

The Combination of the Rexinoid, LG100268, and a Selective Estrogen Receptor Modulator, Either Arzoxifene or Acolbifene, Synergizes in the Prevention and Treatment of Mammary Tumors in an Estrogen Receptor – Negative Model of Breast Cancer

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Abstract Purpose: We tested whether a selective estrogen receptor modulator (SERM) and a rexinoid are active for prevention and treatment in the mouse mammary tumor virus-neu mouse model of estrogen receptor – negative breast cancer.

Experimental Design: For prevention, mice were fed a powdered control diet, the SERM arzoxifene (Arz, 20 mg/kg diet), the rexinoid LG100268 (268, 30 mg/kg diet), or the combination for 60 weeks. In a second prevention study, mice were fed Arz (6 mg/kg diet), 268 (30 mg/kg diet), the combination of Arz and 268, the SERM acolbifene (Acol, 3 mg/kg diet), or the combination of Acol and 268 for 52 weeks. For the treatment studies, mice with tumors were fed combinations of a SERM and 268 for 4 weeks.

Results: The rexinoid 268 and the SERMs Arz and Acol, as individual drugs, delayed the development of estrogen receptor – negative tumors. Moreover, the combination of a SERM and 268 was strikingly synergistic, as no tumors developed in any mouse fed the combination of 268 and a SERM. Moreover, this drug combination also induced significant tumor regression when used therapeutically. These drugs did not inhibit transgene expression *in vitro* or *in vivo*, and the combination of Arz and 268 inhibited proliferation and induced apoptosis in the tumors.

Conclusion: The combination of a rexinoid and SERM should be considered for future clinical trials.

Despite the development of selective estrogen receptor (ER) modulators (SERMs), aromatase inhibitors, and the monoclonal antibody trastuzumab (Herceptin), breast cancer still claims >40,000 lives in the U.S. each year (1). Because of the genetic and epigenetic complexities of an invasive cancer, arresting or reversing carcinogenesis at its earliest stages offers an attractive alternative to treating advanced disease (2, 3). However, the realities of the clinic mean that new drugs and new drug

combinations are desperately needed for both the prevention and treatment of breast cancer.

A number of drugs have been shown to prevent or treat ER+ breast cancer. SERMs such as tamoxifen and raloxifene are effective in women for both prevention (4–8) and treatment (9). Newer SERMs such as acolbifene (Acol, EM-652) and its prodrug (EM-800) have been used to prevent the development of and to treat established mammary tumors in animal models (10–12), and caused the disappearance of 60% of human breast cancer tumors in nude mice (13). Acol also showed positive responses in women who failed tamoxifen treatment (14), suggesting the superiority of Acol over tamoxifen in a series of preclinical studies (15). The SERM arzoxifene (Arz) also prevented mammary carcinogenesis in rats (16) and decreased ER expression in humans in a phase 1 chemoprevention trial (17).

In addition to the SERMs, retinoids, such as 9-*cis*-retinoic acid (18, 19), or rexinoids [selective ligands for the retinoid X receptors (RXRs)], such as LGD1069 (bexarotene, Targretin; refs. 20, 21) and LG100268 (268; refs. 22, 23), have been reported to prevent and treat mammary tumors in animal models of ER+ breast cancer. When tested in patients with metastatic breast disease, bexarotene showed clinical benefit in 20% of patients, without any significant toxicity (24). However, the newer rexinoid, 268 (25), is more potent than bexarotene and has greater specificity for binding to RXRs; bexarotene can

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still bind to retinoic acid receptors (20), which is linked with undesirable toxicity. To date, no prevention trials with rexinoids have been initiated in humans. In addition to the ample evidence that SERMs, retinoids, and rexinoids can prevent and/or treat mammary tumors in experimental animals, preclinical studies establish that combinations of these agents are even more effective than a single drug (18, 19, 21–23).

Despite the ability of SERMs such as tamoxifen to reduce the incidence of ER+ breast cancer in the clinic, they were not effective for preventing or treating ER– disease. Currently, no drugs have been reported to prevent ER– breast cancer in women, and only the retinoid 9-*cis*-retinoic acid (26), the rexinoid bexarotene (27, 28), and the epidermal growth factor receptor inhibitor ZD1839 (gefitinib, Iressa; ref. 29) have been shown to inhibit the formation of ER– mammary tumors in mice. None of these compounds have been tested in combination for the prevention of ER– breast cancer.

We have previously shown that the SERM Arz and the rexinoid 268 prevent and treat mammary carcinogenesis in a rat nitrosomethylurea model of ER+ breast cancer (22, 23). Although there are no functional estrogen receptors in ER– breast cancer epithelium, early treatment with tamoxifen has been reported to decrease the incidence of ER– tumors in mice (30). Moreover, the mammary stroma contains functional estrogen receptors (31), and paracrine regulation from these receptors plays an important role in epithelial growth and differentiation (32, 33). Because the combination of Arz and 268 affects stromal cells which regulate the tumor microenvironment (22), we decided to test this drug combination in a mouse mammary tumor virus (MMTV)-*neu* mouse model of ER– breast cancer. In this commonly used transgenic model, mice develop tumors because of targeted expression of the *neu* gene (*erbB2/HER2*) in the mammary gland. The *neu* gene encodes for a receptor tyrosine kinase member of the epidermal growth factor receptor family; the *neu* protein is overexpressed in 20% to 30% of human breast cancers (34), and *neu* overexpression is inversely correlated with patient survival (35). The validity of *neu* as a drug target is supported by the emerging therapeutic importance of trastuzumab, a recombinant monoclonal antibody that targets *erbB2*, for treating women with HER2-positive breast cancer (36, 37).

In the present experiments, we first tested the combination of Arz and 268 for the prevention of ER– mammary tumors in MMTV-*neu* mice. This prevention protocol was then repeated, and both Arz and Acol were tested alone and in combination with 268. Because of the striking results from these studies, we next allowed tumors to form and then treated the mice with combinations of 268 and a SERM. In both the prevention and treatment experiments, the combinations of either 268 and Arz or 268 and Acol were remarkably effective.

Materials and Methods

Reagents. The synthesis of 268 (25) and Acol (38) have been previously described. Arz (39) was provided by Lilly Research Labs (Indianapolis, IN). The structures for these drugs are shown in Fig. 1.

