Phase II Clinical Trial of *N*-(4-Hydroxyphenyl)retinamide and Tamoxifen Administration before Definitive Surgery for Breast Neoplasia[1]

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ABSTRACT

Purpose: **Surrogate end point biomarkers (SEBs) that can be measured in ductal carcinoma *in situ* or early-stage invasive cancer are needed to improve the efficiency and reduce the cost of chemoprevention trials.**

Experimental Design: **We conducted a prospective study to develop SEBs for tamoxifen and *N*-[4-hydroxyphenyl]retinamide by administering either a placebo or both drugs for 2–4 weeks to women with ductal carcinoma *in situ* or early invasive cancers in the interval between the initial diagnostic core biopsy and definitive surgery. The major statistical end point of the study was pre- *versus* posttreatment change in cell proliferation, as measured by changes in Ki67 labeling indices. In addition, estrogen receptor (ER), HER2/*neu*, p53, retinoid receptors, and DNA index were measured.**

Results: **Between February 1997 and April 200, 52 patients were registered on the study, and 36 (20 in the placebo arm and 16 in the treatment arm) were available for analysis. No statistically significant pre- *versus* posttreatment differences in Ki67 labeling index or in the other markers were observed in the treatment arm compared with the placebo arm. There was a trend toward increased treatment response in ER-positive *versus* ER-negative patients, but this could not be rigorously analyzed because of the low sample size and the unequal distribution of ER-positive patients in the two study arms.**

Conclusion: **Future SEB trials for breast carcinoma must (*a*) incorporate information about patient hormonal status into the study design and (*b*) resolve problems in patient accrual.**

INTRODUCTION

Because up to 25% of women affected with breast cancer still die of their disease, increasing attention has turned to the potential of chemoprevention for decreasing breast cancer incidence. Chemoprevention refers to the administration of drugs to block or reverse carcinogenesis before the development of invasive cancer.

The possibility of chemoprevention in breast cancer was raised by the results of randomized trials of tamoxifen in women with clinically detectable breast cancers. These studies showed a benefit from tamoxifen in reducing tumor recurrence, prolonging survival, and reducing the incidence of contralateral breast cancer, as compared with placebo (1, 2). In the National Surgical Adjuvant Breast and Bowel Project P-1 trial, these results were extended to 13,388 women at increased risk for breast cancer, who received either tamoxifen or placebo daily for 5 years. Tamoxifen reduced the risk of invasive breast cancer by 49% and the risk of noninvasive breast cancer by 50%, compared with placebo (3). At present, tamoxifen is the only Food and Drug Administration-approved drug for risk reduction in women considered to be at increased risk for developing breast cancer.

Another promising drug for the chemoprevention of breast cancer is 4-HPR.[3] This synthetic retinoid has been shown to be more effective and less toxic than other retinoids for chemoprevention of mammary cancer in animals (4). An important characteristic of 4-HPR is its tendency to concentrate in the glandular and fat tissue of the breast instead of in the liver (5). Evidence suggests that 4-HPR is a highly selective activator of retinoid receptors (6) and that this may contribute to its reduced toxicity compared with other retinoids. 4-HPR was the subject of a recent 5-year clinical trial conducted to assess its usefulness in preventing second breast malignancies in women treated for early breast cancer (7). In that study, there was no significant effect seen in the primary end point, the incidence of contralateral or ipsilateral breast cancer 7 years after randomization.

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[3] The abbreviations used are: 4-HPR, *N*-(4-hydroxyphenyl)retinamide; SEB, surrogate end point biomarker; DCIS, ductal carcinoma *in situ*; ER, estrogen receptor; 4-MPR, *N*-(4-methoxyphenyl)retinamide.

However, on *post hoc* analysis, a possible beneficial effect was seen in premenopausal women, with an opposite effect observed in postmenopausal women.

The combined administration of tamoxifen and 4-HPR has been shown to be additive or synergistic in both the growth inhibition of the breast cancer cell line MCF-7 (8) and the prevention of *N*-methyl-*N*-nitrosourea-induced mammary carcinoma in the rat (9, 10). In human studies, the combination of tamoxifen and 4-HPR has been shown to be safe and well tolerated (11, 12).

Chemoprevention trials that use breast cancer incidence as an end point require large sample sizes and long durations of study. The National Cancer Institute has identified many potential chemopreventive agents for clinical testing (13); thus it is important to develop new testing strategies. Neoplastic transformation is accompanied by a number of measurable changes at both the cellular and molecular level, many of which occur long before the appearance of a clinically detectable malignancy. An effective chemopreventive agent should influence these changes through alterations in the expression of specific markers and/or general attributes of malignant transformation. Thus, the measurement of these markers/attributes during the course of a prevention trial may identify SEBs that could provide preliminary evidence of treatment efficacy at a relatively low cost.

