Sensitivity to DNA Damage Is a Common Component of Hormone-Based Strategies for Protection of the Mammary Gland

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Abstract
An early full-term pregnancy significantly reduces the risk of getting breast cancer in women. In animals, this protection can be mimicked by a short-term exposure to physiologic doses of estrogen plus progesterone. Sensitization of p53 and up-regulation of transforming growth factor β are believed to be important aspects of the mechanism by which protection is imparted. Little is known, however, about the use of this pathway in response to other chemopreventive agents. In this article, we investigated the ability of retinoids, such as 9-cis retinoic acid, all-trans retinoic acid, and N-4-hydroxyphenylretinamide (4-HPR), to sensitize the ductal epithelial cells of virgin mammary glands to DNA damage responses. Using a whole-organ culture system, we observed enhanced cell death in response to γ-irradiation in the virgin tissues treated with retinoids for 72 hours. These retinoids were partially dependent on p53 and transforming growth factor β to exert their radiosensitizing effects. However, 4-HPR seemed to sensitize other cells or activate these pathways in a different manner as costimulation with ovarian hormones and 4-HPR was additive, whereas coculture of ovarian hormones and the natural retinoids did not increase amount of death. Taken together, these data suggest that sensitization of the mammary epithelium to p53-dependent apoptosis is a common pathway, which is engaged by retinoids as well as ovarian hormones.

Introduction
Breast cancer is the most common cancer among women in the Western countries. Risk factors include hormonal alterations such as early menarche, late menopause, late first pregnancy, or prolonged use of hormone replacement therapy (1). However, the risk of developing breast cancer can be reduced. A full-term pregnancy before 20 years of age is the most effective natural protection against breast cancer in women. This protection occurs universally among women with different ethnic backgrounds. This indicates that the protective effect does not result from environmental or socioeconomic factors, but from a permanent biological change within the breast caused by pregnancy (2-4). During pregnancy, the mammary gland is exposed to the highest physiologic concentrations of ovarian steroids (estradiol and progesterone), pituitary hormone (prolactin and growth hormone), and placental (hCG as well as placental lactogens) hormone, which cause proliferation and differentiation of the mammary gland (5-7). In rats and mice, the protection of pregnancy can be mimicked by short-term exposure to physiologic doses of estrogen plus progesterone (8). It has been hypothesized that hormone stimulation at a critical stage of mammary development causes cells to change both in the regulatory loops governing proliferation and in the response to DNA damage (9).

Persistent molecular changes induced by parity or hormones in mammary gland have been noted in several laboratories. With the use of DNA microarrays, D'Cruz et al. (2) found that a full-term pregnancy results in the persistent up-regulation of transforming growth factor (TGF) β3, a growth-inhibitory molecule. Ginger et al. (10) noted changes in the chromatin remodeling gene RbAp46 in response to estrogen and progesterone. Sivaraman and coworkers found that in Wistar-Furth rats as well as in BALB/c mice, a 21-day estrogen + progesterone treatment induced the up-regulation and nuclear accumulation of p53 protein. The elevation of p53 was persistent 28 days after hormone withdrawal. Pregnancy induced a similar pattern of expression (11). Minter and colleagues strengthened these findings. They showed that p53-mediated apoptosis in response to γ-radiation was greater in proliferating or estrogen + progesterone–treated mammary glands than in glands of virgin mice (12). Based on these findings, a model has been proposed that p53 activity is impaired during specific periods of mammary gland development, causing the mammary epithelium to be susceptible to cancer. However, prolonged ovarian hormone stimulation, such as during pregnancy, serves to sensitize p53 to the effects of DNA damage. The responsiveness of the p53 pathway to the changes of the endocrine environment associated with pregnancy renders the mammary epithelium refractory to tumours (13).

The main function of activated p53 is to arrest cell cycle and induce apoptosis in response to DNA damage. Medina et al. showed that the deletion of the tumor suppressor gene p53...
results in enhanced tumorigenic risk. They found that the mammary gland from the p53-wild-type and p53-null mice exhibited the same dependence on ovarian hormones for growth. However, estrogen and progesterone strongly enhanced carcinogenesis in the p53-null mammary epithelium (14). This suggests that p53 may be a critical factor for protection of the mammary gland from further accumulation of mutations. Furthermore, recent studies have indicated that pregnancy does not impart protection in p53-null mice, suggesting that p53 is required, in part, for the protective response imparted by hormones (15).