Transgenic mice. MMTV-*neu* transgenic mice (The Jackson Laboratory, Bar Harbor, ME) were developed in the laboratory of William

Muller (McGill University). Wild-type *neu* is expressed in the mammary tissue under the control of the MMTV promoter. Focal mammary tumors begin to appear in the mice at 4 months of age, and these tumors are ER– (28, 40). All animal studies were done in accordance with an institutionally approved protocol.

Prevention of mammary tumors. Beginning at 7 weeks of age (first prevention study) or at 8 weeks of age (second prevention study), female mice were fed a powdered control diet or a diet containing compounds, as previously described (22, 41). When the mice were 4 months old, they were palpated weekly and tumors were measured with calipers. Tumor volume was calculated by multiplying tumor length (*l*) by width (*w*) by height ($h = lw / 2$) and dividing by 2 ($lwh / 2$). Animals were sacrificed if tumor volume exceeded 900 mm³ or at the end of the experiment (60 weeks on diet for the first prevention study and 52 weeks on diet for the second prevention study). Tumors were saved for analysis of transgene expression.

Treatment of mammary tumors. A separate cohort of mice was fed normal rodent chow until tumors developed. Mice were palpated weekly, beginning at 20 weeks of age. When tumors at least 32 mm³ in volume had developed, the mice were fed a powdered diet containing drugs [Arz (20 mg/kg diet), 268 (100 mg/kg diet), Arz + 268, Acol (10 mg/kg diet), or Acol + 268]. Mice were fed treatment diets for up to 4 weeks, but animals were sacrificed early if tumor volume exceeded 900 mm³. Tumors were measured weekly, and tumor regression was defined as at least a 50% decrease in tumor volume. Tumors that did not change (tumor volume increased or decreased less than 50%) were classified as growth-arrested. Active tumor growth was defined as a greater than 50% increase in tumor volume.

Evaluation of transgene expression. Transgene expression was analyzed by PCR, Western blot, and immunohistochemistry. The

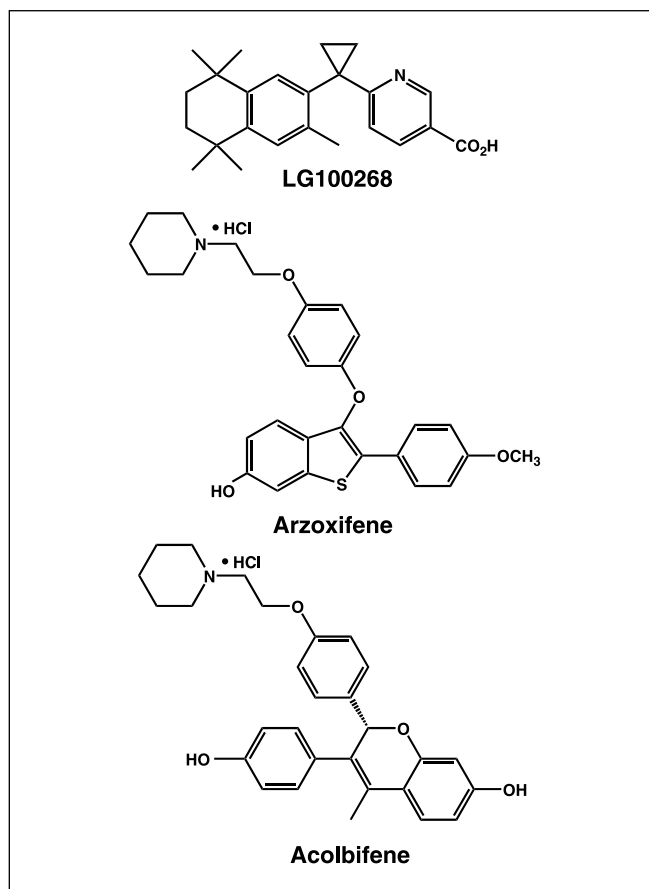


Fig. 1. Structures of 268, Arz, and Acol.

Table 1. Arz and 268 synergize in prevention of ER– mammary gland tumors in MMTV-neu transgenic mice (60-week study)

Treatment	Control	Arz, 20 mg/kg diet	268, 30 mg/kg diet	Arz + 268
No. of mice/group	16	8	8	8
No. of mice with tumors (%)	16 (100)	4* (50)	8 (100)	0*† (0)
Total no. of tumors/group	34	7	11	0
Average no. of tumors/mouse	2.1	0.9*	1.4	0*†

**P* < 0.05 versus control.†*P* < 0.05 versus 268.

E18-14C-27 tumor cell line derived from mammary tumors from MMTV-erbB2 mice were maintained in RPMI + 10% charcoal-stripped fetal bovine serum. Cells were treated with drugs for up to 48 hours and then analyzed by PCR and Western blot. To evaluate transgene expression *in vivo*, 8-week-old MMTV-neu mice (four mice per group) were fed Arz (6 mg/kg diet), 268 (30 mg/kg diet), the combination of Arz and 268, Acol (6 mg/kg diet), or the combination of Acol and 268 for 8 weeks. When the mice were 16 weeks of age, the mammary glands were harvested and analyzed by PCR and immunohistochemistry for erbB2 expression. In separate treatment experiments, tumors at least 45 mm³ in volume were allowed to form, and the mice were then treated with the combination of Arz (20 mg/kg diet) and 268 (100 mg/kg diet) for 1 to 3 weeks, until tumor volume was reduced by ~50%. These tumors and actively growing tumors from mice fed the control diet were harvested and analyzed by Western blot and immunohistochemistry. For Western analysis, cell extracts were separated by SDS-PAGE, transferred to a nitrocellulose membrane, and blotted with antibodies against ErbB2/HER-2/neu (NeoMarkers, Fremont, CA) and actin (Sigma, St. Louis, MO).

Immunohistochemistry and terminal nucleotidyl transferase–mediated nick end labeling staining. Mammary glands or tumors were fixed in 4% paraformaldehyde or neutral-buffered formalin containing additional zinc (Z-fix, Anatech, Ltd., Battle Creek, MI), embedded in paraffin, sectioned, and stained for erbB2, proliferating cell nuclear antigens (PCNA) or terminal nucleotidyl transferase–mediated nick end labeling (TUNEL) as previously described (23, 29). To calculate the percentage of PCNA- or TUNEL-positive cells, two sections on the periphery of each tumor (four tumors per group and >1,000 cells per treatment group) were counted in a blinded fashion.

