Use of Biomarker Modulation in Normal Mammary Epithelium as a Correlate for Efficacy of Chemopreventive Agents Against Chemically Induced Cancers

Ronald A. Lubet[1], Brandy M. Heckman-Stoddard[2], Jennifer T. Fox[1], Fariba Moeinpour[3], M. Margaret Juliana[3], Robert H. Shoemaker[1], and Clinton J. Grubbs[3]

ABSTRACT

In both estrogen receptor/progesterone receptor–positive (ER^+/PR^+) human breast cancer and in ER^+/PR^+ cancers in the methylnitrosourea (MNU)-induced rat model, short-term modulation of proliferation in early cancers predicts preventive/therapeutic efficacy. We determined the effects of known effective/ineffective chemopreventive agents on proliferative index (PI) in both rat mammary epithelium and small cancers. Female Sprague-Dawley rats were treated with MNU at 50 days of age. Five days later, the rats were treated with the individual compounds for a period of 14 days. At that time, normal mammary tissue from the inguinal gland area was surgically removed. After removal, the rats remained on the agents for an additional 5 months. This cancer prevention study confirmed our prior results of striking efficacy with tamoxifen, vorozole, Targretin, and gefitinib, and no efficacy with metformin, naproxen, and Lipitor. Employing a separate group of rats, the effects of short-term (7 days) drug exposure on small palpable cancers were examined. The PI in both small mammary cancers and in normal epithelium from control rats was >12%. In agreement with the cancer multiplicity data, tamoxifen, vorozole, gefitinib, and Targretin all strongly inhibited proliferation (>65%; $P < 0.025$) in the normal mammary epithelium. The ineffective agents metformin, naproxen, and Lipitor minimally affected PI. In the small cancers, tamoxifen, vorozole, and Targretin all reduced the PI, while metformin and Lipitor failed to do so. Thus, short-term changes in the PI in either normal mammary epithelium or small cancers correlated with long-term preventive efficacy in the MNU-induced rat model.

Introduction

The primary use of animal models in the field of chemoprevention is to screen for potential agents that may be useful clinically. However, animal models can also be used to examine surrogate biomarkers that correlate with efficacy. Modulation of surrogate biomarkers is used in early phase prevention studies as a marker of efficacy. In breast cancer treatment and prevention, these markers are often measured using a presurgical or neoadjuvant study, in which women diagnosed with early stage cancer or preneoplastic lesions are treated for a limited length of time with an agent prior to initial surgery (1). The most common biomarkers employed have been proliferation-related biomarkers such as Ki67 or proliferating cell nuclear antigen. Two classes of agents that have proven highly effective as preventive agents in phase III clinical prevention trials, selective estrogen receptor modulators (SERM) and aromatase inhibitors (2, 3), have been shown to significantly reduce Ki67 in presurgical studies as well (4). Approximately 10 years ago, we confirmed that there was a strong correlation between the chemopreventive efficacy of a variety of agents and the agent's ability to inhibit proliferation in small palpable mammary cancers following short-term treatment in the methylnitrosourea (MNU)-induced rat model (5). One alternative approach to examining proliferative changes in tumors would be to test the effects of agents on normal mammary epithelium. Although this approach has certain technical challenges when performed in humans because Ki67 can be low in normal breast epithelium, it has been used in women at high risk of developing breast cancer (6), and has yielded positive results with both SERMs and aromatase inhibitors (7, 8).

Rat models of breast cancer in which cancers are induced by the carcinogens dimethylbenzanthracene or MNU have been employed for many decades (9). The MNU-induced rat model of breast cancer induces estrogen receptor–positive (ER^+) cancers that are similar by array analyses to highly differentiated ER^+ human breast cancers (10). In addition, these

[1]Chemopreventive Agent Development Research Group, Division of Cancer Prevention, NCI, Rockville, Maryland. [2]Breast and Gynecologic Cancer Research Group, Division of Cancer Prevention, NCI, Rockville, Maryland. [3]Department of Surgery, University of Alabama at Birmingham, Birmingham, Alabama.

