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Inhibition of estrogen-induced mammary tumor formation in MMTV-aromatase transgenic mice by 4-chlorophenylacetate

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Abstract

Treatment of estrogen-sensitive breast cancer with selective estrogen selective modulators (SERMs) and, more recently, aromatase inhibitors has met with wide success. However, antagonism of estrogen receptor (ER) activity in breast carcinomas by SERMs such as tamoxifen has been associated with increased risk of cancer in other tissue such as the endometrium. Furthermore, current therapies using aromatase inhibitors have side effects on bone resulting in development of osteoporosis in some patients. We present in this paper the results of a study using 4-chlorophenylacetate (4-CPA), a compound which belongs to a family of small aromatic fatty acids that has been shown to possess anticancer properties, to treat DMBA exposed MMTV-aromatase mice. These animals exhibit elevated levels of estrogen in their mammary glands and develop estrogen-responsive tumors. Consistent with our earlier findings showing that 4-CPA inhibited the growth of ER positive breast cancer cells in vitro, we now demonstrate that this compound inhibits tumor formation in MMTV-aromatase mice. This effect was not associated with reduction of ER expression in their mammary tissue, or to alteration of aromatase levels or activity. The data suggest that 4-CPA is a novel therapeutic agent that could be used in the prevention or treatment of estrogen-sensitive breast cancer.

Keywords

breast cancer; antiestrogen; 4-chlorophenylacetate; estrogen receptor

INTRODUCTION

Breast cancer is the second leading cause of cancer deaths in women. Extensive studies have revealed the importance of estrogen and its receptor (ER) not only in normal breast development but also in the development of breast carcinomas [1–3]. Furthermore, recent evidence suggests that estrogen produced locally in the breast tissue due to the over-expression of aromatase is an important factor in postmenopausal breast cancer [4,5]. Aromatase (encoded by the gene *CYP19A*) is a P450 enzyme that catalyzes the aromatization of androgen to form estrogen, the rate-limiting step in estrogen biosynthesis. Elevated levels of aromatase have been detected in cells adjacent to tumors and have been shown to be present in tumor-containing breast quadrants. Furthermore, aromatase overexpression (under the regulation of the MMTV promoter) in mammary glands of transgenic mice, designated MMTV-aromatase, resulted in extensive hyperplasia, deficient involution, as well as predisposition to tumor development upon treatment with subcarcinogenic doses of DMBA [5–7]. The use of aromatase inhibitors are now used clinically to treat breast cancer, and studies have shown that they are at least as effective as conventional anti-estrogen therapy such as tamoxifen in treating these malignancies [8].

Another class of compounds that has shown early promise in treating breast cancer is certain small aromatic fatty acids exemplified by phenylacetate (PA) as a prototype [9,10]. These compounds have been demonstrated to have low toxicity and antitumor activity in both experimental models and humans [9,11,12]. Furthermore, this class of drugs provides a new avenue of antagonizing the estrogenic pathway without binding to the estrogen receptor [13]. In our recent studies, we have shown that PA and its derivative 4-chlorophenylacetate (4-CPA) inhibited growth of ER positive breast cancer cells but had little effect on ER negative cells, suggesting an anti-estrogenic mechanism of action [10,14]. In support of this contention, PA and 4-CPA reduced the promoter activity of the estrogen-responsive gene cyclin D1 and diminished ER activation of consensus ERE-reporter constructs.

In the current study, we have examined the effects of 4-CPA on tumor formation in an estrogen-dependent in vivo mammary tumor model, namely MMTV-aromatase transgenic mice. Our data have confirmed the antitumor activity of 4-CPA and evaluated the effects of 4-CPA on estrogen receptors and downstream factors. Combined, the data underscore the viability of using the aromatic fatty acid 4-CPA to antagonize the estrogenic pathway for the treatment of breast cancer.

MATERIALS AND METHODS

Maintenance and treatment of mice

The generation and characterization of MMTV-aromatase mice used in these studies have been described previously [5,6,15]. The mice were housed in a centralized animal facility accredited by the AAALAC and USDA and maintained according to the recommendations established in the NIH Guide for the Care and Use of Laboratory Animals.