Statistical analysis. The *in vivo* experiments summarized in Tables 1-3 were analyzed with the Kruskal-Wallis one-way ANOVA on ranks followed by Dunn's method for multiple comparisons (SigmaStat 3.1). Results from the PCNA and TUNEL staining experiments were analyzed by ANOVA and the *post hoc* Student-Newman-Keuls test or the Mann-Whitney rank sum test, respectively.

Results

The combination of 268 and a SERM, Arz or Acol, prevents development of ER– mammary tumors. Beginning at 7 weeks of age, female MMTV-neu mice were fed a powdered control diet or a diet containing Arz (20 mg/kg diet, ~5 mg/kg body weight), 268 (30 mg/kg diet, ~7.5 mg/kg body weight), or the combination. As shown in Fig. 2, 100% of the control mice developed mammary tumors by the 48th week. Notably, no tumors were ever observed in the combination group. At week 38, 25% of the mice in both the 268 and Arz groups had developed tumors compared with 81% in the control group. Although all of the mice on the 268 diet eventually developed tumors, the time required for 100% tumor incidence was delayed by 3 months. After 60 weeks on the diet, only 50% of the mice on the Arz diet had developed tumors. Tumor multiplicity was also reduced in the treated groups (Table 1), and the average number of tumors per mouse at the end of 60 weeks dropped from 2.1 in the control group to 1.4 in the 268 group, 0.9 in the Arz group, and 0 in the combination group (*P* < 0.05 for Arz and Arz + 268 groups versus control).

In order to confirm these striking results, a similar experiment was done with an independent cohort of mice. In this second experiment, the MMTV-neu mice were fed a diet containing Arz (6 mg/kg diet), Acol (3 mg/kg diet), 268 (30 mg/kg diet), the combination of Arz and 268, or the combination of Acol and 268. At the end of 1 year on the diet, 100% of the control mice and 58% of the mice on the 268 or Arz diet had developed tumors (Fig. 3A). When Acol was used in the diet, 67% of the mice developed tumors after 1 year (Fig. 3B); Acol was used at a lower concentration than Arz (3 versus 6 mg/kg diet) because of a lack of weight gain when mice were fed Acol at the higher dose. Remarkably, no tumors

Table 2. The combination of 268, and either Arz or Acol, prevents ER– mammary gland tumors in MMTV-neu transgenic mice (52-week study)

Treatment	Control	Arz, 6 mg/kg diet	268, 30 mg/kg diet	Arz + 268	Acol, 3 mg/kg diet	Acol + 268
No. of mice/group	24	12	12	12	12	12
No. of mice with tumors (%)	24 (100)	7* (58)	7* (58)	0*† (0)	8* (67)	0*† (0)
Total no. of tumors/group	37	7	10	0	12	0
Average no. of tumors/mouse	1.5	0.6*	0.8	0*	1.0	0*‡

**P* < 0.05 versus control.†*P* < 0.05 versus Arz, 268, and Acol.‡*P* < 0.05 versus Acol.

Table 3. The combination of 268 and either Arz or Acol causes regression of ER– mammary gland tumors in MMTV-neu transgenic mice

Treatment	Arz, 20 mg/kg diet	268, 100 mg/kg diet	Arz + 268	Acol, 10 mg/kg diet	Acol + 268
No. of mice in treatment protocol	16	23	70	14	18
No. of tumors in treatment protocol	21	31	84	17	34
No. of tumors showing regression (%)	0 (0)	21* (68)	70* (83)	0 (0)	25* (74)
No. with a 51-75% reduction in tumor volume (%)	0 (0)	5 (16)	7 (8)	0 (0)	2 (6)
No. with a 76-100% reduction in tumor volume (%)	0 (0)	16* (52)	63* [†] (75)	0 (0)	23* (68)
No. of tumors showing arrested growth (%)	2 (10)	5 (16)	10 (12)	2 (12)	3 (9)
No. of tumors showing active growth (%)	19 (90)	5* (16)	4* (5)	15 (88)	6* (18)

**P* < 0.05 versus Arz and Acol.[†]*P* < 0.05 versus 268.

were observed in mice fed either Arz + 268 (Figs. 2A and 3A) or Acol + 268 (Fig. 3B) in either of these year-long prevention studies. The average number of tumors per mouse was again significantly lower (*P* < 0.05) in the groups treated with Arz alone or with the combination of a SERM and 268 compared with the control group (Table 2). The concentrations of drugs used in both of these experiments were well-tolerated by the mice, as determined by continued weight gain throughout the studies.

The combination of 268 and a SERM is effective for the treatment of ER– mammary tumors. Because of the observed synergistic effects when 268 was combined with a SERM in these ER– mammary tumor prevention studies and because this combination was reported to induce apoptosis in breast cancer, partially by inducing transforming growth factor β (23), we next attempted to treat established ER– mammary tumors. For these studies, mice were maintained on a control diet until they had developed tumors with volumes of at least 32 mm³. The mice were then fed a control diet or a diet containing Arz (20 mg/kg diet), 268 (100 mg/kg diet), Arz + 268, Acol (10 mg/kg diet), or Acol + 268; higher concentrations of drugs were used for the treatment studies in order to inhibit tumor cell proliferation or induce apoptosis. All tumors from mice fed the control diet continued to grow (data not shown). As shown in Table 3, 83% of the tumors (*n* = 84) in the mice treated with the combination of Arz + 268 regressed, and tumor volume was

reduced by 76% to 100% in 75% of these tumors. Another 12% of the tumors were growth-arrested, leaving only 5% of the tumors that were resistant to treatment with Arz + 268. In the Acol + 268 combination group, 74% of the tumors regressed, 9% were growth-arrested, but 18% of these tumors continued to grow. As expected, neither Arz nor Acol alone was effective for treatment of ER– tumors, as 90% of the tumors in the Arz group and 88% of tumors in the Acol group continued to grow. Surprisingly, 268 alone caused regression in 68% of the tumors, although 16% of the tumors were resistant to treatment with 268. Moreover, the 75% of tumors that were reduced in volume

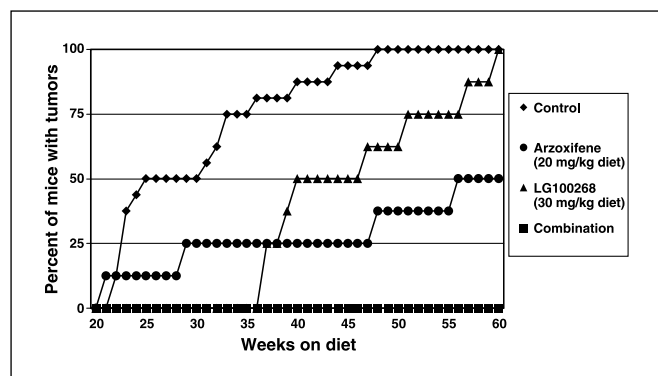


Fig. 2. 268 and Arz synergize in the prevention of ER– mammary gland tumors. MMTV-neu transgenic mice were fed powdered control diet or preventive agents in the same diet, beginning at 7 weeks of age. Mice (*n* = 16 for control group and *n* = 8 for all treatment groups) were palpated weekly for tumors, and none were detected before 28 weeks of age. The experiment was terminated after mice were fed experimental diets for 60 weeks.