The main objective of this trial was to determine whether treatment with tamoxifen and 4-HPR for 2–4 weeks in the interval between diagnostic core biopsy and definitive surgery would cause a significant change in the proliferation marker Ki67 in women with DCIS or early-stage breast cancer. We report here the results obtained after the recruitment of 52 subjects.

MATERIALS AND METHODS

This protocol was approved by the Institutional Review Board of the University of Texas M. D. Anderson Cancer Center.

Patient Selection. From February 1997 to April 2000, we screened 4670 women with a mammogram highly suspicious for DCIS or T_1 or T_2 invasive carcinoma for this study. The minimum criteria for inclusion in the study were as follows: no definitive local therapy; age >35 years (women of child-bearing potential not practicing effective contraception were excluded); Zubrod performance status no greater than 2; absolute granulocyte count >1500/mm^3; platelets >100,000/mm^3; total serum bilirubin <1.5 mg/100 ml; serum creatinine <1.5 mg/100 ml; and fasting serum triglycerides <400 mg/100 ml. Subjects were excluded for any chemotherapy in the preceding 5 years, prior radiation therapy to the chest/breast, vitamin A supplementation, retinoid or tamoxifen therapy in the preceding 12 months, inability to obtain adequate core biopsy, acute intercurrent illness and/or infection that would interfere with administration of proposed chemopreventive agents, or history of thromboembolic disease or degenerative retinal disease. In addition, at the start of the study subjects were excluded for receiving outside needle biopsies, tumor size >2 cm, or estrogen replacement therapy. The protocol was amended 6 months into the study to allow inclusion of patients meeting these last criteria.

Treatment Protocol. Women with an abnormal mammogram or diagnosed breast cancer underwent a preliminary chart screening to determine whether they met the minimum criteria for recruitment to the study. All participants who met the minimum criteria and agreed to participate were asked to sign an informed consent, after which they underwent pretreatment evaluation consisting of (*a*) complete history and physical examination, including history of hormone use and menstrual and obstetric history; (*b*) laboratory testing; and (*c*) core biopsy of the index lesion to confirm histology and to establish baseline measurement of biomarkers. Women who were found to have benign proliferation only without atypia were excluded from the study at this point. All other qualifying women were randomized to receive daily either placebo or tamoxifen (20 mg/day) and 4-HPR (200 mg/day) for 3 weeks (±7 days to allow continuation of treatment to the time of definitive surgery). Tissue obtained at the time of the definitive surgical procedure was used to assess modulation of biomarkers. To minimize the possible effects of intratumoral heterogeneity, special care was taken to ensure that both the tissue sample removed at the initial core biopsy and the sample removed at the time of surgery were from the central portion of the tumor.

Measurement of Biomarkers Selected for Study. Ki67 was chosen as the primary end point for the study because it is a marker of cell proliferation. An essential first step in tumorigenesis is the immortalization process, in which a cell escapes the normal growth control program and becomes capable of uncontrolled proliferation. Numerous studies examining the effects of chemotherapeutic drug regimens have used Ki67 immunoreactivity as an end point, including at least two studies that have looked at a combination of a retinoid drug and tamoxifen (14, 15). Makris *et al.* (16) have demonstrated that a 14-day treatment with 20 mg/day tamoxifen can be effective in reducing the level of Ki67 labeling in breast cancer patients. To our knowledge, there have been no studies looking at the effects of 4-HPR or 4-HPR + tamoxifen on levels of Ki67 labeling in humans over this short time frame. There are, however, both *in vitro* and *in vivo* preclinical studies indicating that a 14-day treatment with 4-HPR may lead to a significant reduction in Ki67 (17–19).

Additional markers measured included ER, HER2/*neu*, p53, nuclear retinoid receptors, and DNA index. ERs in the breast epithelium mediate the induction of proliferation by estrogen. The presence of ERs also appears to be essential for the antineoplastic effect of tamoxifen. Overexpression of HER2/*neu* is one of the more common molecular abnormalities in breast tumors. It is detectable in ~55% of pure DCIS and in >75% of the comedo subtype of this group (20, 21). Mutations in the tumor suppressor gene *p53* are associated with high histological grade and clinical aggressiveness. These mutations generally result in increased half-life and accumulation of the p53 protein, which can be detected immunohistochemically. Nuclear retinoid receptors are central to the action of the retinoids. The two types of receptor (RAR and RXR) exist as several isoforms (α, β, γ). RAR-β is found in most normal mammary epithelial cells, but it is low or suppressed in most human breast cancer cell lines (22). DNA index is a quantitative measure of DNA content and can be used to assess aneuploidy. Aneuploidy is a manifestation of genetic instability, which is characteristic of virtually all

cancers (23). Methods for measuring these markers are presented below.

