N-(4-Hydroxyphenyl)retinamide is more potent than other phenylretinamides in inhibiting the growth of BRCA1-mutated breast cancer cells

Introduction

Germline mutations in the breast cancer susceptibility gene BRCA1 have been detected in ~50% of familial breast cancer cases and in almost all cases of combined familial breast and ovarian cancers (1,2). For BRCA1 mutation carriers, the estimated lifetime risk of developing breast cancer is 56–80% and that of developing ovarian cancer is 16–60% (3–5). BRCA1 mutations in breast tumors are associated with aggressive disease, estrogen receptor (ER)-negative status (6–12), and a high tumor grade (10,11). BRCA1 mutation carriers are at an increased risk of developing ipsilateral breast cancer recurrences, both early (2–5 years) and late (>5 years) after breast-conserving therapy (13,14), and are at an increased risk of developing contralateral breast cancer (40–65% lifetime risk; 13,15–17).

Current strategies to reduce the risk of primary and contralateral breast cancer in BRCA1 mutation carriers include preventive surgical intervention and chemoprevention. Surgical options include prophylactic bilateral mastectomy, which reduces breast cancer risk by 90% (18–21), and oophorectomy, which reduces risk by 50% in mutation carriers younger than 50 years of age (22–24). Currently, the only nonsurgical option available to BRCA1 mutation carriers is the use of tamoxifen, which is the only drug approved for the chemoprevention of breast cancer. In the National Surgical Breast and Bowel Project (NSABP), tamoxifen reduced the risk of non-invasive breast cancer by 50%; however, it was not effective in reducing the incidence of ER-negative tumors. Given that BRCA1-related breast cancers are predominantly ER-negative, it was uncertain whether tamoxifen would be effective as a chemopreventive agent in BRCA1 mutation carriers. Results from the National Surgical Adjuvant Breast and Bowel Project (NSABP-P1) Breast Cancer Prevention Trial indicated that tamoxifen might not be effective among healthy BRCA1 mutation carriers (25). However, definitive conclusions were difficult to draw from that study because of the small number of BRCA1 mutation carriers. But, other studies do suggest that tamoxifen may be effective in preventing breast cancer in BRCA1 mutation carriers. In case-control (26) and retrospective (27) studies of contralateral breast cancer in BRCA1 mutation carriers, tamoxifen reduced the incidence of secondary breast cancers by 50%. However, there was insufficient information available to determine whether the ER status of the primary tumor was related to the contralateral risk reduction associated with tamoxifen in these studies. Some smaller studies have even suggested that tamoxifen may have an efficacy in BRCA1 mutation carriers, irrespective of the ER status (28,29). Thus, the benefit of tamoxifen as a preventive therapy for BRCA1-related breast cancer remains unclear.

N-(4-Hydroxyphenyl)retinamide (4-HPR), a synthetic retinoid, exhibits apoptotic and anti-invasive effects in breast cancer cells in vitro (30–33) and inhibits carcinogen-induced...
mammary cancer in animal models (34). Furthermore, 4-HPR accumulates selectively in breast tissue (35–37). 4-HPR also shows great potential as a chemoprotective agent for breast cancer. 4-HPR is well tolerated by patients (38,39) and has a favorable pharmacokinetic profile (37). In a phase III trial, 4-HPR reduced the incidence of local recurrent and contralateral breast cancer in premenopausal women (40). Mutations in BRCA1 are associated with early-onset breast cancer, usually before menopause (20,41). Given the possible protective effect of 4-HPR in reducing secondary malignancies in premenopausal women, the objective of this study was to determine the efficacy of 4-HPR against BRCA1-mutated breast cancer cells. We also compared and contrasted the growth inhibitory effects of a series of 4-HPR analogues (phenylretinamides) in BRCA1-mutated breast cancer cells.