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Corresponding Author: Ronald A. Lubet, NCI, 9609 Medical Center Drive, MSC 9787, Rockville, MD 20850. Phone: 240-276-5997; Fax: 301-402-0553; E-Mail: ronald.lubet@nih.gov

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tumors respond both in a preventive setting and in a therapeutic setting to hormonal treatments that modulate human ER^+ cancers, including SERMs, aromatase inhibitors, and ovariectomy (11, 12). Since we had previously identified a wide variety of highly effective and ineffective cancer preventive agents in the MNU model, we examined the correlation between short-term proliferative effects on normal mammary epithelium and long-term chemopreventive efficacy. This study was facilitated by the fact that the inguinal mammary tissue adjacent to the linea alba in young rats has a high concentration of terminal end buds and ducts, and that the proliferative rates in these tissues are quite high. Because of these characteristics, we were able to determine the proliferative index (PI) in normal epithelium after 14 days of treatment with the various agents. In brief, we tested a variety of compounds that were known to be effective or ineffective preventive agents based on our prior published data (12–15). Here we sought to correlate the short-term change in proliferation in "high risk" mammary epithelium with the short-term change seen in mammary tumors and the effect of the agent on tumor incidence and multiplicity.

Materials and Methods

Chemicals and animals

MNU was obtained from the NCI Chemical Carcinogen Repository. Teklad diet and female Sprague-Dawley rats were obtained from Envigo, Inc. Gefitinib, Targretin, vorozole, metformin, and Lipitor were supplied by the NCI Cancer Prevention Repository. Naproxen and tamoxifen were purchased from Sigma Chemical Co. Gefitinib [10 mg/kg body weight (BW)], vorozole (1.25 mg/kg BW), and metformin (150 mg/kg BW) were administered by gavage (0.5 mL/gavage) on a daily basis. The vehicle for vorozole and gefitinib was ethanol: polyethylene glycol 400 (10:90; v/v), while metformin was administered in saline. Tamoxifen (3.3 mg/kg of diet), Targretin (150 mg/kg of diet), naproxen (400 mg/kg of diet), and Lipitor (150 mg/kg of diet) were administered in the diet. The agents were incorporated into the feed (Teklad, 4% fat) using a Patterson-Kelly blender with intensifier bar. Fresh diet was provided to the rats 3×/week.

Prevention studies with various agents

All animal experiments were conducted in Association for Assessment and Accreditation of Laboratory Animal Care International–approved facilities following procedures approved by the Institutional Animal Care and Use Committee at the University of Alabama at Birmingham (Birmingham, AL). Treatment of the female Sprague-Dawley rats was as described previously (12, 14). In brief, rats were injected intravenously with MNU (75 mg/kg BW) via the jugular vein at 50 days of age. Treatment of rats with the various agents by gavage or in the diet was initiated 5 days after MNU administration (or at 55 days of age). The number of rats/group was 20. We had previously determined effective daily doses for the various agents (12–15). These doses were all less than or equal to the daily human equivalent dose (HED) based on FDA scaling, with the exception of vorozole and Lipitor, which were slightly higher doses. Two weeks after the initiation of treatment with the various agents, mammary tissue was obtained from the inguinal mammary glands as described below. Treatment with the agents continued for approximately 5 months. The rats were palpated twice a week for the development of mammary tumors. At the end of the study, tumors were weighed and submitted for histologic evaluation. In all studies, rats were weighed 1×/week. The BWs of the control and treated rats did not differ by more than 5% in any of the long-term prevention studies.

To determine proliferative effects in normal epithelium, the rats in the prevention study underwent surgery 14 days after treatment initiation to remove mammary tissue from the inguinal glands. Survival surgery was performed in which rats were anesthetized with isoflurane. Mammary tissue was excised from an area in the inguinal glands (adjacent to the linea alba), which has a high concentration of epithelial cells. Tissues were fixed in 10% formalin for 24 hours and then transferred to 70% ethanol at room temperature. Tissues were then embedded in paraffin blocks and sections cut (4 μm thick).

To determine proliferative effects in small cancers, a separate group of rats (5/group) received MNU at 50 days of age and were palpated 2×/week for the appearance of mammary cancers. When an animal developed a cancer of approximately 100–200 mm^2, the rat received the indicated agent at the dose specified above for 7 days. One day after the last treatment with the agents, the animals were sacrificed, and the mammary cancers were excised. The harvested tumors were fixed in 10% formalin for 24 hours at room temperature and were then transferred to 70% ethanol until histologically processed. Tissues were then embedded in paraffin blocks and sections cut (4 μm thick).