Immunohistochemical Techniques. The immunohistochemical procedures for measuring ER, Ki67, HER2/*neu*, and p53 followed the methodologies recommended by the manufacturers of the antibodies being used. Because slight day-to-day variations in experimental conditions may confound quantitative analysis of immunostaining, the samples were batched, and pre- and posttreatment samples were processed simultaneously. With each batch, we ran known positive and negative controls.

ERs. The antibody used for staining of ER was clone ER 1 D5 (DAKO, Carpinteria, CA), diluted 1:20 in PBS. The percentage of ER nuclear staining was semi-quantitated by two pathologists, and a consensus agreement was recorded. Samples were considered ER-positive when >10% of cells exhibited staining.

Ki67. The antibody used for immunostaining of Ki67 was MIB1 (Immunotech International, Marseilles, France), diluted 1:100 in PBS. The results were expressed as percentage of epithelial cell nuclei in defined preneoplastic (*i.e.*, atypical ductal hyperplasia) and neoplastic (*i.e.*, DCIS or invasive disease) regions that exhibited positive staining.

HER2/*neu*. The antibody used to stain the protein product of the HER2/*neu* gene was clone 3B5 (Oncogene Science, Uniondale, NY) diluted 1:1000 in PBS. Only cells that exhibited complete membranous staining were considered positive. Neu immunostaining was graded semiquantitatively (+ to +++) based on the percentage of positive cells and the intensity of staining.

p53. The antibody used to stain p53 was clone D07 manufactured by Biogenex (San Ramon, CA) and was diluted 1:4 in PBS. Samples were scored as positive when >5% of cells exhibited nuclear staining.

Analysis of Nuclear Retinoid Receptors by mRNA *in situ* Hybridization Using Digoxigenin-labeled RNA Probes. RNA probes were prepared from 1.9-Kb RAR-α and 1.9 Kb RAR-β *Eco*RI cDNA fragments, which span the entire open reading frames of cDNAs cloned from a human breast carcinoma, MCF-7, and T47D cDNA libraries, and 0.615-Kb and 0.650-Kb *Eco*RI fragments, which span all but 120 nucleotides of the 3′ end of the open reading frame of an RAR-β cDNA from a human liver cDNA library. Preparation of the digoxigenin-labeled probes and *in situ* hybridization were performed as described previously (24). The RAR-β staining was graded semiquantitatively as 0 (no cells stained), 0.5 (<20% of cells showing mild to moderate staining), or 1 (all cells showing moderate to intense staining).

DNA Index. DNA index was determined from nuclear absorbance measurements using the Cyto-Savant image analysis system (Xillix Technologies Corp., Vancouver, British Columbia, Canada). For each sample, the pathologist first identified areas of interest on H&E-stained slides, and these areas were scored on thionin-stained slides. Nuclear images from 50–200 tumor cell nuclei, 50–100 adjacent normal epithelial cells, and 50 lymphocytes were manually collected and stored in the computer memory. Multiple fields were scanned across the slide. Only nonoverlapping nuclei were selected. No additional procedure was used to make the selection random. Automated segmentation was used to delineate nuclei from background. Manual correction of contour was applied in the instances of touching, but not overlapping nuclei. Lymphocytes were used as an internal control to normalize for DNA index.

Measurement of Serum Retinol, 4-HPR, and 4-MPR. Serum retinol, 4-HPR, and 4-MPR were measured by high-performance liquid chromatography assay, according to the procedure recommended by the National Institute of Standards and Technology. After addition of a butanoic solution of the 4-ethylphenoxyretinamide internal standard, the analytes were extracted into hexane, which was removed under vacuum, and redissolved in mobile phase for injection onto a C-18 reversed-phase column. Elution was accomplished by gradient programming. The mobile phase A was acetonitrile:butanol:water (55:10:35, v/v/v) containing 0.01 M ammonium acetate salt; mobile phase B components was acetonitrile:butanol:water (85:10:5, v/v/v). The chromatogram was recorded at 364 and 325 nm. The elution times were as follows: 4-HPR (13.05 min), retinol (14.00 min), 4-MPR (15.4 min), and 4-ethylphenoxyretinamide (16.12 min). Sensitivity of the method is <0.01 ng/ml for the analytes.