To determine the effects of 4-CPA on histological and biochemical changes in mammary glands of aromatase transgenic mice, females were divided into two groups, with one group receiving 4-CPA (6 mg/ml) continuously in their drinking water starting at 6 weeks of age until they were sacrificed. Our previous work has shown that this method of long-term 4-CPA administration for many months has no adverse effects on the mice and achieves a mean 4-CPA plasma concentration (~0.8 mM) that is antiproliferative against ER+ breast cancer cells in vitro [10,16]. Both groups were exposed to DMBA (Sigma, St. Louis, MO) at 7–8 weeks of age. DMBA (1.0 mg) dissolved in 100 μ l of corn oil was delivered via orogastric tube to mildly anesthetized mice once per week for four weeks. One month after the administration of

DMBA the mice were palpated for tumors and weekly observations were continued up until 4 months after DMBA treatment. Mice were sacrificed shortly after tumor identification or at the termination of the experiment.

Histological assessment of mammary glands and tumors

After the mice were sacrificed, tumors or mammary glands were dissected free from skin and processed for histology as previously described [17]. Routine sections of mammary tissues were prepared after fixation in 10% neutral buffered formalin by embedding in paraffin, sectioning at 5 μ m, and staining with H&E. Tumors were identified as described previously [17].

Animal treatment for the measurement of ethoxyresorufin O-deethylase (EROD)-CYP1B1 activity

Female Balb/C mice (wild-type control for the aromatase transgenic mice) were purchased from Charles River (Wilmington, MA). The mice were housed, maintained, and treated in accordance with NIH guidelines for animal use and care under the supervision of Laboratory Animal Resources, Emory University. All mice used in these studies were at least 2 months of age. As in the tumor induction studies described above, groups of mice were either untreated or treated with 4-CPA by receiving the compound continuously in their drinking water at a concentration of 6 mg/ml. Seven days later, the mice were gavaged with either DMBA (Sigma, St. Louis, MO) at 1 mg/mouse or solvent control (corn oil). Mice were sacrificed 24 hours after gavage treatment and the livers and mammary glands were quick frozen in liquid nitrogen and stored at -80°C until used. For the assay, liver or mammary glands were lysed in homogenization buffer (0.1 M Tris-OAC, pH 7.4, 1 mM EDTA, 0.1 M KCl) and homogenized by overhead stirrer. The lysate was centrifuged at 10,000g for 25 min to remove mitochondria and other debris, and the supernatant was then centrifuged at 100,000g for 90 min to pellet the microsomal fraction. Microsomal protein was resuspended in storage buffer (10 mM Tris-OAC, pH 7.4, 0.1 mM EDTA, 23% glycerol). Protein concentration was determined according to the Sigma-BCA kit procedure (Sigma, St. Louis, MO).

P450 enzyme activity assays

To measure aromatase enzymatic activity, we used the tritiated water-release assay, using [^3H] androstenedione as substrate [18]. For this assay, we used a cell line clone of MCF-7 that overexpresses aromatase, designated MCF-7Ca, that has been characterized previously to have higher aromatase activity than the parent cell line [19]. This clone was kindly provided by Dr. Shuan Chen (Beckman Research Institute, City of Hope, CA). MCF-7Ca cells grown in phenol-free media/charcoal-stripped FBS were suspended in assay mixture containing 0.1% BSA, 67mM KPO_4 (pH 7.4), and 2.0 μM progesterone. After sonication, 100 nM of [^3H] androstenedione (25.3 ci/mmol, Perkin Elmer, NET-962,) was added and mixture was incubated for 10 minutes at room temperature. NADPH was then added to a final concentration of 1.2 mM, followed by 37°C incubation and addition of equal volume of 5% TCA. Supernatant was collected and extracted with equal volume of chloroform. Dextran-coated charcoal was added to the assay mixture, which was then vortexed and centrifuged. The supernatant was then added to scintillation fluid and measured in a scintillation counter.

To measure CYP1B1 activity, the EROD assay was used [20]. Reactions were performed in a total volume of 250 μL in microfuge tubes at 37°C at a final substrate concentration of 4 μM . Microsomal protein (100–250 μg) in storage buffer (10 mM Tris OAc, pH 7.4, 0.1 mM EDTA, 23% glycerol) was added to 7-ethoxyresorufin dissolved in DMSO and then diluted in double distilled water (final DMSO concentration of 0.08% v/v). The reaction mixture was allowed to equilibrate for 5 min at 37°C . Reactions were initiated by timed additions of 50 μL NADPH (10 mM). After 10 min incubation at 37°C , the reaction was stopped by timed additions of 250

μ L ml of ice-cold acetonitrile. EROD activity was determined by measuring the fluorescence of resorufin at 545 nm (excitation) and 610 nm (emission) in a microplate reader. Resorufin standard curves were used for the determination of the activities.