Materials and methods

Reagents

4-HPR and Triton X-100 were purchased from Sigma Chemical Co. (St Louis, MO). Nitric oxide synthase (NOS) NOSII and NOSIII antibodies were purchased from BD Transduction Laboratories (San Diego, CA). The Vectastain Elite ABC Kit, the M.O.M. (mouse on mouse) Immunodetection Kit, hematoxylin, and 3-amino-9-ethyl-carbazole (AEC) were purchased from Vector Laboratories (Burlingame, CA). The NOS inhibitor N\(^5\)-monomethyl-L-arginine (L-NMMA) was purchased from Cayman Chemical (Ann Arbor, MI). Aqua-Mount was purchased from Lerner Laboratories (Pittsburgh, PA). N\(^3\)-Hydroxyphenyl)retinamide (3-CPR), N\(^3\)-carboxyphenyl)retinamide (3-HPR), N\(^3\)-carboxyphenyl)retinamide (3-CPR), N\(^3\)-carboxyphenyl)retinamide (4-CPR) and N\(^3\)-methoxyphenyl)retinamide (4-MPR) were obtained from the National Cancer Institute. Stock solutions (10 mM) of 4-HPR and the 4-HPR analogues were prepared in dimethyl sulfoxide and stored at –20°C. Stock solutions (10 mM) of L-NMMA were prepared in phosphate-buffered saline (PBS) and stored at –20°C. All the reagents were diluted in culture medium to the indicated final concentration.

Cell lines and culture conditions

The human BRCA1-mutated cell line HCC1937 was obtained from American Type Cell Culture (Manassas, VA). This cell line is homozygous for the BRCA1 5382C mutation and has acquired a p53 mutation with wild-type allele loss. Since the HCC1937 cell line is the only human BRCA1-mutated cell line that is commercially available, we also used murine BRCA1-mutated breast tumor cell lines (W0069 and W525) that were established by Dr Deng. These murine cell lines were established from mammary tumors arising from Brca1 conditional knockout mice in a heterozygous p53\(^{-/-}\) background (42,43). These mice carried one conditional and one knockout Brca1 allele (Brca1\(^{KO\text{p}53^C\text{O}}\)). Alternative splicing at exon 11 of Brca1 generates two major transcripts of ~7.2 and 3.9 kb, respectively. The 7.2-kb full-length transcript in the mouse genome was specifically deleted, while leaving the 3.9-kb-exon 11 transcript intact. The murine BRCA1-mutated cell lines also display genetic alterations of p53 at the DNA and protein levels. Cells were cultured in Dulbecco’s modified Eagle medium (DMEM/F12) supplemented with 5% heat-inactivated fetal bovine serum (FBS) at 37°C under 5% CO\(_2\) in a humidified incubator.

Cell growth and nitric oxide (NO) assay

HCC1937, W525 and W0069 breast cancer cells were plated in 1.5 × 10\(^4\), 5 × 10\(^4\), and 2.5 × 10\(^4\) cells/well, respectively, in six-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. After 24 h, the cells were treated with 4-HPR at doses of 1 and 2.5 μM. HCC1937 cells were also treated with the 4-HPR analogues (2-HPR, 3-HPR, 2-CPR, 3-CPR, 4-CPR and 4-MPR) at identical doses. After 5 days of incubation, cell growth was determined by total live cell counts using trypan blue exclusion. Supernatants were collected from untreated and 4-HPR-treated HCC1937, W525 and W0069 cells. Supernatants were also collected from HCC1937 cells treated with the 4-HPR analogues. Aliquots of the supernatants were stored at –80°C for NO determination. Total NO was determined by quantifying nitrite, the stable end product of NO oxidation, spectrophotometrically, using a Colori- metric Non-enzymatic Nitric Oxide Assay Kit (Oxford Biomedical Research, Oxford, MI) as described previously (44). Briefly, 100-μl samples were incubated with 0.5 g of cadmium beads overnight. Cadmium was used to catalyze the reduction of nitrate to nitrite, thus allowing for the measurement of total NO present in the samples. The samples were reacted with an equal volume of Greiss reagent (1% sulfanilamide and 0.1% naphthylethenediamine), and the absorbance was measured at 540 nm in a microplate reader. Sodium nitrite was used as a standard. Nitrite values were normalized for total cell counts and expressed as μM/l million cells. The values were reported as the means (±SD) of experiments performed in triplicate.