Statistical Considerations. The main statistical end point was pre- *versus* posttreatment change in Ki67 in the treatment group compared with the placebo group. A number of other exploratory markers were also examined (see above). Data consisted of pairs of pre- and posttreatment values for the markers. Pre-post differences were calculated and then compared between treatment and placebo by use of a Wilcoxon rank-sum test. This nonparametric test was used rather than a *t* test because graphical examination of the data suggested the differences were not normally distributed. The Wilcoxon test considers both the direction and the magnitude of change. The analyses were performed using the JMP statistics package from SAS Institute, Inc. (Cary, NC).

In the original study design (25), a sample size of 50 patients per arm was selected, based on the hypothesis that the proportion of cases with changes of interest would be 5% in the placebo arm and at least 20% in the treatment arm. When 50% of this target sample had been accrued, an interim analysis was undertaken to determine whether the data supported continued patient accrual. By prior agreement among investigators and the granting agency, patient accrual would be suspended if results of the interim analysis did not support the study hypothesis.

It was initially proposed that additional variables of interest would be used as factors in covariate analysis, but the low patient number achieved ultimately precluded this. Instead, *post hoc* analyses of the relationship between change in Ki67 labeling index and other population and experimental variables (patient age, days in treatment, and serum levels of 4-HPR and metabolites) were carried out using the Wilcoxon rank-sum test. The relationship between change in Ki67 and ER status was examined qualitatively and using χ^2 analysis.

RESULTS

Patient Characteristics. During the 39-month period from February 1, 1997 to April 30, 2000, a total of 4670 women who had either an abnormal mammogram or a diagnosed breast cancer underwent preliminary screening for the study. From this initial group, 1565 met study criteria, and 52 (3%) of these

Table 1 Patient and tumor characteristics of evaluable breast cancer patients treated with placebo or with 4-HPR and tamoxifen

Characteristics	Treatment: Placebo (n = 20), n (%)	Treatment: 4-HPR and tamoxifen (n = 16), n (%)
Age (yrs)		
Median	56	50
Range	39–73	29–76
ER status		
Positive	17 (85)	9 (56)
Negative	3 (15)	7 (44)
Initial T stage		
T_{is}	3 (15)	3 (19)
T_1	13 (65)	9 (56)
T_2	4 (20)	3 (19)
Atypical ductal hyperplasia	0 (0)	1 (6)
Nodal status		
N_0	20 (100)	15 (94)
N_1	0 (0)	1 (6)
Pathological tumor size		
No greater than 1 cm	8 (40) [3 DCIS]	2 (12) [2 DCIS]
Greater than 1 cm	12 (60)	14 (88)
No. of positive nodes by pathology		
0	13 (65)	8 (50)
1–3	2 (10)	5 (31)
4–10	2 (10)	1 (6)
>10	1 (5)	0 (0)
No ALND[a]	2 (10)	2 (13)
Modified Black's nuclear grade[b]		
1	1 (5)	1 (6)
2	13 (65)	7 (44)
3	6 (30)	7 (44)
Not gradable[c]	0 (0)	1 (6)
Histology of primary tumor		
Invasive ductal with or without DCIS	13 (65)	10 (62)
Invasive lobular	1 (5)	1 (6)
DCIS	3 (15)	3 (19)
Other[d]	3 (15)	2 (13)

[a] Axillary lymph node dissection (ALND) not performed in these patients.

[b] 3 = most anaplastic.

[c] Atypical ductal hyperplasia.

[d] Placebo arm: mucinous carcinoma; mixed invasive ductal, invasive lobular, DCIS; mixed invasive ductal and invasive lobular. Treatment arm: atypical ductal hyperplasia; mucinous carcinoma.

patients were registered on the study. [For a detailed discussion of the accrual process in this study, see Singletary *et al.* (25).]