IHC

The mammary gland and tumor tissues from 10 and 5 rats/group, respectively, were deparaffinized with xylene and placed in ethanol. Antigen retrieval employed boiling in sodium citrate (pH, 6.0) for 20 minutes. Slides were then covered with peroxidase block for 3 hours and washed with tris buffer. The tissues were incubated with Ki67 primary antibody (Abcam) for 1 hour at room temperature. Processing and staining of tissue were performed according to the manufacturer's procedures (DAKA Envision + Kits). Tissues were then washed and dehydrated in ethanol and xylene. The images were captured and counted using the Aperio Scan Scope Imaging System (Aperio Imaging). For counting the cells, each area containing mammary ductal epithelial cells was randomly analyzed (stained cells/total cells counted) by a program within Scan Scope. Approximately 15 areas/slide were analyzed to ensure that a minimum of 1,000 cells/tissue were counted.

Statistical analysis

Final mammary cancer multiplicities and weights were compared statistically using a nonparametric Mann–Whitney rank analysis test because the data does not follow a "normal" curve. PIs are presented as mean ± SE and were compared statistically using one-way ANOVA. Correlation coefficients were calculated using Microsoft Excel. $P < 0.05$ was used to determine a statistically significant difference.

Results

Chemopreventive effects of various agents

We had previously identified a variety of agents that were either highly effective (tamoxifen, vorozole, Targretin, and gefitinib) or ineffective (metformin, Lipitor, and naproxen) in long-term prevention studies in the MNU rat model of mammary cancer (refs. 12–16; **Table 1**). We had also previously shown that tamoxifen, vorozole, and Targretin reduced both cancer multiplicity and PI, and increased apoptotic index in a dose-dependent manner in a short-term study in the same animal model (ref. 5; Supplementary Table S1). To determine whether there was a correlation between the long-term efficacy of these agents and their short-term effects on cellular proliferation in normal epithelium, we repeated these chemoprevention studies in the MNU rat model, taking biopsies of normal mammary tissue from the inguinal gland 14 days after initiating treatment with the agents. After removal of mammary tissue from the inguinal gland, drug treatment of rats continued for another 5 months. Consistent with our previous findings, tamoxifen, vorozole, gefitinib, and Targretin were profoundly effective in reducing cancer development and final tumor weight; and naproxen and metformin both significantly increased cancer multiplicity and final tumor weights. Lipitor had no statistically significant effect (**Fig. 1A** and **B**; **Table 2**; Supplementary Table S2).

Effects of preventive agents on the PI in normal mammary epithelium and correlation with long-term preventive effects

To correlate these efficacy data with the PI in the normal mammary epithelium, we stained the biopsied tissue samples collected 14 days after treatment initiation for Ki67 expression (**Fig. 2A**). A minimum of 1,000 cells/tissue were counted using the Scan-scope instrument described above. The normal epithelium from the mammary gland of control rats had a relatively high number of proliferating cells that approached the values observed in cancers. This PI was significantly ($P < 0.05$) reduced (ANOVA) by all of the agents that showed chemopreventive efficacy; vorozole and Targretin both reduced the PI in normal epithelium by >80%, while tamoxifen and gefitinib reduced the PI by approximately 75% and 60%, respectively (**Fig. 2B**). The two agents that did not demonstrate long-term chemopreventive efficacy (naproxen and metformin) marginally altered the PI in normal epithelium, respectively (**Fig. 2B**); neither effect was statistically significant. Lipitor, which reduced tumor multiplicity by 17%, reduced the PI by approximately 35%, although this reduction failed to achieve statistical significance ($P > 0.05$; **Fig. 2B**). The correlation between the effects of short-term treatment on the proliferation of normal mammary epithelium and the long-term effects on cancer multiplicity was strong, with a Pearson correlation coefficient (R) of 0.87 and a coefficient of determination (R^2) of 0.76 ($P < 0.01$; **Fig. 2C**).

Effects of preventive agents on the PI following the short-term treatment of small cancers and correlation with long-term preventive effects

To correlate the long-term efficacy data with the PI in mammary cancers, we injected a separate group of rats with MNU and let individual animals develop small palpable

Table 1. Description of the agents used in these studies.