Apoptosis assay

HCC1937 cells were plated in 1.5 × 10\(^5\) cells/well, in six-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. After 24 h, the cells were treated with 4-HPR at doses of 1 and 2.5 μM. After 4 days of incubation, cells were harvested and prepared for flow cytometry as described previously (44). Approximately 1 × 10\(^5\) cells were trypsinized, collected by centrifugation at 1500 r.p.m. for 5 min, washed in PBS, and resuspended in 1 ml of PBS. The cell suspension was added to 1 ml of cold 70% ethanol and incubated overnight at –20°C. The cells were then centrifuged at 1500 r.p.m. for 10 min at 4°C and washed twice in PBS, and the pellet was left loose. Approximately 0.5–1 ml of PBS containing RNase (20 μg/ml) and propidium iodide (50 μg/ml) was added to each pellet followed by a 20-min incubation period at room temperature. Flow cytometric analysis was performed using a Coulter Epics profile 488 laser. Apoptotic cells were defined as cells in the sub-G1 phase.

Immunohistochemistry

Immunohistochemistry was used to determine the expression of NOSII and NOSIII in untreated and 4-HPR-treated BRCA1-mutated breast cancer cells. HCC1937 and W0069 cells were plated in 1.5 × 10\(^5\) and 2.5 × 10\(^5\) cells/well, respectively, in six-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS and treated the next day with 4-HPR at doses of 1 and 2.5 μM. After 3 days of incubation, the cells were harvested and suspended in 1 × 10\(^5\) cells/ml in PBS. Cytofins for each treatment were prepared by using 100 μl of the appropriate cell suspension. Slides were quick-fixed in –20°C acetone and stored at –20°C until immunostaining, according to the protocol of Simeone et al. (44). Briefly, the slides were fixed in acetone at –20°C and then incubated in 3% hydrogen peroxide in methanol to block the endogenous peroxidase activity. Next, the slides were incubated in PBS containing 0.05% Triton X-100 to permeabilize the cells. The Vectastain Elite ABC Kit was then used to detect primary NOSII and NOSIII antibody staining in HCC1937 cells. The M.O.M. immunodetection kit was used to detect primary antibody staining in W0069 cells. The M.O.M. kit was used to eliminate any background staining associated with using mouse primary antibodies on mouse tissues. Immunostainings were developed using AEC as a chromogen. Slides were counterstained with hematoxylin and mounted with Aqua-Mount. The slides were analyzed for both intensity and percentage of NOS immunostainings. The intensity of NOS immunostain- ing was classified as follows: -, none; +, light; ++, moderate; or ++++, intense. The percentage of immunostaining (i.e. percentage of positive cells) was classified as follows: -, <5%; 5–25%; 26–50%; 51–75%; 76–95%; or >95%.

Inhibition of NO production

The NOS inhibitor L-NMMA was used to block NO synthesis. HCC1937, W525 and W0069 cells were plated in 1.5 × 10\(^5\), 5 × 10\(^4\) and 2.5 × 10\(^4\) cells/well, respectively, in six-well plates in 2 ml of DMEM/F12 medium supplemented with 5% FBS. The next day, the cells were treated with 4-HPR (1 μM) in the absence and presence of L-NMMA (1, 10, 100 μM) for 5 days. After incubation, the extent of cell growth and NO production was determined as described above.

Statistical analysis

For statistical analysis of the NOS inhibitor experiments, the Shapiro–Wilk test was first performed to assess the normality assumption of the data. The data were normally distributed, and therefore, two-sample t-tests were performed to compare the cell counts and NO production between treatments for each cell line. For each cell line, using the two-sample t-test, the 1 μM 4-HPR + 1 μM L-NMMA, 1 μM 4-HPR + 10 μM L-NMMA and 1 μM 4-HPR + 100 μM L-NMMA groups were compared with the group of untreated cells, as well as the group of cells treated with 1 μM 4-HPR alone. The significance level for each individual comparison was adjusted by the Bonferroni method to account for multiple testing. All analyses were performed using SAS statistical soft- ware at an overall significance level of 0.05.