Although an early full-term pregnancy can reduce the risk of women developing breast cancer, it is not a practical approach to breast cancer protection. Chemoprevention is defined as the use of specific chemical substances (natural or synthetic) or their mixtures to suppress, retard, or reverse the process of carcinogenesis. Among these chemicals, the retinoids (derivatives of vitamin A) have shown great promise in the area of cancer chemotherapy and chemoprevention (16). The most extensively studied native retinoids in cancer medicine are all-trans retinoic acid (RA) and 9-cis RA. In humans, all-trans RA has been used to treat acute promyelocytic leukemia with great success. All-trans retinoids, alone or in combination with other drugs, also showed activity in juvenile chronic myeloid leukemia (17). Retinoids inhibit breast cancer growth and augment the action of other breast cancer cell growth inhibitors both in vivo and in vitro (18). However, retinoids cause serious side effects at high doses (19), and thus more selective retinoids have been designed (20). Among these is N-4-hydroxyphenylretinamide (4-HPR), a synthetic retinoid that has been examined as a cancer chemopreventive agent (21). A phase III clinical breast cancer trial found a beneficial trend with 4-HPR in the protection of premenopausal women from contralateral and ipsilateral breast cancer (22).

![Graph A: No irradiation vs. Irradiation](image1)

**FIGURE 1.** The effect of retinoids on sensitivity to γ-irradiation--induced death. A. Mammary glands from mice were treated in whole-organ culture (WOC) with different hormones in the media. After 72 hours of incubation in 37°C incubator, experimental whole-organ cultures (black columns) were treated with 5 Gy γ-irradiation, whereas control whole-organ cultures (white columns) were not. Six hours after the γ-irradiation, all tissues were processed for immunohistochemical staining. TUNEL assays were used to identify the apoptotic cells. Compared with unirradiated treatments, ethanol cannot sensitize cells to death in response to irradiation (P > 0.05), whereas estrogen + progesterone or RAs can sensitize significantly more cells to apoptosis in response to γ-irradiation (*, P < 0.05). However, there is no significant difference among the effects of drug treatments. Among the irradiated treatments (black columns), estrogen + progesterone or RAs can sensitize more cells to death than ethanol. Without irradiation (white columns), 9-cis RA can cause more baseline death than others. Exposure time: 72 hours. Concentrations: 9-cis RA 1 μmol/L, all-trans RA 10 μmol/L, 4-HPR 20 μmol/L, estrogen (E) 3.7 × 10⁻³ μmol/L, progesterone (P) 3.2 μmol/L. B. p53 wild-type mammary glands were treated with ovarian hormones or RAs for 72 hours. Glands of experimental groups were γ-irradiated (black columns), whereas control group glands were not (white columns). Six hours after irradiation, all tissues were fixed and processed and paraffin embedded. p53 staining was done and p53-positive cells were counted. Estrogen + progesterone or RA can induce more p53 nuclear sequestration in response to irradiation (*, P < 0.05). C. Mammary glands from mice were treated in whole-organ culture with different hormones in the media. After 72 hours of incubation in 37°C incubator, experimental whole-organ cultures (black columns) were treated with 5 Gy γ-irradiation, whereas control whole-organ cultures (white columns) were not. Six hours after irradiation, all tissues were processed for immunohistochemical staining. TUNEL assays were used to identify the apoptotic cells. The results were very similar with those observed in A (*, P < 0.05). D. The same was done as in B except that the concentrations of all-trans RA and 4-HPR were lowered to 1 μmol/L. p53 staining was done and p53-positive cells were counted. Estrogen + progesterone or 1 μmol/L RA also can induce more p53 nuclear sequestration in response to irradiation (*, P < 0.05).
Retinoids signal through multiple pathways and seem to exert protection at numerous levels. Pathways shared by the natural and synthetic retinoid family seem to include an increased expression and activation of TGF-β family members (23, 24). Activation of p53 is another pathway by which retinoids may exert their effects. Numerous examples have been cited which indicate that retinoid exposure leads to the induction of p53 stability and transcriptional activities (25-28).

In this article, we addressed the question of whether natural or synthetic retinoids can sensitize the ductal epithelial cells to p53-dependent death induced by irradiation. We showed that DNA damage–induced death by these retinoids is partially dependent on p53 and TGF-β.

**Results**

**Retinoids Sensitize Ductal Epithelial Cells to Apoptosis in Response to Radiation**

Ionizing radiation is a complete mutagen and is a known carcinogen of human breast and rodent mammary glands (29). Here, we treated mouse mammary glands with γ-radiation to damage DNA and impart a response.