RNA and protein analysis

Mammary gland samples were homogenized and total RNA was isolated by Tri-reagent (Sigma, MO). For mRNA evaluation, reverse transcription was used to synthesize cDNA from RNA template as described previously [15]. For real-time PCR, a total of 25 μ L reaction mix was prepared using Omni-beads (TAKARA), 0.25X Syber Green dye (Fisher, Pittsburgh PA) and specific primer sets (0.3 μ M each). Primer sequences used for the evaluation of ER α , ER β , progesterone receptor (PR), and aromatase gene expression were described previously [5]. The PCR reaction was set for 40 cycles in a SmartCycler real-time thermocycler (Cepheid). The data was analyzed after normalization with GAPDH RNA levels, using the formula $2^{-\Delta\Delta ct}$, where ct is the cycle threshold [16].

Expression of aromatase protein was assessed by Western Blot analysis. Total protein was isolated from the mammary tissue by homogenization in lysis buffer. Equal amount of protein (60 μ g) from representative samples were separated on a denaturing polyacrylamide gel and transferred to a nylon membrane. Non specific binding of antibodies was blocked by incubation (overnight, 4°C) in Tris-buffered saline containing 0.1% Triton X-100 (TBST) and 5% non fat dry milk. Membranes were then incubated with anti-aromatase antibody (Santa Cruz Biotechnology, Santa Cruz, CA) in TBST and 5% milk overnight at 4°C. Specific binding to aromatase protein was visualized by chemiluminescent detection and exposure to ECL hyper film (Amersham, Piscatawa, NJ). Quantification of relative band densities was achieved by densitometry and normalized to GAPDH protein obtained from reprobed blots.

RESULTS

4-CPA suppresses DMBA-induced hyperplasia/tumor induction in MMTV-Aromatase mice

In our previous study, we showed that 4-CPA is a potent inhibitor of the growth of ER positive breast cancer cell lines and it interferes with the activation of ER-sensitive promoters, suggesting that one possible mode of action of 4-CPA in suppression of tumor cell growth is via antagonizing the estrogenic pathway [10]. To examine this hypothesis in vivo, we used MMTV-aromatase transgenic mice, which have been previously shown to possess elevated mammary tissue estrogen and ER levels and produce premalignant mammary phenotypes, including hyperplasia and dysplasia, which are abrogated by aromatase inhibitors [21]. These mice are also susceptible to tumor formation upon treatment with sub-carcinogenic doses of DMBA [7]. Thus, as part of this study, we tested the effects of 4-CPA on DMBA-induced tumors. Data in table 1 show that 85% of DMBA treated mice (n=13) exhibited microscopic tumors and 46% developed palpable tumors. 4-CPA significantly reduced tumor formation due to DMBA treatment, with only 8% developing microscopic tumors and none developing palpable tumors (Table 1). The corresponding wildtype controls did not develop tumors, as expected. Nonetheless, 4-CPA abrogated DMBA-induced ductal hyperplasia in wild-type mice. Histology slides showing effects of 4-CPA on reducing tumor formation and preneoplasia are presented in Fig 1.

4-CPA does not interfere with activity of P450 enzymes CYP19 (aromatase) and CYP1B1

The data demonstrated that 4-CPA is a potent inhibitor of tumor formation in DMBA-treated MMTV-aromatase mice. This inhibition may be attributed to suppression of aromatase expression in the transgenic mice or to interference with aromatase activity. To test these possibilities, we examined aromatase expression in 4-CPA treated and untreated animals. The data in Fig 2A shows that 4-CPA did not affect aromatase expression at the mRNA or protein

levels in the mammary tissue of the mice. Furthermore, 4-CPA did not affect aromatase activity in MCF-7Ca, a previously characterized clone of MCF-7 breast cancer cell line that over expresses aromatase (Fig. 2B). We therefore conclude that inhibition of tumor growth by 4-CPA in DMBA-treated MMTV-aromatase mice is not due to effects on aromatase expression or activity.