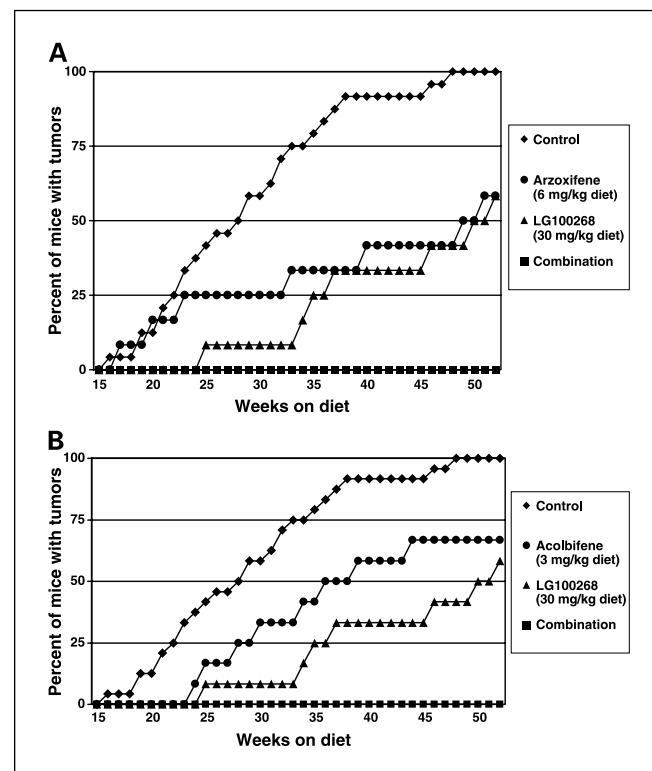


Fig. 3. Arz and 268 (A, repeat experiment) and Acol and 268 (B) synergize in the prevention of ER– mammary gland tumors. MMTV-neu transgenic mice were fed powdered control diet or preventive agents in the same diet, beginning at 8 weeks of age. Mice (*n* = 24 for control group and *n* = 12 for all treatment groups) were palpated weekly for tumors, and none were detected before 24 weeks of age. The experiment was terminated after mice were fed experimental diets for 1 year.

by 76% to 100% were significantly ($P = 0.03$) higher in the Arz + 268 combination group than the 52% of tumors that regressed in the 268 alone group.

268, alone or in combination with a SERM, does not reduce *c-neu* transgene expression *in vitro* or *in vivo*. To verify that these significant results did not occur because of a reduction in

transgene expression, *c-neu* expression was analyzed by PCR, Western blot, and immunohistochemistry. First, E18-14C-27 tumor cells from MMTV-erbB2 mice were treated with various concentrations (0.01-1 $\mu\text{mol/L}$) of 268, Arz, Acol, or their combinations for 24 to 48 hours. Transgene expression did not change either at the mRNA or protein level, as shown by