Agent	Description	Chemopreventive activity in prior mammary cancer studies (12–15)
Tamoxifen	SERM that binds ER alpha and inhibits the stimulatory effects of estrogen in ER^+ breast cancers. Has both therapeutic and preventive activity clinically. The dose employed in these studies is at the HED.	Yes
Vorozole	Small-molecule competitive inhibitor of aromatase (CYP 27) that is similar to clinically employed letrozole and anastrozole. Aromatase inhibitors (letrozole and exemustane) are used clinically in therapy and are effective in clinical prevention studies.	Yes
Targretin	Pure RXR agonist employed clinically in the treatment of CTCL. The dose used in these studies is less than the HED for CTCL.	Yes
Gefitinib	Small-molecule competitive inhibitor of EGFR1. Effective in human ER^+ tumors and effective in MNU model at roughly half of the HED.	Yes
Metformin	Used to control blood sugar levels in type 2 diabetics. Its mechanism of action is unclear. The dose employed in these studies is approximately equivalent to the commonly employed human dose of 1,500 mg.	No
Lipitor	Competitive HMG CoA reductase inhibitor used to lower cholesterol levels. The dose employed in these studies is approximately 2–4× higher than that typically used in humans.	No
Naproxen	NSAID that competitively inhibits COX 1/2. It is a highly effective chemopreventive agent in colon and bladder cancer models. The dose employed in these studies is approximately equivalent to 350 mg in humans.	No

Abbreviation: CTCL, cutaneous T-cell lymphoma.

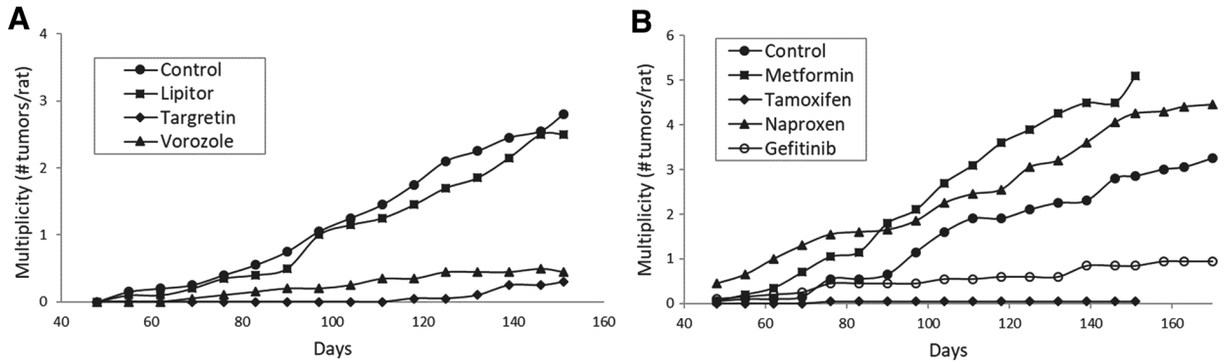

Figure 1.

Effect of various agents on the appearance of palpable mammary cancers in MNU-treated rats. **A,** Five days after MNU injection, rats (20/group) were treated daily with vorozole (1.25 mg/kg BW by gavage), Targretin (150 mg/kg of diet), or Lipitor (150 mg/kg of diet). **B,** In a separate study, the rats received naproxen (400 mg/kg of diet), gefitinib (10 mg/kg BW by gavage), tamoxifen (3.3 mg/kg of diet), or metformin (150 mg/kg BW by gavage). The rats were palpated twice a week for the development of mammary cancers.

mammary cancers before treating them with the various agents for 7 days. The cancers were then collected from these animals and stained for Ki67 expression (**Fig. 3A**). As observed in the normal epithelium, the effective chemopreventive agents (vorozole, tamoxifen, and Targretin) significantly reduced proliferation in the tumors by approximately 70%–80% ($P < 0.05$; **Fig. 3B**). Consistent with their failure to reduce cancer multiplicity, neither Lipitor nor metformin significantly altered the PI (**Fig. 3B**). Again, the correlation between the effects of short-term treatment on the proliferation of small palpable cancers and the long-term effects on cancer multiplicity was strong, with an R value of 0.97 and an R^2 value of 0.95 ($P < 0.01$; **Fig. 3C**).

Discussion

One of the primary objectives of preclinical models is to identify surrogate endpoints that can be used in early phase prevention trials that are predictive of cancer preventive efficacy in phase III trials. Phase II trials have often employed Ki67 as the biomarker of interest. In the area of breast cancer, the two classes of agents that have proven effective in large phase III prevention clinical trials are the SERMs (tamoxifen and raloxifene) and the aromatase inhibitors (letrozole and exemestane; refs. 2, 3). Both classes of agents have been shown to significantly reduce Ki67 in normal mammary epithelium in humans (7, 8), and have proven to be highly effective in reducing the proliferation of breast cancer cells in a presurgical trials (4). This efficacy in early tumors is not surprising because these classes of agents are clinically effective therapies in ER^+/PR^+ breast cancer. Here, we sought to investigate the correlation between long-term chemopreventive efficacy and short-term PI in normal mammary epithelium and palpable cancers in the MNU-induced rat model using a series of agents that are known to be effective or ineffective in this model. The question arises whether one might use another biomarker either in addition to or instead of Ki67. In fact, in a recent study with the aromatase inhibitor vorozole, we employed a variety of proliferation-related proteins as potential biomarkers in both normal epithelia and in tumor lesions (17). The reason we have not employed any of these in the current studies is that they are not standardized or virtually ever employed clinically. Thus, there is no data to tell us whether comparable results can be obtained in the human. In contrast, for Ki67, there is significant human data available for comparison.

