in SC of tumors from animals treated with high dose of bexarotene. No difference was also found in pRb expression between SC and non-SC (p > 0.5).

Bb: p21 expression in breast cancer cell lines after treatment for 24 hrs with bexarotene, 1.0 umol/L. p21 increased in MDA-MB-231 and BT474 cells, did not change in T47D cells and decreased in MCF-7 cells.

Bc: Bexarotene at 1.0 and 5.0 umol/L induced pan-cytokeratin and p21 expression in MDA-MB-231 and BT-474 cells.

**Fig. 5: Bexarotene induces RARβ in MEC and decreases RXRα expression in mammary tumors.**

Aa: RARβ expression in MEC of lobules and ducts (arrows) of a control animal. In tumor (T) there is lack of RARβ positive cells. RARβ is predominantly expressed in MEC of lobules and ducts (arrows). The slide is counterstained by hematoxylin. x 200.
Ab: RARβ expression (brown-stained) among tumor cells of an animal treated with bexarotene. RARβ is localized in the nucleus only.

Ac: RXRα expression in a duct (D) and in two tumors (T) of a control animal. There is a clear decrease in RXRα expression in tumors as compared to ductal MEC. x 200.

Ad: RXRα expression is decreased in mammary tumor of animal treated with bexarotene, as compared to its expression in tumors of control animals. x 200.

Fig. 5B: (left) – Percent of RARβ positive cells among MEC of ducts and lobules and in tumors (T). Bexarotene induced RARβ expression among MEC ($p < 0.01^*$) and in 3 of 9 mammary tumors. However, in 6 tumors only single RARβ positive cells were detected. B (right), RXRα decreased in mammary tumors of bexarotene-treated animals ($p < 0.02^*$) but not among MEC of ducts and lobules.

Fig. 5C (upper figure): Effects of bexarotene on RARβ2 expression in MDA-MB-231 cells. Cells were treated for 24 hrs with 1.0 uM or 5.0 uM 4-HPR, bexarotene (Bx) and atRA. The alterations in RARβ2 expression as compare to the values of control cells are given below the line of doses. 4-HPR did not induce RARβ2, whereas bexarotene at 1.0 uM and atRA at both doses increased RARβ2 expression (western blots).

Fig. 5C (lower figure): Effects of bexarotene (Bx) on RXRα expression in MDA-MB-231 and BT474 cells. Bexarotene decreased RXRα expression in MDA-MB-231 cells but had no effect in BT474 cells (western blots).
Effects of Bexarotene on RARβ and RXRα expression

Fig. 5
Bexarotene induces cellular senescence in the MMTV-Neu mouse model of mammary carcinogenesis

Anne Shilkaitis, Laura Bratescu, Albert Green, et al.

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