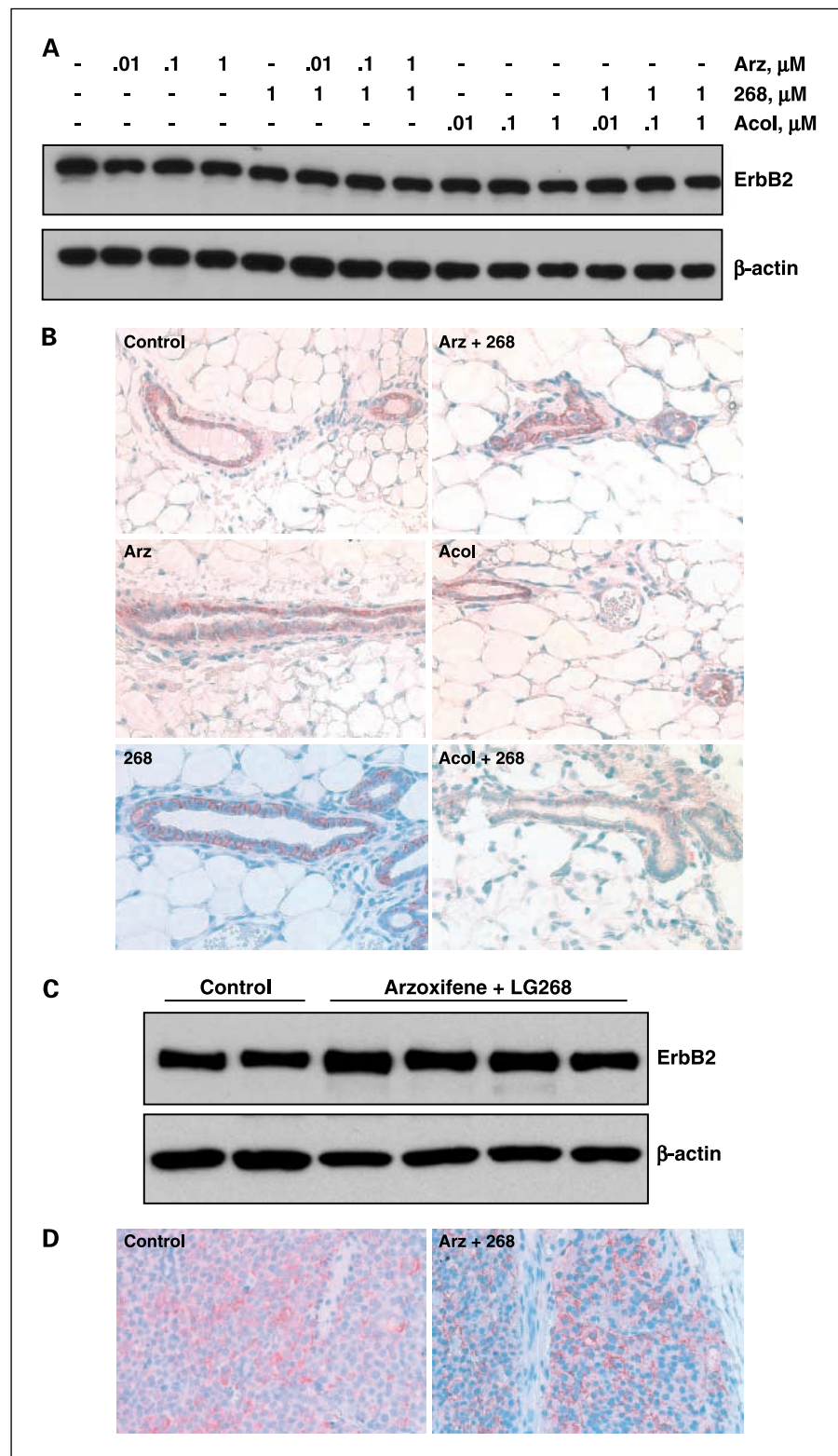


Fig. 4. Drugs do not affect erbB2 transgene expression *in vitro* or *in vivo*. *A*, E18-14C-27 cells were treated for 48 hours with various concentrations and combinations of drugs. Cell lysates were immunoblotted with ErbB2 and actin antibodies. *B*, MMTV-neu mice (four mice per group) were fed Arz (6 mg/kg diet), 268 (30 mg/kg diet), Arz + 268, Acol (6 mg/kg diet), or Acol + 268 in the diet for 8 weeks. Mammary glands were harvested and analyzed by immunohistochemistry for erbB2 expression. *C* and *D*, in separate treatment experiments, tumors were allowed to form, and the mice were then treated with the control diet or the combination of Arz (20 mg/kg diet) and 268 (100 mg/kg diet) for 1 to 3 weeks. Tumors then were harvested and analyzed by Western blot (*C*) or immunohistochemistry (*D*) for ErbB2 (positive cells are reddish-brown).

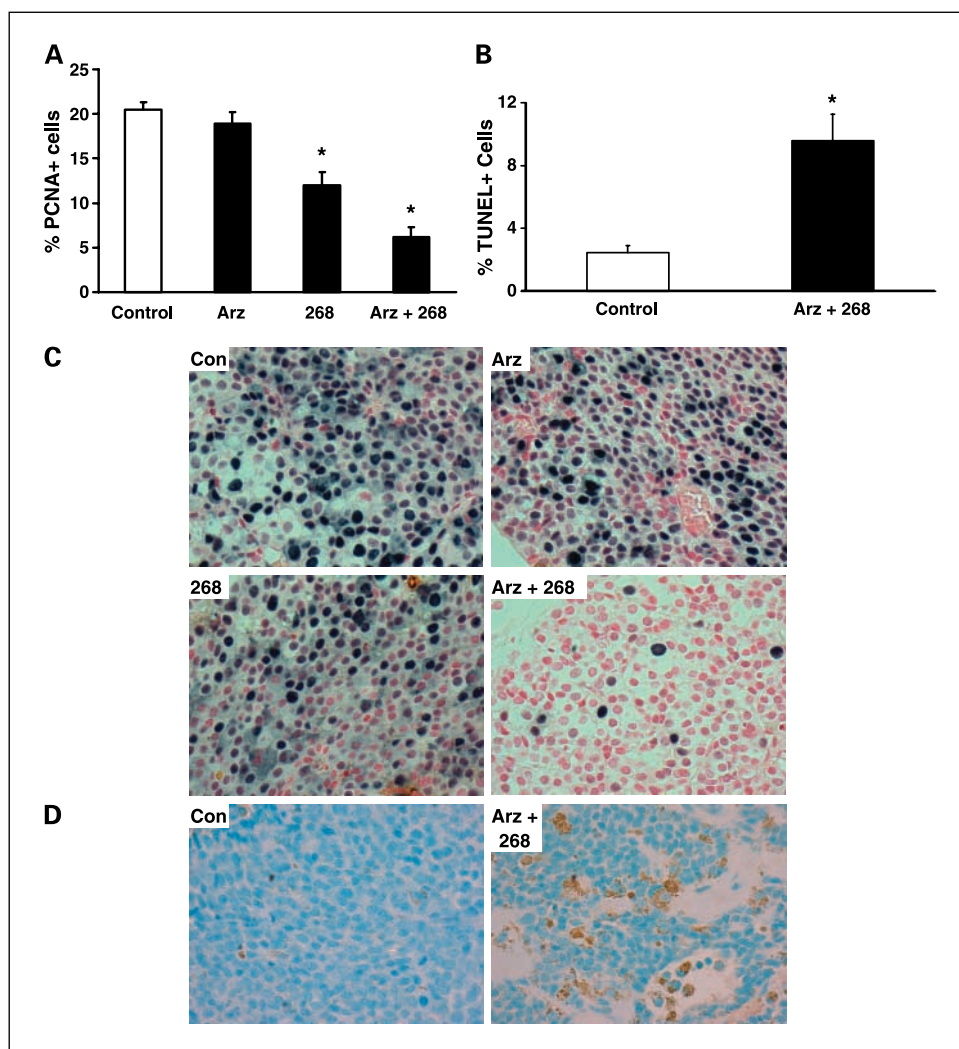


Fig. 5. 268 or the combination of 268 and Arz decreases proliferation and increases apoptosis in tumors. Tumor sections from MMTV-neu mice (four per group) were analyzed for cell proliferation by PCNA staining (A and C, positive SG-black signal contrasted with nuclear red counterstaining of PCNA-negative cells) and for apoptosis by TUNEL staining (B and D, positive cells brown with 3,3'-diaminobenzidine and methyl green as the counterstain). Columns, mean of >1,000 cells from four tumors per treatment group; bars, \pm SD. Drug concentrations were 20 mg/kg diet for Arz and 100 mg/kg diet for 268. *, $P < 0.05$ versus control (and Arz in A). Magnification, $\times 400$.