Table 2. Effect of various agents on final mammary cancer multiplicity and weight.

Agent	Multiplicity (# of cancers/rat)[a]	Cancer weight (g)
Control	2.58	4.58
Vorozole	0.25 (90% decrease, $P = 1e^{-5}$)	0.36 (92% decrease, $P = 6e^{-5}$)
Targretin	0.20 (92% decrease, $P = 6e^{-6}$)	0.30 (93% decrease, $P = 2e^{-5}$)
Lipitor	2.15 (17% decrease, $P = 0.6$)	5.11 (12% increase, $P = 0.7$)
Control	2.90	6.79
Naproxen	4.20 (45% increase, $P = 0.02$)	10.49 (54% increase, $P = 0.05$)
Gefitinib	0.20 (93% decrease, $P = 2e^{-6}$)	0.14 (98% decrease, $P = 1e^{-6}$)
Tamoxifen	0.00 (100% decrease, $P = 1e^{-9}$)	0.00 (100% decrease, $P = 1e^{-7}$)
Metformin	4.95 (71% increase, $P = 0.01$)	9.71 (43% increase, $P = 0.33$)

[a]On the basis of histopathologic analysis following animal sacrifice; P values were calculated using the Mann–Whitney test.

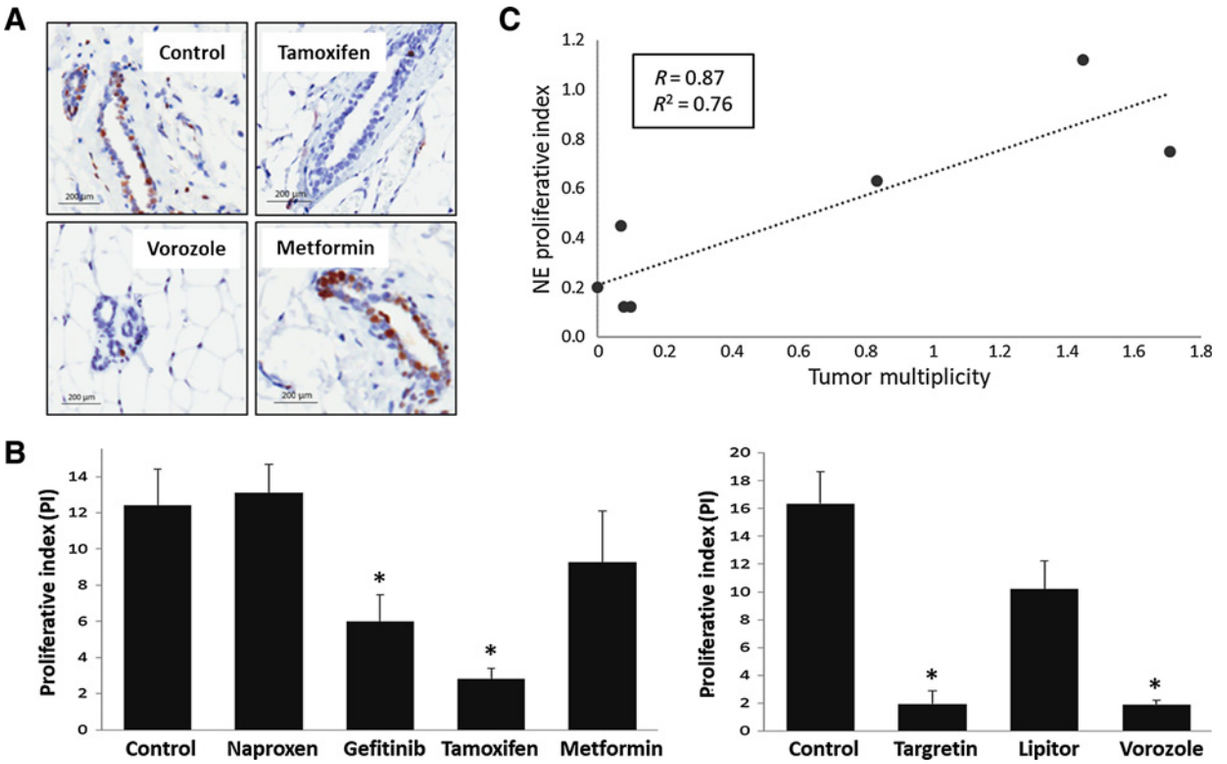

Figure 2.