In Fig. 1A, terminal deoxynucleotidyl transferase–mediated dUTP nick-end labeling (TUNEL) assays were used to detect apoptotic cells. In response to DNA damage, virgin BALB/c-p53+/+ mammary glands treated with ethanol failed to induce a significant apoptotic response following irradiation compared with unirradiated tissues (P > 0.05). However, when the glands were treated with estrogen + progesterone or with all-trans (10 μmol/L), 9-cis RA (1 μmol/L), or 4-HPR (20 μmol/L) for 72 hours, the percentage of apoptotic cells in the irradiated glands was increased significantly compared with the irradiated ethanol-treated glands. There was, however, no difference among effects of drug treatments (P > 0.05). Interestingly, in the absence of irradiation, 9-cis RA caused more baseline death than others, suggesting that it can cause DNA damage or stress response which can mediate death. However, irradiation still significantly further increased death. All the treatments caused a sensitization to radiation-induced death that was statistically significant (P < 0.05). This suggests that all three RAs can sensitize ductal epithelial cells to respond to DNA damage.

To determine if p53 can respond to radiation in virgin tissue exposed to retinoids, we examined the nuclear accumulation of p53 in the treated glands. The results showed that p53 is activated in the presence of retinoids and radiation. This suggests that p53 is a target for the sensitizing effects of retinoids to radiation-induced death.

**FIGURE 1 continued.**

Photos were taken of the glands with the treatments described in A and B. The dark brown stained cells were TUNEL- or p53-positive cells; the green cells were negative cells counterstained with methyl green.

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p53 protein in response to radiation through immunohistochemistry. In the unirradiated tissues, nuclear accumulation of p53 was not observed. However, in the 9-cis RA–, all-trans RA–, and 4-HPR–treated groups, subsequent radiation caused significant nuclear accumulation of the p53 protein ($P < 0.05$) compared with the ethanol-treated group. No significant difference was found when comparing the efficiencies of all-trans RA and estrogen + progesterone or 9-cis RA and estrogen + progesterone, but 4-HPR was found to be more effective than estrogen + progesterone ($P < 0.05$). Because the pharmacologic significances of high doses of retinoids (10 μmol/L all-trans RA and 20 μmol/L 4-HPR) were not clear, we investigated the effects of retinoids at lower doses (1 μmol/L all-trans RA and 4-HPR). In Fig. 1C and D, we can see the same effect of retinoids at lower doses.

4-HPR and Estrogen + Progesterone Work Additively

Because all three retinoids, as well as the ovarian hormones, sensitize glands to cell death in response to DNA damage, we were interested to determine whether overlapping or distinct pathways were used to sensitize p53 and the cells to DNA damage–induced cell death. If the pathways were distinct, then we would expect to see an additive response. However, if they were similar, we would expect to see no augmentation of the response when both stimuli were presented together. We treated virgin mammary glands with retinoids and ovarian hormones for 72 hours, and then irradiated the tissue. Figure 2 shows that, in response to radiation, estrogen plus progesterone sensitized 5.7% cells to apoptosis in ductal epithelial cells. All-trans RA or 9-cis RA alone sensitized 7.1% and 7.6% cells to death, respectively; however, the levels of apoptosis did not increase when glands were treated with estrogen plus progesterone along with all-trans and 9-cis RAs. In contrast, the response to 4-HPR with estrogen + progesterone seemed to be additive. When we treated glands with estrogen + progesterone and 4-HPR, 9.4% cells were apoptotic, whereas only 6.9% cells (statistically significant, $P < 0.05$) were apoptotic when glands were treated with 4-HPR alone and only 5.7% cells (statistically significant, $P < 0.05$) were apoptotic when glands were treated with estrogen + progesterone. This suggests that 4-HPR sensitizes cells to death through a distinct pathway from the one used by the natural retinoids and ovarian hormones.