Studies in mice have demonstrated that the P450 enzyme CYP1B1 is the predominant enzyme for metabolic activation of DMBA to produce the proximate carcinogenic metabolite 3,4-dihydrodiol [22]. In contrast, CYP1A1, although also contributing to the metabolism of DMBA, is thought to only play a minor role in this process. To test whether 4-CPA might affect the carcinogenic potential of DMBA through alteration of its metabolism, we utilized the EROD assay to measure CYP1B1 activity [20]. Initial experiments indicated that the rates of EROD production were constant at least up to 10 min and that the addition of 4-CPA up to 10 mM directly in the assay did not inhibit the activity (not shown). Figure 3 shows that DMBA stimulated the metabolism of ethoxyresorufin by liver microsomal protein approximately 2.5-fold in both control and 4-CPA-treated mice. 4-CPA treatment of the mice had no significant effect on either basal or DMBA-induced EROD activity. Measurement of EROD activity in the presence of mammary microsomal protein was more difficult due to the lower yield of microsomes and CYP1 levels [23]. Therefore, we pooled mammary derived microsomes obtained from 5 mice in each treatment group in order to obtain enough protein for detecting EROD activity. Results from two pooled samples in each treatment group (total of 10 mice/group) also showed no effect of 4-CPA treatment with mean values (in pmol/mg protein-min) of 0.06, 0.06, 0.30, and 0.31 in the control, 4-CPA, DMBA, and DMBA + 4-CPA groups, respectively. Thus, 4-CPA treatment of the mice did not appear to alter CYP1B1 hepatic or mammary enzyme activities.

4-CPA has little effect on receptor activity

To evaluate whether 4-CPA affects the estrogenic pathway by altering the expression of ER α , ER β , we evaluated the relative mRNA levels of these receptors in 4-CPA treated and untreated mice. Using both semi-quantitative and real time RT-PCR, we found no significant changes in the expression of these receptors in the mammary glands of DMBA-treated MMTV-aromatase mice (Fig. 4). Since PR is known to be transcriptionally regulated by estrogen in an ER-dependent manner [24], we also tested whether mRNA levels of PR were altered in the 4-CPA treated mice. Unexpectedly, 4-CPA did not significantly affect mammary PR expression in these in vivo studies in contrast to previous results showing 4-CPA-mediated inhibition of PR in MCF-7 breast cancer cells in vitro [25].

DISCUSSION

The strong correlation between breast cancer development and estrogen signaling has prompted the development of anti-estrogen therapies that modulate ER activity, such as tamoxifen. However, a large percentage of breast tumors develop resistance to known anti-estrogen therapies after prolonged use [26,27]. Moreover, tamoxifen and similar drugs have been shown to have estrogen agonistic effects in the endometrium leading to increased risk of endometrial cancer [27]. Recently, aromatase inhibitors have been developed in response to the observations that local estrogen production due to in situ aromatase over-expression may promote breast cancer development [28]. We have previously shown that 4-CPA is a potent inhibitor of estrogen signaling in ER+ breast cancer cell lines. Treatment of these cell lines with 4-CPA resulted in diminished cellular growth and a reduction in the expression of estrogen responsive genes, suggesting that 4-CPA antagonizes cell growth in an ER dependent manner [10,25]. In this paper, we have examined the effects of 4-CPA on the mammary glands of MMTV-aromatase transgenic mice, an in vivo model of estrogen-induced breast cancer. These

mice exhibit estrogen-dependent premalignant changes in the architecture of their mammary glands, such as hyperplasia and dysplasia. Suppression of estrogen synthesis by using aromatase inhibitors results in abrogation of these premalignant changes [21]. MMTV-aromatase mice are also susceptible to DMBA exposure, which results in microscopic as well as palpable tumor formation [7]. We have previously determined that long-term addition of 4-CPA to the drinking water of the mice can achieve therapeutic plasma concentrations without any adverse effects [16]. In this study, treatment of DMBA-primed MMTV-aromatase transgenic mice with 4-CPA abrogated tumor formation induced by DMBA in these mice. This inhibitory effect of 4-CPA on tumor development in vivo is consistent with its antiproliferative effects on ER+ breast cancer cell lines in vitro [10] and suggest that this novel class of compounds may be useful in targeting estrogen-dependent breast cancer. Previous studies of DMBA-mediated carcinogenesis in rodent models have demonstrated inhibition of mammary tumor induction by SERMs [29]. Although a comparison of such results in other rodent models to the present study is at best tenuous, our findings suggest that in comparison to conventional SERMs like tamoxifen, 4-CPA is an especially potent compound for inhibiting mammary oncogenesis.