Effect of various agents on the PI of normal mammary epithelium from MNU-treated rats. Fourteen days after the initiation of treatment with the agents, mammary tissue was removed from the inguinal mammary glands of the MNU-treated rats in **Fig. 1**, and stained for epithelial cell proliferation. **A,** Representative images of Ki67 staining are shown. **B,** The PI was determined by calculating the percentage of Ki67-positive cells; a minimum of 1,000 cells/tissue were counted. The data represent the average from 10 rats/group ± SE. The * indicates statistical significance ($P < 0.05$) as determined by ANOVA. **C,** The average PIs in the normal mammary epithelium (NE) were normalized to their respective controls (average PI of control = 1.0) and were plotted as a function of the normalized average cancer multiplicities (average multiplicity of control = 1.0) reported in **Table 2**. The Pearson correlation coefficient (R) and coefficient of determination (R^2) are shown on the graph.

In agreement with prior studies from our laboratory and others (11, 12), and consistent with the clinical trials discussed above, we demonstrated that tamoxifen and vorozole are highly effective in reducing mammary cancer multiplicity and weight in the ER^+ model. It was found that short-term treatment with these agents also reduced proliferation in the inguinal mammary gland as well as in small palpable cancers. Because the normal mammary epithelium and cancers of this model are both ER^+, it is not surprising that a SERM (tamoxifen) and an aromatase inhibitor (vorozole) were highly effective in reducing the PI in both.

In addition to testing tamoxifen and vorozole, we confirmed our previous results (13, 14) demonstrating that gefitinib and Targretin are highly effective as preventive agents in the MNU model. Chemopreventive activity correlated well with the PI in both the normal mammary epithelium and in cancers, despite the fact that neither gefitinib (an EGFR inhibitor) nor Targretin (an RXR agonist) directly target the hormonal axis. Interestingly, two clinical studies have shown the efficacy of EGFR inhibitors against ER^+/PR^+ breast cancers in women by employing either proliferation (18, 19) or therapeutic efficacy in a neoadjuvant setting (20). In prior studies, we have also shown that one can achieve a dose-dependent response to with either vorozole (12) or wide variety of other agents (5), when examining proliferation in lesions. We summarize some of this data in the Supplementary Table S1. In contrast to tamoxifen, vorozole, gefitinib, and Targretin, the agents naproxen, metformin, and Lipitor showed no significant chemopreventive effects in the long-term cancer study, and likewise did not significantly reduce the PI of normal mammary epithelium or small cancers in the short-term biomarker study. In fact, naproxen and metformin both increased tumor multiplicity. However, we have routinely not given emphasis to the tumor-promoting effects of agents in the MNU model because the studies were performed in carcinogen-treated Sprague-Dawley rats, whereas carcinogenesis assays performed by the National Toxicology Program use rodents that have not previously been exposed to a carcinogen.

The primary objective of this article was to examine the relationship between short-term effects on Ki67 in the normal epithelium and small palpable tumors with long-term chemopreventive activity in the MNU breast cancer model.