PCR (data not shown) or Western blot (Fig. 4A). Moreover, MMTV-neu mice from the prevention study were fed Arz, 268, or the combination in diet for 8 weeks, and PCR (data not shown) and immunohistochemistry (Fig. 4B) revealed no apparent changes in neu expression in the mammary glands of these mice. Finally, tumors from mice that were treated with the combination of Arz and 268 for 1 to 3 weeks, and that had regressed by at least 50% were harvested and analyzed by Western blot and immunohistochemistry for neu expression. The erbB2 protein was expressed in 11 tumors from the mice fed Arz + 268 and was indistinguishable from the levels in 7 control tumors. An additional 17 tumors were analyzed by immunohistochemistry, and the average erbB2 immunostaining score from eight control tumors was 3 versus 2.5 for nine tumors from the combination group; this difference is not statistically different ($P = 0.13$). Representative Western blot and immunohistochemistry staining results are shown in Fig. 4C and D, respectively. Taken together, these experiments suggest that these compounds do not inhibit the expression of the neu transgene.

The combination of 268 and Arz inhibits proliferation and induces apoptosis in mammary tumors. Because of the dramatic reduction in tumor volume in mice treated with the combination of Arz and 268, tumors were analyzed by PCNA and

TUNEL staining. As shown in Fig. 5A and C, the percentage of PCNA-positive cells was similar in tumors from the control and Arz groups (20.5% and 18.9%, respectively). However, the percentage of cells that were positive for PCNA staining was significantly lower ($P < 0.05$) in both the 268 group (12.0%) and in the Arz + 268 tumors (6.2%). Moreover, 9.6% of the cells in the Arz + 268 tumors stained positive for TUNEL versus only 2.5% ($P < 0.05$) of cells in the control group (Fig. 5B and D).

Discussion

The results described here clearly show that the combination of a SERM (either Arz or Acol) and the rexinoid 268 is extremely effective for the prevention and treatment of tumorigenesis in a mouse model of ER- breast cancer. Although all three individual compounds significantly delayed the development of mammary tumors in the prevention studies, the combination was remarkably potent, as no tumors were detected in any mouse fed a combination diet in two independent, year-long studies. Moreover, the combination of a SERM and 268 also induced marked regression of established ER- mammary gland tumors.

In addition to our important results with the combination treatments, we also show that 268 alone caused tumor regression. The efficacy of the 268 treatment was unexpected, based on our previous studies with this drug in the nitro-somethylurea rat model of ER+ breast cancer. In these studies (22, 23), 268 alone reduced tumor volume, but the combination of Arz and 268 was markedly synergistic. Bexarotene, the only rexinoid approved for use in the clinic, inhibited the development of both ER- and ER+ mammary tumors (20, 27) and caused the regression of ER+ mammary tumors (21, 42), but it was unable to reduce the size of ER- MDA-MB-231 xenografts unless used in combination with standard chemotherapeutic agents (43). Our studies also show that 268 decreased the percentage of PCNA-positive cells in the tumors, and a recent study reports that 268 inhibits the growth of breast epithelial cells by decreasing cyclin D1 protein and thus Rb phosphorylation (44). It will be important to examine cyclin D1 levels and proteins in the apoptotic pathway in tumors from mice treated with 268; these studies will be described in a future report. Furthermore, the receptors (RXR- α , RXR- β , and RXR- γ) for rexinoids such as 268 heterodimerize with many other members of the nuclear receptor superfamily (45, 46). Because the rexinoids interact with numerous networks that regulate growth and apoptosis, our results are likely the integration of diverse signals from these multiple pathways and thus will be difficult to define.

The ability of Arz or Acol alone to inhibit the formation of ER- tumors in the prevention studies, whereas having no effect in the treatment studies is also notable. Tamoxifen has been reported to reduce tumor incidence (47) or to delay the development of tumors (30) in two different mouse models of ER- breast cancer. In both of these models, the beneficial effects of tamoxifen were only observed if the treatment was started early, before tumors could be detected. Tamoxifen, however, had no effect on established tumors. These animal studies support our findings and suggest either that SERMs inhibit cell proliferation and thus decrease the number of cells available to form a tumor or that ER- tumors require estrogen during their early development. Therefore, it may be possible to prevent both ER+ and ER- breast cancer with a SERM, alone or

in combination, if given at an appropriately early time before cells transition to an ER- phenotype.

Although we did not observe any decrease in PCNA staining in tumors from the Arz group, SERMs can induce the expression of transforming growth factor β in breast cancer cells or fibroblasts (22, 23, 48–50). Transforming growth factor β has growth-inhibitory and proapoptotic effects on cancer cells (51–54) and might contribute to the chemopreventive properties of the SERMs *in vivo*. Moreover, we have previously reported that the combination of Arz and 268 synergistically enhances transforming growth factor β production in Swiss-3T3 cells and inhibits iNOS expression in primary rat embryo fibroblasts (22). The marked synergy between the SERMs and 268 in a number of previous studies (22, 23) and in the current experimental model of ER- breast cancer probably involves several different signaling pathways, including the transforming growth factor β network (55) and stromal-epithelial interactions (33, 56). Defining these pathways and mechanisms will require additional experiments; these studies are important but will require a systems approach (55, 57, 58) to the tissue networks and thus will be described in future publications. Moreover, a recent article reported that bexarotene inhibits angiogenesis (59), and future studies will examine the effects of 268 and the combination of 268 and a SERM on angiogenesis in tumors in the MMTV-neu model. Finally, the ability of multifunctional drugs such as SERMs and rexinoids to act on multiple pathways has important clinical implications, and our data suggests that the combination of a SERM and the rexinoid 268 should be tested in patients for both the prevention and/or treatment of breast cancer.