Of the 52 patients who were recruited into the study, 16 additional patients were excluded after recruitment: 3 refused to take some or all of their medication; 3 experienced a delay in surgery; 5 had no residual cancer on final pathology, 3 had insufficient tissue in the biopsy sample; 1 was misdiagnosed at an outside institution; and 1 received 4-HPR alone at the beginning of the study. This left a total of 36 patients evaluable for biomarker changes (placebo arm, n = 20; treatment arm, n = 16). Table 1 summarizes the characteristics of the 36 patients. There were no significant differences between the two study arms in median age, ER status, initial or pathological tumor size, initial or pathological nodal status, nuclear grade, or histology of the primary tumor. The median age of patients was 53 years (range, 29–76 years). Initial clinical tumor size was T_{is} in 15–19%, T_1 in 56–65%, and T_2 in 19–20% of patients. Nearly all patients were clinically node negative. The most frequent histological class of primary tumor was invasive ductal carcinoma with or without DCIS. Slightly more than half of the patients in the treatment arm were ER positive compared with 85% of patients in the placebo arm, and the treatment arm also tended to have larger tumors (pathological tumor size), but these differences were not significant.

Toxicity. Of the 52 patients who were recruited into the study, 7 experienced mild to moderate toxicity from the combination of tamoxifen and 4-HPR. One each experienced mild dizziness, headache, or nausea (alone), three experienced mild hot flashes, and one experienced a moderate skin reaction (a transient red truncal rash). No patient left the study as a result of toxicity reactions to the drug regimen.

Ki67 Labeling Index: The Main Statistical End Point. The main statistical end point was pre- *versus* posttreatment change in Ki67 labeling index in the treatment arm compared with the placebo arm. No pretreatment Ki67 labeling indices were available for 2 patients, one from each study arm, leaving an evaluable sample size of 34 for this marker (19 in the placebo arm and 15 in the treatment arm). Of those patients who showed change in Ki67 labeling index, the majority of cases in both the treatment arm and the placebo arm (70–85%) showed a negative change (Table 2; Fig. 1). Considering all cases, the average change in Ki67 labeling index was −2.42 (SD = 7.02) for the placebo arm *versus* −5.67 (SD = 9.42) for the treatment arm. This difference was not statistically significant.

Time in Treatment. Although the goal of the study was to treat patients with either placebo or tamoxifen and 4-HPR for 3 weeks, actual treatment times varied ±7 days to allow continuation of treatment to the time of definitive surgery. Thus, the number of days on treatment varied from 14 to 28 (median, 18.5) in the placebo arm and from 15 to 28 (median, 19) in the treatment arm. There was no significant association between change in Ki67 labeling index and number of treatment days for either study arm.

Serum Levels of 4-HPR, 4-MPR, and Retinol. Serum levels of 4-HPR, its major metabolite 4-MPR, and retinol were measured in all patients to validate that 4-HPR was present in pharmacologically appropriate levels in patients in the treatment arm. None of the placebo patients had detectable serum concentrations of 4-HPR. The subjects in the placebo arm had baseline retinol values within the expected limits of 360-1200 ng/ml (26). One patient had a baseline value just above the upper limit. at 1467 ng/ml. For most of the placebo subjects, the posttreatment measurement of retinol agreed well with the pretreatment value. One subject showed a 38% increase, and three subjects showed a decrease (all <30%). Standard oral doses of 4-HPR produced a large range of serum levels in subjects in the treatment arm (4-HPR, 26–463 ng/ml; 4-MPR, 46–430 ng/ml; see Table 3). These levels are consistent with those reported in other studies (11, 27) and are expected to be clinically efficacious based on preclinical studies in breast carcinoma cell lines (17). The baseline retinol values for this group were consistent with the expected values for healthy subjects, and all but two posttreatment values demonstrated a decrease of 40–80% from the pretreatment values, consistent with reports from the literature for

Table 2 Pre-*versus* posttreatment differences in SEBs for patients treated with placebo or with 4-HPR and tamoxifen

SEB	Pre-*versus* post-treatment change: Placebo (n = 20), n (%)	Pre-*versus* post-treatment change: 4-HPR and tamoxifen (n = 16), n (%)	P^a
Ki67 labeling index			
No change	8 (40)	8 (50)	
Positive change	3 (15)	1 (6)	
Negative change	8 (40)	6 (38)	
Unknown[b]	1 (5)	1 (6)	0.49
ER			
No change	14 (70)	10 (63)	
Positive change	2 (10)	1 (6)	
Negative change	3 (15)	4 (25)	
Unknown[b]	1 (5)	1 (6)	0.87
HER2/*neu*			
No change	18 (90)	15 (94)	
Positive change	1 (5)	1 (6)	
Negative change	1 (5)	0 (0)	
Unknown[b]	0 (0)	0 (0)	0.55
p53			
No change	17 (85)	12 (75)	
Positive change	1 (5)	2 (13)	
Negative change	2 (10)	1 (6)	
Unknown[b]	0 (0)	1 (6)	0.81
RAR-β			
No change	8 (40)	10 (62)	
Positive change	5 (25)	3 (19)	
Negative change	4 (20)	3 (19)	
Unknown[b]	3 (15)	0 (0)	0.78
DNA index			
No change[c]	5 (25)	1 (6)	
Positive change	2 (10)	0 (0)	
Negative change	11 (55)	14 (88)	
Unknown[b]	2 (10)	1 (6)	0.68

[a] Wilcoxon rank-sum test, which takes into consideration magnitude of the changes seen as well as the direction (increase or decrease).
[b] Insufficient tissue or assay failure.
[c] Defined as pre- *versus* post-treatment difference no greater than 0.05.