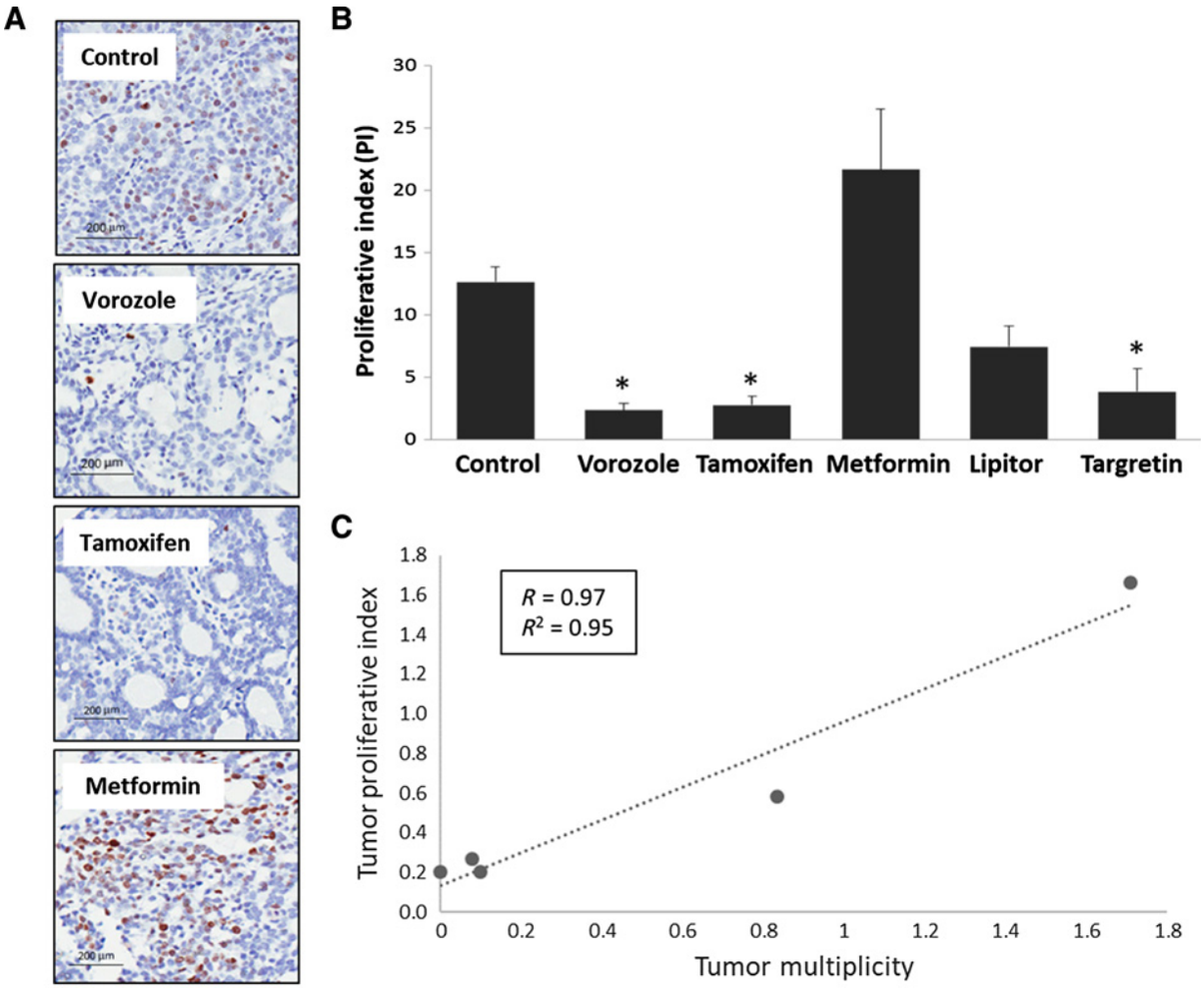

Figure 3.

Effect of various agents on the PI in small palpable cancers. A separate group of MNU-treated rats (5/group) were allowed to develop mammary cancers. When an animal developed a cancer of approximately 100–200 mm^2, the rat was treated with vorozole (1.25 mg/kg BW by gavage), Targretin (150 mg/kg of diet), Lipitor (150 mg/kg of diet), tamoxifen (3.3 mg/kg of diet), or metformin (150 mg/kg BW by gavage) for 7 days. Upon termination of the experiment, the cancers were removed and stained for Ki67. **A,** Representative images of Ki67 staining are shown. **B,** The PI was determined by calculating the percentage of Ki67-positive cells; a minimum of 1,000 cells/tissue were counted. The data represent the average from 5 rats/group ± SE. The * indicates statistical significance ($P < 0.05$) as determined by ANOVA. **C,** The average PIs in the cancers were normalized to the control (average PI of control = 1.0) and were plotted as a function of the normalized average cancer multiplicities (average multiplicity of control = 1.0) reported in **Table 2**. The Pearson correlation coefficient (R) and coefficient of determination (R^2) are shown on the graph.