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References

- Jemal A, Murray T, Ward E, et al. Cancer statistics, 2005. *CA Cancer J Clin* 2005;55:10–30.
- Sporn MB, Suh N. Chemoprevention: an essential approach to controlling cancer. *Nat Rev Cancer* 2002;2:537–43.
- Sporn MB, Liby KT. Cancer chemoprevention: scientific promise, clinical uncertainty. *Nat Clin Pract Oncol* 2005;2:518–25.
- Fisher B, Costantino JP, Wickerham DL, et al. Tamoxifen for prevention of breast cancer: report of the National Surgical Adjuvant Breast and Bowel Project P-1 Study. *J Natl Cancer Inst* 1998;90:1371–88.
- Cummings SR, Eckert S, Krueger KA, et al. The effect of raloxifene on risk of breast cancer in postmenopausal women: results from the MORE randomized trial. Multiple Outcomes of Raloxifene Evaluation. *JAMA* 1999;281:2189–97.
- Vogel VG, Costantino JP, Wickerham DL, et al. Effects of tamoxifen vs raloxifene on the risk of developing invasive breast cancer and other disease outcomes: the NSABP study of tamoxifen and raloxifene (STAR) P-2 trial. *JAMA* 2006;295:2727–41.
- Martino S, Cauley JA, Barrett-Connor E, et al. Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst* 2004; 96:1751–61.
- Fabian CJ, Kimler BF. Selective estrogen-receptor modulators for primary prevention of breast cancer. *J Clin Oncol* 2005;23:1644–55.
- Osborne CK, Zhao H, Fuqua SA. Selective estrogen receptor modulators: structure, function, and clinical use. *J Clin Oncol* 2000;18:3172–86.
- Luo S, Stojanovic M, Labrie C, Labrie F. Inhibitory effect of the novel anti-estrogen EM-800 and medroxyprogesterone acetate on estrone-stimulated growth of dimethylbenz[a]anthracene-induced mammary carcinoma in rats. *Int J Cancer* 1997;73: 580–6.
- Luo S, Labrie C, Belanger A, Candas B, Labrie F. Prevention of development of dimethylbenz(a)anthracene (DMBA)-induced mammary tumors in the rat by the new nonsteroidal antiestrogen EM-800 (SCH57050). *Breast Cancer Res Treat* 1998;49:1–11.
- Gutman M, Couillard S, Roy J, Labrie F, Candas B, Labrie C. Comparison of the effects of EM-652 (SCH57068), tamoxifen, toremifene, droloxifene, idoxifene, GW-5638 and raloxifene on the growth of human ZR-75-1 breast tumors in nude mice. *Int J Cancer* 2002;99:273–8.
- Roy J, Couillard S, Gutman M, Labrie F. A novel pure SERM achieves complete regression of the majority of human breast cancer tumors in nude mice. *Breast Cancer Res Treat* 2003;81:223–9.
- Labrie F, Champagne P, Labrie C, et al. Activity and safety of the antiestrogen EM-800, the orally active precursor of acolbifene, in tamoxifen-resistant breast cancer. *J Clin Oncol* 2004;22:864–71.
- Labrie F, Labrie C, Belanger A, et al. EM-652 (SCH 57068), a third generation SERM acting as pure anti-estrogen in the mammary gland and endometrium. *J Steroid Biochem Mol Biol* 1999;69:51–84.
- Suh N, Glasebrook AL, Palkowitz AD, et al. Arzoxifene, a new selective estrogen receptor modulator for chemoprevention of experimental breast cancer. *Cancer Res* 2001;61:8412–5.
- Fabian CJ, Kimler BF, Anderson J, et al. Breast cancer chemoprevention phase I evaluation of biomarker modulation by arzoxifene, a third generation selective estrogen receptor modulator. *Clin Cancer Res* 2004;10:5403–17.
- Anzano MA, Byers SW, Smith JM, et al. Prevention