Results

4-HPR induced apoptosis in BRCA1-mutated breast cancer cells

To assess the effect of 4-HPR on the growth of BRCA1-mutated breast cancer cells, HCC1937, W525 and W0069
cells were treated with 4-HPR. The two experimental doses 1 and 2.5 μM were chosen because they are clinically achievable (37). 4-HPR inhibited the growth of both human and murine BRCA1-mutated breast cancer cells in a dose-dependent manner (Figure 1A). At the 1 μM dose, 4-HPR inhibited the growth of HCC1937, W0069 and W525 cells by 51, 64, and 62%, respectively. The growth of HCC1937, W0069 and W525 cells was inhibited by 86, 90, and 83%, respectively, at the 2.5 μM dose of 4-HPR.

To determine whether apoptosis is related to the growth inhibition found in 4-HPR-treated BRCA1-mutated breast cancer cells, HCC1937 cells were treated with 4-HPR and then assessed for the percentage of apoptotic cells. 4-HPR increased the proportion of apoptotic cells in a dose-dependent manner in HCC1937 cells, as shown by an increase in the sub-G1 peak (Figure 1B). At the 1 and 2.5 μM doses, 4-HPR increased the percentage of apoptotic cells from 2.6% to 21.9 and 46%, respectively. These data indicate that BRCA1-mutated breast cancer cells are sensitive to pharmacologically achievable doses of 4-HPR.

4-HPR was the most potent of the phenylretinamides in inhibiting the growth of HCC1937 breast cancer cells

In addition to 4-HPR, several novel phenylretinamides were tested for their ability to inhibit the growth of HCC1937 cells. The structures of the 4-HPR analogues used in this study are shown in Figure 2A. These new phenylretinamides have hydroxyl, carboxyl or methoxyl residues on carbons 2, 3 and 4 of the terminal phenylamine ring (2-HPR, 3-HPR, 2-CPR, 3-CPR, 4-CPR and 4-MPR). 2-HPR, 3-HPR, 4-CPR and 4-MPR showed no significant inhibitory activity against HCC1937 cells (Figure 2B). 2-CPR and 3-CPR did not significantly inhibit the growth of HCC1937 cells at the 1 μM dose, but did inhibit their growth by 57 and 41%, respectively, at the 2.5 μM dose (Figure 2B). However, 2-CPR and 3-CPR were less potent than 4-HPR at both doses.

4-HPR-induced inhibition was directly correlated with NO production and NOS expression in BRCA1-mutated breast cancer cells

Being the most potent of the phenylretinamides tested against BRCA1-mutated breast cancer cells, 4-HPR was then tested for its mechanism(s) of action. We previously reported that the main mechanism by which 4-HPR, at clinically relevant doses, induces apoptosis in breast cancer cells, is by inducing NOS-mediated NO production (44). NO production was increased in a dose-dependent manner in the three BRCA1-mutated cell lines in response to treatment with 4-HPR (Figure 3A). At the 1 μM dose, 4-HPR increased NO production by 2.2-, 3-, and 3.5-fold in HCC1937, W0069 and W525 cells, respectively. At the 2.5 μM dose, 4-HPR induced 6.2-, 12-, and 8-fold higher levels of NO in HCC1937, W0069 and W525 cells, respectively. The growth inhibitory effects of 4-HPR were directly correlated with increases in NO production in BRCA1-mutated breast cancer cells.

We also reported previously that 4-HPR increases NO production in breast cancer cells by increasing the expression of NOSII and NOSIII (44). To determine which of the NOS isoforms mediated the increase in NO production in BRCA1-mutated breast cancer cells, HCC1937 cells were treated with 4-HPR and subjected to immunohistochemical staining for NOSII and NOSIII. The intensity and percentage of NOSII and NOSIII immunostainings in HCC1937 cells is shown in Figure 3B. NOSII expression was not detected in untreated HCC1937 cells; and <5% of the cells exhibited light NOSII staining when treated with 1 μM 4-HPR. At the 2.5 μM concentration, 4-HPR substantially increased the percentage of NOSII-positive HCC1937 cells. 5–25% of untreated HCC1937 cells exhibited light NOSII staining, whereas it was exhibited in a significantly higher percentage (51–75%) of HCC1937 cells treated with 1 μM 4-HPR. However, increasing the dose of 4-HPR to 2.5 μM did not enhance this effect. Similar effects of 4-HPR on NOSII and NOSIII expression were observed in W0069 cells (data not shown). Thus, 4-HPR mediates the increases in NO production in BRCA1-mutated cells by increasing NOSII and NOSIII expression.
**NO production is essential for 4-HPR-induced growth inhibition of BRCA1-mutated breast cancer cells**