Retinoids Are Partially Dependent on p53 to Induce Cell Death in Response to Irradiation

Previous studies have suggested that the hormonal prevention of breast cancer is mediated by active p53. The protein level and nuclear compartmentalization of the p53 gene are largely up-regulated as a result of estrogen and progesterone stimulation followed by γ-radiation (9, 12, 30). The requirement for p53 activity in the sensitizing effects of the retinoids to DNA damage–induced death was tested using p53-null mice. In Fig. 3, we can see that when 9-cis RA–, all-trans RA–, and 4-HPR–treated glands were irradiated, the apoptotic cell numbers of p53-null glands were approximately half of those of p53-wild-type glands exposed to the same retinoid treatments. In the ethanol group, only 0.27% p53-null cells commit apoptosis in response to irradiation, whereas retinoids can still sensitize p53-null cells to commit apoptosis (around 3% apoptotic cells), suggesting that 9-cis RA, all-trans RA, and 4-HPR may be only partially dependent on p53 to sensitize cells to death, indicating other pathways to death are involved. In the case of 4-HPR, we see higher apoptotic cells in p53-null glands with no irradiation treatment than in p53-wild-type glands without irradiation treatment, suggesting that p53 may inhibit the pathway induced by 4-HPR alone ($P < 0.05$).
TGF-β Is Involved in 9-cis RA– and all-trans RA–Mediated Sensitivity to γ-Irradiation

γ-Radiation has been shown to activate TGF-β, and the signaling through this pathway has in turn been shown to activate p53 in mammary epithelium (31). TGF-β has been shown to require estrogen + progesterone induction for p53 sensitization to irradiation (32). Retinoids have been shown to induce the secretion of several isotypes of TGF-β from some cell lines (33, 24). Furthermore, all-trans RA and its analogue BMS453 were shown to increase active TGF-β in human mammary epithelial cells. Growth arrest by BMS453 is believed to occur predominantly through TGF-β–induced mechanism (34). To investigate if TGF-β is involved in the retinoid-mediated sensitivity to γ-irradiation, we added a neutralizing pan-TGF-β antibody to the retinoid-treated media to block the function of TGF-β1, 2, and 3. In Fig. 4A, the 9-cis RA–induced death in the absence of radiation is strikingly reduced, suggesting the addition of this retinoid induces death on its own through a TGF-β–mediated pathway. TUNEL assays also indicate that in the presence of anti-TGF-β antibodies, the radiation-induced death of ductal epithelial cells in mammary glands treated with 9-cis RA, all-trans RA, and 4-HPR was partially reduced (for 9-cis RA–treated group, apoptotic cells dropped from 7.6% to 2%; for all-trans RA–treated group, apoptotic cells dropped from ~7.1% to 3.6%; for 4-HPR–treated group, apoptotic cells dropped from 6.9% to 3.8%). In Fig. 4B, we observed that the neutralizing antibody to TGF-β also reduced the number of p53-positive staining cells in the retinoid-treated tissues, demonstrating that TGF-β is required for the accumulation of p53 under these circumstances.

Discussion

Hormone stimulation at a critical stage of mammary development is an effective protection against breast cancer (9). The mechanism of the protection seems to be related, in part, to the sensitization of p53 in response to DNA damage. Retinoid derivatives have been shown to be effective in preventing contralateral and ipsilateral breast cancer in premenopausal women. The mechanism differs among the retinoid derivatives. Natural retinoids have been shown to activate retinoid receptors and strongly induce growth arrest. The growth arrest seems to be due to a rapid decline of cyclin D1 protein through a proteolytic mechanism (35, 36). Some studies show that retinoid treatment induces a senescence-like phenotype in tumor cells (37). 4-HPR, however, inhibits cell growth through the induction of apoptosis rather than differentiation, likely resulting from an increase of reactive oxygen species and apoptosis-related proteins.

In the present study, we showed that both natural (9-cis RA and all-trans RAs) and synthetic RAs (4-HPR) can sensitize ductal epithelial to death in response to DNA damage caused by γ-irradiation (Fig. 1A). More apoptotic cells were detected in mammary glands treated with estrogen plus progesterone or retinoids in response to radiation. Previous studies had suggested that estrogen and progesterone treatment caused accumulation and nuclear localization of p53 after irradiation (9, 12). Our study extended these findings and showed enhanced DNA damage–induced p53 staining in the nuclei of the cells treated with retinoids as well as estrogen plus progesterone (Fig. 1B).