However, there is at least some basis for comparing the Ki67 results in rats and humans. Examining Ki67 in lesions, we observed an approximately 70%–80% reduction in proliferation in both the normal rat epithelium and in tumors following treatment with either tamoxifen or an aromatase inhibitor. These results parallel those observed following the similar treatment of ER^+/PR^+ human tumors in a neoadjuvant setting (4) and in two studies with normal epithelium and fine-needle aspirates (6, 7). Furthermore, the EGFR inhibitor erlotinib showed striking inhibition of proliferation in ER^+/PR^+ tumors (18), which is similar to our current data in normal rat epithelium and parallels our prior data in rat tumors (5, 14). Whereas our own Ki67 data with metformin in either tumors or normal epithelium was negative corresponding to our prevention results, Ki67 data in humans have been more mixed. Four trials have examined the short-term effects (1–4 weeks) of various doses of metformin on cell proliferation (as assessed by the expression of Ki67) in tissues from women awaiting surgery for breast cancer (presurgical trials; refs. 21–24). In the largest randomized, double-blind, placebo-controlled study of the effect of metformin on Ki67 in breast cancer, the change in Ki67 between diagnostic biopsy and surgical specimen was not significant relative to placebo (21). However, women with higher insulin resistance (HOMA-IR > 2.8) had a nonsignificant 10.5% decrease in Ki67. While a recently completed study in a

majority Hispanic population with a historic control group matched for age, body mass index, and stage showed no reduction in Ki67 in the metformin arm or in the untreated control group (24), two single-arm trials (metformin baseline vs. presurgery; refs. 22, 23) resulted in limited, but statistically significant, decreases in proliferation (<10%). Thus, none of the studies yielded the striking inhibitions achieved by tamoxifen, anastrozole, or erlotinib in humans (4, 7, 8, 18).

The results presented in this article confirm the reproducibility of our data with respect to both effective and ineffective cancer chemopreventive agents. More importantly, they show that there is a strong correlation between a reduced PI in normal epithelium in the mammary gland or in palpable lesions and long-term cancer preventive activity with a variety of agents. This supports the use of this biomarker in prevention trials. However, when considering the translation of our data with normal epithelium to human trials, there are several points that should be noted. First, the mammary gland biopsied from a 70-day-old rat has a high concentration of terminal end buds, resulting in a high concentration (~15%) of normal epithelial cells in the gland. This is much higher than the concentration of normal epithelial cells observed in mammary glands of a mature rat, and (more importantly) much higher than that in adult humans where epithelial cells may represent only $\leq$3% of the cells obtained following a fine-needle aspirate of a normal gland. Second, because 70-day-old rats still have developing mammary glands, approximately 12%–16% of epithelial cells in the inguinal mammary gland are proliferating based on Ki67 staining. Thus, the PI in the normal mammary epithelium of these rats is almost as high as that in the mammary cancers produced in this model, and is approximately equal to the PI in human $ER^+/PR^+/Neu^-$ breast cancers (4). In adult women, the mammary gland has both a low incidence of epithelial cells and a low PI. As a result, most of the measurements of proliferation in humans are determined in lesions (hyperplasia, ductal carcinoma *in situ*, or early tumors). The final limitation of using normal epithelium involves loss of specificity of agents. For example, SERMs and aromatase inhibitors are generally effective with tumors that are $ER^+/PR^+/Neu^-$, but they are much less effective against $ER^+/PR^+/Neu^+$ cancers (4). We have employed small tumors in the MNU model to examine the efficacy of the farnesyl transferase inhibitor tipifarnib (25, 26) and found that lesions with an HRAS mutation are more susceptible than tumors without these mutations. This specific approach involving sequencing of mutated genes or array analysis can only be performed in clear lesions, not in normal epithelium. However, despite these challenging hurdles, this study showed it is possible to biopsy animals to determine the correlation between surrogate biomarkers and tumor endpoints. Ki67, which is used in many phase II studies, is a reasonable surrogate marker based on these studies.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Authors' Contributions

Conception and design: R.A. Lubet, B.M. Heckman-Stoddard, R.H. Shoemaker, C.J. Grubbs
Development of methodology: B.M. Heckman-Stoddard, C.J. Grubbs
Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): B.M. Heckman-Stoddard, C.J. Grubbs
Analysis and interpretation of data (e.g., statistical analysis, biostatistics, computational analysis): R.A. Lubet, B.M. Heckman-Stoddard, F. Moeinpour, M.M. Juliana R.H. Shoemaker, C.J. Grubbs
Writing, review, and/or revision of the manuscript: R.A. Lubet, B.M. Heckman-Stoddard, J.T. Fox, R.H. Shoemaker
Administrative, technical, or material support (i.e., reporting or organizing data, constructing databases): F. Moeinpour
Study supervision: B.M. Heckman-Stoddard, C.J. Grubbs

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