To determine the importance of NO in 4-HPR-induced inhibition, the NOS competitive inhibitor L-NMMA was used to inhibit NO production. HCC1937, W0069 and W525 cells were treated with 4-HPR (1 μM) in the presence and absence of L-NMMA. L-NMMA, at the concentrations used (1, 10, 100 μM), was not cytotoxic to the cells (Table I). L-NMMA effectively suppressed 4-HPR-induced NO production in a dose-dependent manner in the three cell lines (Table I). Viable cell counts were statistically lower ($P < 0.05$) in HCC1937 cells treated with 4-HPR than for those treated with 4-HPR in the presence of 1, 10 and 100 μM L-NMMA (Table I). In HCC1937 cells, L-NMMA at a concentration of 100 μM was able to return NO levels and cell counts to the levels seen in untreated breast cancer cells. Compared with 4-HPR alone, the addition of 10 or 100 μM L-NMMA led to significantly ($P < 0.05$) increased cell counts in W0069 and W525 cultures (Table I). Suppression of NO production desensitized the BRCA1-mutated breast cancer cells to the inhibitory effects of 4-HPR.

**Discussion**

Here we report that 4-HPR, at clinically relevant doses, is a potent inducer of apoptosis in BRCA1-mutated breast cancer cells. Several new phenylretinamide analogues have been developed that show varying degrees of anti-proliferative and apoptotic activity, depending on the cell type treated. In the present study, we found that 4-HPR was the most active of the various phenylretinamide analogues tested in inhibiting the growth of human HCC1937 BRCA1-mutated breast cancer cells.
cancer cells. Although 2-CPR and 3-CPR significantly inhibited the growth of HCC1937 cells at the highest dose (2.5 μM), they were still less potent than 4-HPR. However, 2-CPR was more potent than 4-HPR at inducing apoptosis in several head and neck cancer cell lines (45), in at least 1 lung cancer cell line (45), and in oral squamous carcinoma cells (46). 3-HPR has been found to have growth inhibitory effects comparable to or greater than those of 4-HPR in bladder cancer cell lines (47) and in oral squamous carcinoma cells (46). 4-MPR is the most abundant metabolite of 4-HPR in the circulation and in the breast adipose tissue of patients treated with 4-HPR (36, 37), and it has been suggested that 4-MPR may contribute to the apoptotic effects of 4-HPR. In the present study, however, 4-MPR had no activity against BRCA1-mutated breast cancer cells. These results are in agreement with those of Sheikh et al. (30) and Fanjul et al. (48), who also reported no effect of 4-MPR on the growth of breast cancer cells. Although sensitivity to 4-HPR has been correlated with the appearance of 4-MPR as a metabolite in a panel of breast carcinoma and melanoma cell lines, 4-MPR did not display any growth inhibitory activity in these cell lines (49). In contrast, Kazmi et al. (50) reported that 4-MPR is active against breast cancer cells, but at higher doses of 4-MPR (5–10μM).

In the present study, NO production was essential for 4-HPR to induce apoptosis in BRCA1-mutated breast cancer cells. Indeed, the inhibition of NO production completely blocked the inhibitory effects of 4-HPR in human HCC1937 cells and to a lesser, though still marked, extent in murine BRCA1-mutated cell lines. Since NO production was not returned to
Table I. Effect of nitric oxide inhibition on 4-HPR-induced growth inhibition in BRCA1-mutated breast cancer cells

<table>
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<tr>
<th>4-HPR</th>
<th>l-NMMAa</th>
<th>Cell countb</th>
<th>Nitritec</th>
<th>HCC1937 cells</th>
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<td>(1 μM)</td>
<td>(μM)</td>
<td>(×10⁶)</td>
<td>(μM × 10⁶ cells)</td>
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<td>59.8 ± 1.9</td>
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<td>−</td>
<td>28.9 ± 0.61d</td>
<td>25.0 ± 0.32d</td>
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<td>−</td>
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<td>61.5 ± 1.8</td>
<td>12.4 ± 0.32</td>
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<td>W525 cells</td>
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aNOS competitive inhibitor.
bTotal viable cell counts were determined by trypan blue exclusion. Values shown are means ± SD of experiments performed in triplicate. cTotal NO production was determined by measuring levels of its stable end product nitrite. Nitrite values were normalized to cell number. Values shown are means ± SD of experiments performed in triplicate. dP < 0.05 compared with untreated cells.