Interestingly, 4-HPR was found to work additively with ovarian hormones whereas 9-cis RA and all-trans RAs did not show the additive effect (Fig. 2). This suggests that 4-HPR may use a pathway that differs from that used by hormones to sensitize cells to death in response to DNA damage, but 9-cis RA and all-trans RAs may use overlapping pathways. Retinoids have previously been shown to cause the accumulation of p53. In a normal human mammary epithelial cell line, p53 protein expression was enhanced after 72 hours of all-trans RA treatment (38). The human carcinoma cell line NT2/D1 also responds with an up-regulation of p53 that is retinoid receptor dependent (25). Correlative data suggest that retinoids may induce death through the activation of p53. 9-cis and all-trans RAs were found to induce apoptosis in human p53-wild-type soft tissue sarcoma cells lines HTB-82 and HTB-93. However, 9-cis and all-trans RAs were not able to sensitize cells to death in the other soft tissue sarcoma cell lines with mutated p53 (39).
A study done with the subclones of the retinoid-sensitive acute myeloblastic leukemia (OCI/AML-2) cell lines showed 9-cis RA had the most prominent effect of inducing apoptosis and inhibiting cell growth compared with all-trans and 13-cis RAs and that 9-cis RA was most effective at causing the translocation of p53 from cytosol to nucleus (27). Because the p53 pathway is involved in the protective response imparted by hormones (14), and retinoids in cell lines have been shown to induce p53 stability and transcriptional activity (38), we further investigated the relationship between p53 and retinoids in a whole-organ culture system. Mammary glands from wild-type and p53 knockout mice were pretreated with retinoids and then irradiated. The numbers of apoptotic cells were compared with those of the p53-wild-type mammary glands given the same treatments. From Fig. 3, we can see that without p53, apoptosis was greatly reduced in response to γ-radiation. However, all three retinoids could still induce death in ~3% of ductal epithelial cells. This suggests that 9-cis RA, all-trans RA, and 4-HPR are able to sensitize cells to irradiation-induced death through p53-dependent and -independent pathways.

TGF-β inhibits cell proliferation and has been shown to be associated with death in many kinds of epithelial cells. TGF-β1 has been found to be rapidly activated in response to ionizing radiation. Barcellos-Hoff et al. found that radiation-induced apoptosis was a TGF-β1–dependent process in the mammary epithelium. In addition, they found that glands from TGF-β1 knockout animals or addition of TGF-β1 neutralizing antibodies resulted in less p53 activation in response to γ-radiation (31). Previous studies in cell lines have indicated that retinoids can also induce the activity of TGF-β (34, 40). All-trans RA and its analogue BM453 were found to inhibit proliferation and induce more total and active TGF-β in normal human mammary epithelial cells (34). To investigate the involvement of TGF-β in the cell death induced by RAs, we used neutralizing TGF-β antibody to block the TGF-β functioning to see if there were less apoptotic cells and p53-positive cells. In Fig. 4A, we observed that with neutralizing TGF-β antibody, the background death observed in response to 9-cis RA was reduced, suggesting that 9-cis RA causes activation of TGF-β in the mammary gland and that this is responsible for the death observed in the absence of radiation. Reduced cell death was observed in response to radiation with all the retinoid treatments. p53 staining on the same samples indicated that the neutralizing TGF-β antibody decreased the p53-positive cell numbers of 9-cis RA—, all-trans RA—, and 4-HPR—treated tissues followed by irradiation. This suggests that all these retinoids can stabilize or up-regulate p53 through TGF-β in the gland and that this pathway is partially responsible for the increased amount of death. Examples of other pathways which might be involved in the induction of death include the up-regulation of reactive oxygen species, retinoic acid receptor β, and other apoptosis-related genes such as c-myc and c-jun (36, 41).

In summary, our study showed that sensitization of the mammary gland to radiation-induced responses may be a very important pathway shared by numerous agents that act in protecting the mammary gland. Retinoids, as well as ovarian hormones, can sensitize p53 such that radiation will activate TGF-β and result in the accumulation and activation of the p53 protein and death. Unlike ovarian hormones, retinoids may also contain the ability to sensitize cells via other pathways, particularly in the case of 4-HPR. The ability of 4-HPR to enhance the death response in combination with other agents suggests that it may use a different pathway or can affect cells which are not responsive to the other agents. This is an important observation because it indicates that 4-HPR may be used in combination with other chemopreventive agents to enhance protection of the mammary gland. This requirement for both p53 and TGF-β in the death induced by 4-HPR suggests that the activation of p53 by TGF-β is likely to be downstream of the convergence of the retinoid pathway on the sensitization of p53. Future studies will determine if ovarian hormones use the retinoid pathway for the sensitization of p53 or if the ovarian hormones and retinoids are acting on similar effectors.

In conclusion, we are able to show that multiple chemopreventive agents are able to sensitize p53 in the mammary gland to death induced by radiation. This suggests that the responsiveness of p53 is a critical protein target for chemopreventive strategies.