MMTV-aromatase mice are deficient in post-lactation involution of their mammary glands and maintain extensive ductal branching after weaning the pups [5]. Because this effect is thought to be due to sustained elevation of estrogen levels and resultant signaling in post lactation mammary tissue [5], we examined whether 4-CPA could restore this involution. However, we have determined that this is not the case; 4-CPA-treated MMTV-aromatase mice maintained extensive mammary ductal branching after weaning (data not shown). The differences between the anti-estrogenic action of 4-CPA on tumor formation versus involution may be attributed to differences in pathways regulating cellular growth during these two processes. Ductal growth and branching during pregnancy leads to a highly differentiated state of cellular development. On the other hand, tumor development is caused by uncontrolled and undifferentiated growth of cells. Thus, the growth factors, cytokine dependence, and estrogen requirements of the two modes of mammary growth may differ, leading to differential responses to 4-CPA.

The observed anti-tumorigenic effects of 4-CPA in MMTV-aromatase mice raises the possibilities that 4-CPA may interfere with aromatase activity and/or may repress aromatase expression in these mice. These two possibilities were shown to be unlikely since 1) 4-CPA did not inhibit aromatase activity in MCF-7Ca, an aromatase over-expressing MCF-7 breast cancer cell line clone, and 2) aromatase mRNA and protein were not altered due to 4-CPA treatment in MMTV-aromatase mice. Another possibility is that 4-CPA may alter DMBA metabolism in these mice in such a way as to suppress conversion to its carcinogenic metabolite 3,4-dihydrodiol. However, this too seems unlikely since 4-CPA treatment did not interfere with the activity of CYP1B1, the major P450 enzyme responsible for DMBA activation. Furthermore, it is noted that DMBA resulted in a marked increase in CYP1B1 activity that was unaffected by 4-CPA treatment. This observation suggests a mechanism by which DMBA induces tumors in the aromatase mice but only hyperplasia in the nontransgenic wildtype controls. In the former, mammary estrogen levels are elevated, which provide a substrate for CYP1B1 and could then result in increased formation of estrogen quinone and other free-radical compounds that are genotoxic estrogen metabolites [30]. Since less estrogen is available for conversion to these metabolites in nontransgenic mice, less mutational events leading to malignant transformations would be expected. Thus, the cooperation of these genotoxic effects and estrogen-mediated mitogenic activity through transcriptional transactivation could lead to tumor formation in DMBA treated aromatase mice, with 4-CPA more likely affecting the latter pathway.

The observation that PA derivatives suppress ER α /estrogen response element (ERE) interactions in breast cancer cell lines [25] suggests that 4-CPA can directly interfere with the estrogenic pathway by affecting ER α activity. Unlike our previous in vitro studies showing that ER α and PR expression could be inhibited by PA derivatives, we could not document significant changes in the expression of these receptors in the mammary glands of 4-CPA-treated MMTV-aromatase mice. The reason(s) for these divergent effects is unknown but may be due to inherent differences between homogenous in vitro grown cells compared to the more complex in vivo environment of mammary tissue, which contains epithelial and stromal cells undergoing both paracrine and autocrine interactions. Previous investigations of animal treated with a variety of classical antiestrogenic compounds such as tamoxifen and its derivatives have also shown little or no changes in the expression of estrogen and progesterone receptors in their mammary tissue after treatment [31].

The mechanism(s) of the anti-estrogenic activity of 4-CPA is not known. Unlike most known antiestrogens, 4-CPA does not seem to bind to ER and compete with estrogen for interaction with its receptor [13]. Our recent studies have illustrated that PA and other piperidinedione derivatives can directly bind to canonical ERE motifs through intercalation into 5'-dTdG-3': 5'-dCdA-3' sites and that the energetic fit of the compounds is correlated with their potency to inhibit ERE reporter gene activity and proliferation of breast cancer cells [14]. Especially potent derivatives included those that formed two stereospecific hydrogen bonds with phosphate groups on adjacent DNA strands of the ERE complex. Thus, the ability of 4-CPA to block estrogen signaling may, at least partly, be due to its direct binding to DNA at certain critical sites within the ERE, thereby interfering with ER-ERE interactions. Such a mechanism of action has recently been demonstrated in the case of the anticancer agent XR5944 which has been shown to bis-intercalate into sequence specific DNA motifs such as TG sequences found in AP-1 and NF-1 regulatory sites [32]. This intercalation was shown to directly interfere with AP-1 protein (i.e. fos, jun dimers)-DNA binding which resulted in inhibition of AP-1-mediated transcriptional activation.