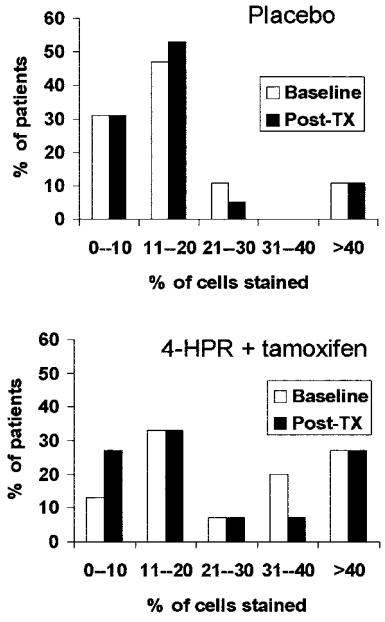

Fig. 1 Ki67 labeling index before and after treatment with placebo or tamoxifen (*TX*) and 4-HPR. Labeling index is defined as percentage of epithelial cell nuclei in defined region that exhibit positive staining.

Table 3 Plasma concentrations of 4-HPR, 4-MPR, and retinol in patients treated with placebo or 4-HPR and tamoxifen

	Retinoid level (ng/ml) by treatment arm, median (range): Placebo	Retinoid level (ng/ml) by treatment arm, median (range): 4-HPR and tamoxifen
4-HPR (posttreatment)	ND[a]	159 (26–463)
4-MPR (posttreatment)	ND	181 (46–430)
Retinol (pretreatment)	753 (408–1467)	720 (459–929)
Retinol (posttreatment)	640 (436–1425)	362 (141–743)

[a] ND, none detected.

patients taking 4-HPR (28, 29). There was no significant association between change in Ki67 labeling index and plasma levels of 4-HPR or 4-MPR in the treatment arm.

Tumor Size and Patient Age. There was no significant association between change in Ki67 labeling index and pathological tumor size or patient age. There was also no significant association with menopausal status, which was approximated by dichotomizing patients into groups ≤50 years *versus* >50 years.

ER Status. The relationship between change in Ki67 labeling index and baseline ER status is shown in Fig. 2. In both study arms, change in Ki67 labeling index was more likely to occur in ER-positive cases compared with ER-negative cases. In the treatment arm, five of seven (71%) ER-negative cases showed no change in Ki67 labeling index, compared with three of eight (38%) ER-positive cases. This compares with the placebo arm, where 2 of 3 (67%) ER-negative cases showed no change compared with 6 of 16 (38%) ER-positive cases. This same picture emerged when the data were analyzed by examining Ki67 positivity (defined as >10% of cells labeled) in the treatment group *versus* the placebo group (data not shown). There were no significant differences in Ki67 labeling index as a function of ER status, although in both arms there was a higher percentage of Ki67-negative patients in the ER-positive group compared with the ER-negative group. Unfortunately, formal stratification of the patient sample into ER-positive and -negative subgroups was not possible because of the low sample size, precluding any meaningful statistical analysis.

Changes in Other SEBs as a Function of Treatment Arm. There were no significant differences between the placebo arm and the treatment arm in the direction or magnitude of change in ER, HER2/*neu*, p53, RAR-β, or DNA index. Table 2 shows the number and percentage of patients in each study arm who showed no change, positive change, or negative change for each of the biomarkers. For all markers except DNA index, the majority of cases showed no change. For HER2/*neu*, this undoubtedly reflects the fact that 75–85% of cases were initially HER2/*neu* negative at baseline, so little or no change would have been predicted. A significant proportion of cases were also p53 negative (35–45%), ER negative (defined as <10% of cells stained; 44% of the treatment arm), and RAR-β negative (59–75%) at baseline. DNA index measurements tended to decrease