Materials and Methods

Animals, Treatments, and Tissue Procedures

All procedures were conducted in accordance with the NIH Guide for the Care and Use of Laboratory Animals. Virgin BALB/c female mice, Trp53+/+ or Trp53−/−, 8 weeks of age, were housed in plastic cages under a 12-hour dark/light cycle, and were permitted free access to food and water. Fourth inguinal mammary glands were removed from these mice and were maintained in whole-organ culture for 72 hours. Then, the glands were treated with 5 Gy of γ-radiation using a 137Cs irradiator. Six hours after γ-radiation, tissues were processed with gradient alcohol and paraffin embedded.

Whole-Organ Culture

Age-matched 8-week-old virgin BALB/c-p53+/+ and BALB/c-p53−/− mice were sacrificed with CO2 and fourth inguinal mammary glands were isolated aseptically, cut in half longitudinally, and then floated on siliconized lens paper in 60 mm tissue culture dishes containing 2 mL of medium (12). Basic medium was made of serum-free DMEM/F12 buffer with HEPES and NaHCO3, plus insulin (5 μg/mL; Sigma, St. Louis, MO), penicillin (100 units/mL), streptomycin (100 μg/mL), and gentamycin (10 μg/mL; Life Technologies, Grand Island, NY). Hormone-supplemented media contained 1 ng/mL (3.7 × 10−3 μmolar/L) estradiol (Sigma) and 1 μg/mL (3.2 μmolar/L) progesterone. RA-supplemented medium was prepared by adding 1 μmol/L 9-cis RA, 20 μmol/L 4-HPR (Sigma), or 10 μmol/L all-trans RA (Sigma) to the basic medium, respectively. The retinoid plus neutralizing TGF-β antibody–supplemented medium was prepared by adding 0.1 μg/mL pan-specific TGF-β antibody (R&D systems, Inc., Minneapolis, MN) to the RA-supplemented medium at respective concentrations. Negative control medium was prepared by adding a similar percentage of ethanol to the basic medium. Whole-organ cultures were maintained in an incubator supplied with 5% CO2 in air. Seventy-two hours later, whole-organ cultures
were subjected to 5 Gy of γ-radiation using a $^{137}\text{Cs}$ irradiator, whereas control whole-organ cultures were not irradiated. Six hours after irradiation, tissues were fixed in 10% neutral-buffered formalin and then embedded in paraffin.

**Immunohistochemistry**

To detect p53, 4-μm-thick slides were baked at 37°C overnight and were deparaffinized in xylene, rehydrated in graded ethanol, rinsed in TBS, and then subjected to microwave antigen retrieval in 0.1 mol/L citrate buffer for 5 minutes with two sets of 1-minute intervals. Slides were stained by a Dako autostainer with Dako Envision Anti-rabbit kit (Dako Cytomation, Carpinteria, CA). The primary antibody used was rabbit polyclonal CM5 anti-p53 antibody (1:200, Novacatra, Newcastle upon Tyne, United Kingdom). Slides were counterstained with methyl green, dehydrated through 100% ethanol and xylene, and coverslipped. Internal negative controls were treated identically, omitting primary antibody labeling on the tissues from age- and treatment-matched p53-wild-type mice. A minimum of 3,600 mammary gland epithelial cells were counted per treatment (1,200 cells per culture/triplicate cultures per treatment). Differences in the percentage of cells with p53 staining with or without γ-radiation were determined using a test of two portions with a 95% confidence interval. Differences of the effects among drug treatments were tested by ANOVA and Tukey’s multiple comparison procedure at 0.05 α level.

**TUNEL Assay**

Four-micrometer-thick slides were baked at 37°C overnight and were deparaffinized in xylene, rehydrated in graded ethanol, rinsed in TBS, and incubated in 3% hydrogen peroxide in methanol. Terminal deoxynucleotidyl transferase dUTP was used to label the nick ends of fragmented DNA (FragEL DNA Fragmentation Kit, Oncogene Research Products, Cambridge, MA). Sections were stained with 3,3’-diaminobenzidine and counterstained with methyl green. A minimum of 3,600 mammary gland epithelial cells was counted per treatment (1,200 cells per culture/triplicate cultures per treatment). Differences in the percentage of TUNEL-positive cells with or without γ-radiation were determined using a test of two portions with a 95% confidence interval. Differences of the effects among drug treatments were tested by ANOVA method and Tukey’s multiple comparison procedure at 0.05 α level.

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