- of breast cancer in the rat with 9-*cis*-retinoic acid as a single agent and in combination with tamoxifen. *Cancer Res* 1994;54:4614–7.
19. Anzano MA, Peer CW, Smith JM, et al. Chemoprevention of mammary carcinogenesis in the rat: combined use of raloxifene and 9-*cis*-retinoic acid. *J Natl Cancer Inst* 1996;88:123–5.
 20. Gottardis MM, Bischoff ED, Shirley MA, Wagoner MA, Lamph WW, Heyman RA. Chemoprevention of mammary carcinoma by LGD1069 (Targretin): an RXR-selective ligand. *Cancer Res* 1996;56:5566–70.
 21. Bischoff ED, Gottardis MM, Moon TE, Heyman RA, Lamph WW. Beyond tamoxifen: the retinoid X receptor-selective ligand LGD1069 (TARGRETIN) causes complete regression of mammary carcinoma. *Cancer Res* 1998;58:479–84.
 22. Suh N, Lamph WW, Glasebrook AL, et al. Prevention and treatment of experimental breast cancer with the combination of a new selective estrogen receptor modulator, arzoxifene, and a new retinoid, LG 100268. *Clin Cancer Res* 2002;8:3270–5.
 23. Rendi MH, Suh N, Lamph WW, et al. The selective estrogen receptor modulator arzoxifene and the retinoid LG100268 cooperate to promote transforming growth factor β -dependent apoptosis in breast cancer. *Cancer Res* 2004;64:3566–71.
 24. Esteva FJ, Gaspy J, Baidas S, et al. Multicenter phase II study of oral bexarotene for patients with metastatic breast cancer. *J Clin Oncol* 2003;21:999–1006.
 25. Boehm MF, Zhang L, Zhi L, et al. Design and synthesis of potent retinoid X receptor selective ligands that induce apoptosis in leukemia cells. *J Med Chem* 1995;38:3146–55.
 26. Wu K, Kim HT, Rodriguez JL, et al. 9-*cis*-Retinoic acid suppresses mammary tumorigenesis in C3(1)-simian virus 40 T antigen-transgenic mice. *Clin Cancer Res* 2000;6:3696–704.
 27. Wu K, Kim HT, Rodriguez JL, et al. Suppression of mammary tumorigenesis in transgenic mice by the RXR-selective retinoid, LGD1069. *Cancer Epidemiol Biomarkers Prev* 2002;11:467–74.
 28. Wu K, Zhang Y, Xu XC, et al. The retinoid X receptor-selective retinoid, LGD1069, prevents the development of estrogen receptor-negative mammary tumors in transgenic mice. *Cancer Res* 2002;62:6376–80.
 29. Lu C, Speers C, Zhang Y, et al. Effect of epidermal growth factor receptor inhibitor on development of estrogen receptor-negative mammary tumors. *J Natl Cancer Inst* 2003;95:1825–33.
 30. Medina D, Kittrell FS, Hill J, Shepard A, Thordarson G, Brown P. Tamoxifen inhibition of estrogen receptor- α -negative mouse mammary tumorigenesis. *Cancer Res* 2005;65:3493–6.
 31. Cunha GR, Young P, Hom YK, Cooke PS, Taylor JA, Lubahn DB. Elucidation of a role for stromal steroid hormone receptors in mammary gland growth and development using tissue recombinants. *J Mammary Gland Biol Neoplasia* 1997;2:393–402.
 32. Bissell MJ, Radisky D. Putting tumours in context. *Nat Rev Cancer* 2001;1:46–54.
 33. Wiseman BS, Werb Z. Stromal effects on mammary gland development and breast cancer. *Science* 2002;296:1046–9.
 34. Harari D, Yarden Y. Molecular mechanisms underlying ErbB2/HER2 action in breast cancer. *Oncogene* 2000;19:6102–14.
 35. Slamon DJ, Clark GM, Wong SG, Levin WJ, Ullrich A, McGuire WL. Human breast cancer: correlation of relapse and survival with amplification of the HER-2/neu oncogene. *Science* 1987;235:177–82.
 36. Romond EH, Perez EA, Bryant J, et al. Trastuzumab plus adjuvant chemotherapy for operable HER2-positive breast cancer. *N Engl J Med* 2005;353:1673–84.
 37. Piccart-Gebhart MJ, Procter M, Leyland-Jones B, et al. Trastuzumab after adjuvant chemotherapy in HER2-positive breast cancer. *N Engl J Med* 2005;353:1659–72.
 38. Gauthier S, Caron B, Cloutier J, et al. (S)-(+)-4-[7-(2,2-Dimethyl-1-oxopropoxy)-4-methyl-2-[4-2-(1-piperidinyl)-ethoxy]phenyl]-2H-1-benzopyran-3-yl]-phenyl 2,2-dimethylpropanoate (EM-800): a highly potent, specific, and orally active nonsteroidal antiestrogen. *J Med Chem* 1997;40:2117–22.
 39. Palkowitz AD, Glasebrook AL, Thrasher KJ, et al. Discovery and synthesis of [6-hydroxy-3-[4-2-(1-piperidinyl)ethoxy]phenoxy]-2-(4-hydroxyphenyl)]-benzo[b]thiophene: a novel, highly potent, selective estrogen receptor modulator. *J Med Chem* 1997;40:1407–16.
 40. Guy CT, Webster MA, Schaller M, Parsons TJ, Cardiff RD, Muller WJ. Expression of the neu protooncogene in the mammary epithelium of transgenic mice induces metastatic disease. *Proc Natl Acad Sci U S A* 1992;89:10578–82.
 41. Moon RC, Thompson HJ, Becci PJ, et al. N-(4-Hydroxyphenyl)retinamide, a new retinoid for prevention of breast cancer in the rat. *Cancer Res* 1979;39:1339–46.
 42. Bischoff ED, Heyman RA, Lamph WW. Effect of the retinoid X receptor-selective ligand LGD1069 on mammary carcinoma after tamoxifen failure. *J Natl Cancer Inst* 1999;91:2118.
 43. Yen WC, Lamph WW. The selective retinoid X receptor agonist bexarotene (LGD1069, Targretin) prevents and overcomes multidrug resistance in advanced breast carcinoma. *Mol Cancer Ther* 2005;4:824–34.
 44. Wu K, Dupre E, Kim H, et al. Receptor-selective retinoids inhibit the growth of normal and malignant breast cells by inducing G1 cell cycle blockade. *Breast Cancer Res Treat* 2006;96:147–57.
 45. Mangelsdorf DJ, Evans RM. The RXR heterodimers and orphan receptors. *Cell* 1995;83:841–50.
 46. Shulman AI, Mangelsdorf DJ. Retinoid X receptor heterodimers in the metabolic syndrome. *N Engl J Med* 2005;353:604–15.
 47. Menard S, Aiello P, Tagliabue E, et al. Tamoxifen chemoprevention of a hormone-independent tumor in the proto-neu transgenic mice model. *Cancer Res* 2000;60:273–5.
 48. Knabbe C, Lippman ME, Wakefield LM, et al. Evidence that transforming growth factor- β is a hormonally regulated negative growth factor in human breast cancer cells. *Cell* 1987;48:417–28.
 49. Liang Y, Hou M, Kallab AM, Barrett JT, El Etreby F, Schoenlein PV. Induction of antiproliferation and apoptosis in estrogen receptor negative MDA-231 human breast cancer cells by mifepristone and 4-hydroxytamoxifen combination therapy: a role for TGF β 1. *Int J Oncol* 2003;23:369–80.
 50. Butta A, MacLennan K, Flanders KC, et al. Induction of transforming growth factor β 1 in human breast cancer *in vivo* following tamoxifen treatment. *Cancer Res* 1992;52:4261–4.
 51. de Caestecker MP, Piek E, Roberts AB. Role of transforming growth factor- β signaling in cancer. *J Natl Cancer Inst* 2000;92:1388–402.
 52. Derynck R, Akhurst RJ, Balmain A. TGF- β signaling in tumor suppression and cancer progression. *Nat Genet* 2001;29:117–29.
 53. Muraoka-Cook RS, Dumont N, Arteaga CL. Dual role of transforming growth factor β in mammary tumorigenesis and metastatic progression. *Clin Cancer Res* 2005;11:937–43s.
 54. Tang B, Vu M, Booker T, et al. TGF- β switches from tumor suppressor to prometastatic factor in a model of breast cancer progression. *J Clin Invest* 2003;112:1116–24.
 55. Barrios-Rodiles M, Brown KR, Ozdamar B, et al. High-throughput mapping of a dynamic signaling network in mammalian cells. *Science* 2005;307:1621–5.
 56. Barcellos-Hoff MH, Medina D. New highlights on stroma-epithelial interactions in breast cancer. *Breast Cancer Res* 2005;7:33–6.
 57. Janes KA, Albeck JG, Gaudet S, Sorger PK, Lauffenburger DA, Yaffe MB. A systems model of signaling identifies a molecular basis set for cytokine-induced apoptosis. *Science* 2005;310:1646–53.
 58. Janes KA, Albeck JG, Peng LX, Sorger PK, Lauffenburger DA, Yaffe MB. A high-throughput quantitative multiplex kinase assay for monitoring information flow in signaling networks: application to sepsis-apoptosis. *Mol Cell Proteomics* 2003;2:463–73.
 59. Yen WC, Prudente RY, Corpuz MR, Negro-Vilar A, Lamph WW. A selective retinoid X receptor agonist bexarotene (LGD1069, targretin) inhibits angiogenesis and metastasis in solid tumours. *Br J Cancer* 2006;94:654–60.