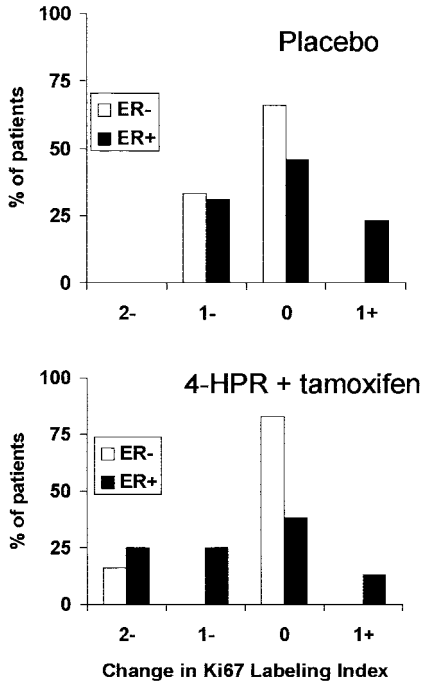

Fig. 2 Change in Ki67 labeling index after treatment with placebo or tamoxifen and 4-HPR in ER-positive (*ER+*) *versus* ER-negative (*ER−*) patients. 1+, labeling index increased by 1–10; 0, no change; 1−, labeling index decreased by 1–10; 2−, labeling index decreased by 11–20.

in most subjects (55% of placebo arm *versus* 88% of treatment arm), with a median change of 0.18 in the placebo arm compared with 0.22 in the treatment arm.

DISCUSSION

Ki67 Labeling Index as a SEB for Short-Term Treatment with Tamoxifen and 4-HPR. It was hypothesized that patients in the treatment arm of this study would show decreases in Ki67 labeling index and that these changes would be significantly greater than those seen in the placebo arm. That this was not the case was somewhat surprising given that apparently similar studies had shown such a difference (16, 30, 31). Why was a significant difference in Ki67 labeling index not seen in the present study?

A series of collaborative studies between the Royal Marsden Hospital in London and the University of Texas Health Science Center in San Antonio examined changes in Ki67 labeling index as a function of short-term hormonal or chemohormonal treatment. Makris *et al.* (16) looked at changes in Ki67 labeling index as a function of tamoxifen treatment in 21 patients, with fine-needle aspiration samples taken on days 1 and 14 of treatment. At 14 days, they found significant changes in Ki67 labeling index in responders (*i.e.*, those who showed >50% reduction in primary tumor size as a function of tamoxifen treatment), but not in nonresponders. Response was highly correlated with ER status; thus a significant decrease in Ki67 labeling index was seen in ER-positive but not ER-negative patients. The following year, Makris *et al.* (30) published a study that examined changes in Ki67 labeling indices in patients before and after treatment with mitomycin C and tamoxifen with or without mitoxantrone. Again, changes were significant only in responders. The majority of patients in this study (~75%) were ER positive. Chang *et al.* (31) updated the 1998 study, increasing the sample size to 54. They verified that ER-positive tumors were more likely to show a decrease in Ki67 labeling index on day 14 as a predictor of response to tamoxifen. Of interest, they saw changes in Ki67 at day 14, but not at day 60, indicating that sampling time may be an important factor in the measurement of biomarkers. These results were supported in a related study originating at Northwestern University Medical School in Chicago (32). This study found no difference in Ki67 labeling index between pre- and post-tamoxifen treatment in ER-negative breast tumor samples, although there was a significant decrease in ER-positive samples.

The lack of response of Ki67 labeling index to tamoxifen in ER-negative breast tumor samples was anticipated in our original study design. Our original patient accrual goal, had it been successful, would have allowed for statistical assessment of the effects of ER status. In addition, a specific reason for combining tamoxifen with 4-HPR was the additive or synergistic effects reported for the combined administration of the two drugs in both *in vitro* and *in vivo* test systems (8–10). Early reports (33, 34) indicated that retinoids do not require ERs for their action and should be able to affect biomarker expression in ER-negative cells. If this is true, why was a significant difference in Ki67 labeling index not seen as a result of 4-HPR treatment?