untreated levels in the murine cell lines and the toxicity of the NOS inhibitor prevented us from using higher doses, we could not rule out the possibility that 4-HPR might use other mechanisms, in addition to NO production, to inhibit the growth of the murine cell lines. The growth inhibitory effects of the phenylretinamide analogues in HCC1937 cells were also associated with the production of NO. The phenylretinamide analogues 2-HPR, 3-HPR, 4-CPR and 4-MPR did not induce NO production (data not shown) or significantly inhibit growth in HCC1937 cells. In contrast, 2-CPR and 3-CPR increased NO production in HCC1937 cells at a dose of 2.5 μM but not 1 μM (data not shown), which was in direct correlation with the growth inhibition. This suggests that the ability to induce NO is important for the induction of apoptosis in BRCA1-mutated breast cancer cells.

Although 4-HPR increased NO production in BRCA1-mutated cells, the increase was not of the magnitude we typically observe in breast cancer cells that are highly responsive to the growth inhibitory effects of 4-HPR. Previously, we reported that ER-positive breast cancer cells highly sensitive to 4-HPR, produced 24-fold more NO when treated with 2.5 μM 4-HPR (44). In the present study, BRCA1-mutated cells that displayed a similar sensitivity to 4-HPR inhibition and treated at the same dose produced only 6- to 12-fold more NO. BRCA1 is believed to maintain genomic integrity (51) through its involvement in DNA repair and cellular stress responses (52-54). Indeed, it has been shown to modulate the apoptotic response to a variety of chemotherapeutic agents. BRCA1 renders breast cancer cells less sensitive to DNA-damaging agents such as cisplatin, etoposide and bleomycin, but more sensitive to antimicrotubule agents, such as paclitaxel and vinorelbine (55-57). Loss of BRCA1 function leads to defective DNA damage repair, genetic instability, increased apoptosis and tumorigenesis (51). NO is known to affect cellular membranes, proteins and DNA. The reaction of NO with oxygen and oxygen-derived species generates a variety of reactive nitrogen oxide species that are capable of producing a number of cellular effects, including DNA damage, lipid peroxidation and protein degradation (58). Mutations in BRCA1 may enhance the sensitivity of breast cancer cells to 4-HPR and as a result, lower levels of NO may be sufficient to induce an apoptosis in breast cancer cells exhibiting these mutations.

The lifetime risk of breast cancer is increased up to 20-fold in women with BRCA1 germline mutations (59). BRCA1 mutation carriers have higher rates of local relapse and a higher annual risk of contralateral breast cancer (75% VS 0.5-1%) when compared with women with sporadic breast cancer (6,13-15,17,20). Current strategies to reduce breast cancer risk in BRCA1 mutation carriers are limited. The invasive prophylactic bilateral mastectomy and oophorectomy procedures effectively reduce the risk of breast cancer in BRCA1 mutation carriers (19,21-24), but are not widely accepted by women (60). Furthermore, studies have shown that among mutation carriers, prophylactic oophorectomy, which affords less risk reduction, is utilized substantially more often than prophylactic mastectomy (60,61). Nonsurgical preventive therapy in BRCA1 mutation carriers is limited to tamoxifen, whose use in BRCA1 mutation carriers is still controversial, and warrants further investigation. Therefore, there is an urgent need to develop effective chemopreventive strategies to prevent or reverse the development of breast cancers in these women. 4-HPR is one of the most promising retinoids for breast cancer chemoprevention because of its favorable toxicity profile (35) and potent apoptosis-inducing activity (30-32,44). The results of our present in vitro studies indicate the potential of 4-HPR as a chemopreventive agent against BRCA1-mutated breast cancers and warrant its further evaluation in in vivo chemoprevention studies.

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4-HPR inhibits BRCA1-mutated breast cancer cell growth


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