In summary, we have shown that 4-CPA is an effective drug against estrogen-induced mammary tumorigenesis and is associated with decreased estrogen signaling via ER. 4-CPA is likely to interfere with the estrogen/ERE pathway by a novel mechanism that is different from that of classical ER antagonists and SERMs like tamoxifen. Such a finding could pave the way to a new class of drugs for the treatment of breast cancer and other estrogen-dependent diseases.

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Figure 1.

4-CPA reduces tumor formation in DMBA-treated MMTV-aromatase mice. Palpable tumors formed in mammary glands of MMTV-aromatase treated with DMBA. Segments of the tumors were embedded in paraffin and sectioned for H&E staining as described in Materials and Methods. A representative sample is shown in A. Microscopic tumors were also observed in DMBA-treated mice. Representative microscopic tumor growth in MMTV-aromatase DMBA-treated mice is shown in B and C. Representative ductal hyperplasia and dysplasia for MMTV-aromatase mice treated with 4-CPA and DMBA are shown in D–F.

Figure 2.

4-CPA does not affect aromatase expression/activity. A, Aromatase mRNA and protein expression were measured using real-time RT-PCR and Western blotting, respectively, in DMBA-exposed MMTV-aromatase transgenic mice with or without 4-CPA treatment. Values are expressed relative to untreated control mice. B, Aromatase enzymatic activity in MCF-7Ca cells was measured as described in Materials and Methods after 72 hr treatment with 3 mM of 4-CPA or vehicle. Results in each figure show the mean $\pm$ s.d. of three determinations.

Figure 3.

4-CPA does not affect hepatic EROD activity. Untreated mice or mice given 4-CPA (6 mg/ml) continuously in their drinking water for 7 days were gavaged with vegetable oil (Con and 4-CPA groups, respectively) or 1 mg DMBA (DMBA and DMBA + 4-CPA groups, respectively). Twenty-four hours later, the mice were sacrificed and their livers assayed for EROD activity as described in Materials and Methods. Values represent the mean $\pm$ s.d. where $n = 8$ for all groups.

Figure 4.

Expression of estrogen and progesterone receptors in MMTV-aromatase transgenic mice. Total RNA was isolated from the mammary glands of solvent-control treated mice (Cont) or those treated with DMBA or DMBA + 4-CPA (DMBA/4-C) as described in “Materials and Methods”, and then subjected to RT-PCR analysis using ER α , ER β , or PR primers. Primers for GAPDH were used as an internal control for normalization purposes. This representative example of receptor expression in the mammary glands of mice from each group shows that significant changes were not induced by the treatments. The bottom graphic was obtained by quantitative densitometry of the example shown.

Table 1

Effect of 4-CPA on the prevention of DMBA-induced mammary tumors in aromatase transgenic mice

Genotype/Treatment	Ductal Hyperplasia (%)	Microscopic Tumors (%)	Palpable Tumors (%) [*]
Aromatase/DMBA	100 (13/13) ^a	85 (11/13) ^{***b}	46 (6/13) ^{***c}
Aromatase/DMBA +CPA	34 (4/12) ^a	8 (1/12) ^b	0 (0/13) ^c
Wild type/DMBA	16 (3/19)	0 (0/19)	0 (0/19)
Wild type/DMBA +CPA	0 (0/11)	0 (0/11)	0 (0/11)

^{*} Palpable tumors observed until the time of termination of experiment (4 months after the last dose of DMBA).

^{**} Tumor types observed: DCIS 40%; adenocarcinoma 60%; Serous-Papilloma 15%. Values with the identical superscript letter are significantly different from each other with the following p values:

^a p<.0005

^b p<.0002,

^c p<.02 (two-tailed Fisher's exact test).