Several *in vitro* studies originating from the University of Maryland School of Medicine have reported that retinoids may be largely ineffective in altering cell proliferation in ER-negative breast cancer cell lines. Rishi *et al.* (35) showed that the putative ER-negative cells lines MDA-MB-231 and MDA-MB-468 were not growth inhibited by retinoic acid or any of a series of other retinoids tested. Shao *et al.* (36) demonstrated that mRNA levels for the RAR-α receptor are strongly correlated with ER status. They found that transfecting RAR-α cDNA into the retinoic acid-resistant ER-negative HPB cell line caused it to become sensitive to growth inhibition by retinoids. It should be noted that several early studies reported that they did see growth inhibitory effects of retinoic acid, 13-*cis*-retinoic acid, and retinol in MDA-MB-231 cells (33, 34). Coradini *et al.* (37), on the other hand, showed that high levels of 4-HPR inhibited growth in the ER-negative BT20 cell line, but not in MDA-MB-231. The effect on BT20 cells was confirmed by Kazmi *et al.* (38). These conflicting results may be explained in a recent report by Aldous *et al.* (39), who reported that MDA-MB-231 cells, previously thought to be ER negative, were positive for ER-β. Thus, there is a very complex picture developing about the role of ER status in proliferative response to retinoids. At best, it would be predicted that only a fraction of ER-negative patients might show a decrease in proliferation as a result of retinoid treatment.

On the basis of these findings, it seems likely that our failure to demonstrate a statistically significant decrease in Ki67 labeling index in the treatment arm was attributable, in part, to the fact that nearly half of the patients randomized to that arm of the study were ER negative. Decreases in Ki67 as a result of short-term treatment with tamoxifen, 4-HPR, or tamoxifen and 4-HPR would be most likely in ER-positive patients. In both arms of this study, at least two-thirds of ER-negative patients showed no change in Ki67 labeling index.

Patient Accrual into Nontherapeutic Clinical Trials. Analysis of the influence of ER status on response to chemopreventive drug therapy was hampered in this study by low patient accrual rates. Difficulty in the accrual of patients for clinical trials is a serious impediment to clinical research in cancer (40). Accrual into nontherapeutic SEB protocols is particularly challenging for a variety of reasons. Only a fraction of women with a suspicious mammogram will prove to have a malignancy, and only a fraction of those will ultimately participate in a clinical trial (41). There are no potential direct benefits to the participants to counterbalance the possible side effects from the drugs and the delay in definitive surgery. Given these difficulties, this study was designed to minimize patient accrual problems. For example, a two-arm combination regimen (control *versus* tamoxifen + 4-HPR) was used instead of a four-arm design (control *versus* tamoxifen *versus* 4-HPR *versus* tamoxifen + 4-HPR) because a significantly larger number of patients would have been required to assure statistical validity in a four-arm design. During the course of the study, several exclusion requirements were eliminated (outside needle biopsy, tumor size >2 cm, and estrogen replacement therapy) to maximize the patient base. Even so, it was quite difficult to recruit patients into this study, as discussed in detail in a preliminary report by Singletary *et al.* (25). In addition to the lack of therapeutic benefits, patients expressed concern about side effects from the drug treatment, and many did not want to wait for surgery. In addition, this study was carried out in a tertiary referral cancer center, where many competing clinical trials, many with therapeutic benefit, are presently recruiting patients.

It should be noted that several recent studies with a study design similar to ours have been able to accrue moderate to large numbers of patients (42–45). An important distinction is that these studies all involved only a single agent (a selective estrogen response modifier); none involved 4-HPR or any other retinoid. In addition, most or all of the patients recruited for these studies were postmenopausal women. We believe that the wide usage of estrogen replacement therapy and the press coverage that has been given to tamoxifen and raloxifene have made patients, especially those who are postmenopausal, relatively comfortable with the idea of taking a selective estrogen response modifier. Retinoid drugs, on the other hand, have been presented in the public media as representing significant toxicity, especially to the liver. Thus, we suspect that patient accrual into the present study may have been more difficult because (*a*) patients were averse to taking two drugs, and (*b*) patients were specifically averse to taking a drug that they believed to have the potential for severe toxicity.

The largest impediment to patient accrual may be overly restrictive eligibility criteria (40, 46–48). In addition to making patient accrual more difficult and generally making a study more complex and costly, overly exclusionary eligibility criteria generate problems in generalizing data from a study. The practicing clinician must determine whether the published results of clinical trials will be relevant to a specific patient who may not meet study criteria.

On the other hand, reducing the number of eligibility criteria may mean that additional stratification and/or covariate analyses will be needed to interpret the results of the clinical trial. These more sophisticated analyses require larger sample sizes, which must be weighed against the advantages of easier recruitment and wider applicability of the trial results in the general population.

The development of future SEB trials will be of critical importance to the growing field of chemoprevention in breast cancer. The results of this trial, although largely negative, serve as a caution for future studies to (*a*) carefully incorporate information about patient hormonal status into the study design, and (*b*) anticipate difficulties in patient accrual into nontherapeutic trials.

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