Genistein Prevents *BRCA1* CpG Methylation and Proliferation in Human Breast Cancer Cells with Activated Aromatic Hydrocarbon Receptor

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Abstract

Background: Previous studies have suggested a causative role for agonists of the aromatic hydrocarbon receptor (AhR) in the etiology of breast cancer 1, early-onset (BRCA-1)–silenced breast tumors, for which prospects for treatment remain poor.

Objectives: We investigated the regulation of *BRCA1* by the soy isoflavone genistein (GEN) in human estrogen receptor α (ERα)–positive Michigan Cancer Foundation-7 (MCF-7) and ERα-negative sporadic University of Arizona Cell Culture-3199 (UACC-3199) breast cancer cells, respectively, with inducible and constitutively active AhR.

Methods: In MCF-7 cells, we analyzed the dose- and time-dependent effects of GEN and (–)-epigallocatechin-3-gallate (EGCG) control, selected as prototype dietary DNA methyltransferase (DNMT) inhibitors, on BRCA-1 expression after AhR activation with 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) and in TCDD-washout experiments. We compared the effects of GEN and EGCG on *BRCA1* cytosine-phosphate-guanine (CpG) methylation and cell proliferation. Controls for DNA methylation and proliferation were changes in expression of DNMT-1, cyclin D1, and p53, respectively. In UACC-3199 cells, we compared the effects of GEN and α-naphthoflavone (αNF; 7,8-benzoflavone), a synthetic flavone and AhR antagonist, on *BRCA1* expression and CpG methylation, cyclin D1, and cell growth. Finally, we examined the effects of GEN and αNF on *BRCA1*, AhR-inducible cytochrome P450 (*CYP*)-1A1 (*CYP1A1*) and *CYP1B1*, and *AhR* mRNA expression.

Results: In MCF-7 cells, GEN exerted dose- and time-dependent preventative effects against TCDD-dependent downregulation of BRCA-1. After TCDD washout, GEN rescued BRCA-1 protein expression while reducing DNMT-1 and cyclin D1. GEN and EGCG reduced *BRCA1* CpG methylation and cell proliferation associated with increased p53. In UACC-3199 cells, GEN reduced *BRCA1* and estrogen receptor-1 (*ESR1*) CpG methylation, cyclin D1, and cell growth while inducing BRCA-1 and *CYP1A1*.

Conclusions: Results suggest preventative effects for GEN and EGCG against *BRCA1* CpG methylation and downregulation in ERα-positive breast cancer cells with activated AhR. GEN and flavone antagonists of AhR may be useful for reactivation of *BRCA1* and ERα via CpG demethylation in ERα-negative breast cancer cells harboring constitutively active AhR. *Curr Dev Nutr* 2017;1:e000562.

Keywords: genistein, *BRCA1*, *ESR1*, AhR, DNA methylation, epigenetics, breast cancer, flavonoids, prevention

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Abbreviations used: AhR, aromatic hydrocarbon receptor; *BRCA1*, breast cancer 1, early-onset; CpG, cytosine-phosphate-guanine; *CYP*, cytochrome P450; DNMT, DNA methyltransferase; EGCG, (–)-epigallocatechin-3-gallate; ER, estrogen receptor; *ESR1*, estrogen receptor-1; E2, 17β-estradiol; GEN, genistein; IC_{50}, half maximal inhibitory concentration; M, methylated; MCF-7, Michigan Cancer Foundation-7; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; RPMI, Roswell Park Memorial Institute; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin; U, unmethylated; UACC-3199, University of Arizona Cell Culture-3199; αNF, α-naphthoflavone.

Introduction

The breast cancer 1, early-onset (*BRCA1*) gene encodes a tumor suppressor protein involved in DNA repair and cell cycle control (1). In women who carry a mutated *BRCA1* copy ($BRCA1^{+/-}$), the silencing of the wild-type allele creates a BRCA-1–deficient phenotype, which is associated with a high probability (~60–80%) of developing breast cancer (2, 3). On the other hand, sporadic breast cancers, which represent the majority (~90%) of breast

tumor cases, do not have mutations in *BRCA1* (*BRCA1*$^{+/+}$) but display a "BRCAness" phenotype commonly observed in hereditary *BRCA1* tumors. This phenotype includes absent or markedly reduced concentrations of BRCA-1 (4, 5), loss of estrogen receptor α (ERα), and basal-like pathology subtype (6). Therefore, elucidating the nonmutational mechanisms that contribute to silencing of *BRCA1* has important implications for the prevention of both hereditary and sporadic breast cancers.

Epigenetics refers to modifications in chromatin structure [i.e., histone posttranslational modifications and DNA cytosine-phosphate-guanine (CpG) methylation] and noncoding RNAs (7). Sporadic breast cancers that have hypermethylated *BRCA1* share features with hereditary *BRCA1* mutation tumors [i.e., they tend to be triple-negative with reduced or absent expression of ERα, progesterone receptor (PR), and human epidermal growth factor receptor 2] (8). CpG methylation of *BRCA1* is associated with reduced BRCA-1 expression in 50–60% of higher-histologic-grade sporadic tumors (9, 10). A high degree of correlation (~75%) is generally observed between hypermethylation of the *BRCA1* and estrogen receptor-1 [*ESR1* (ERα)] promoters and reduced expression of BRCA-1 and ERα protein (11, 12), which are invariably associated with resistance to endocrine therapies based on antagonists of the ERα (i.e., tamoxifen) (13). Therefore, main objectives in breast cancer research are to identify the mechanisms linking silencing of *BRCA1* to the development of ERα-negative breast cancers, and clarify whether or not opportunities exist for the prevention of these tumors with dietary components.

Agonists of the aromatic hydrocarbon receptor (AhR) are ubiquitous in the environment and include dietary compounds, metabolites of FAs, industrial xenobiotics, and photoproducts generated in the skin from UV radiation (14). Results from our laboratory document that the *BRCA1* gene is a target for epigenetic regulation by AhR. In the absence of exogenous ligands, AhR forms a transcription complex with ERα and various cofactors (p300, steroid receptor coactivator-1) (15) contributing to the transcriptional activation of *BRCA1* by 17β-estradiol (E2) (16). Conversely, in the presence of agonists, AhR binds to xenobiotic response elements (XRE) with consensus 5′-GCGTG-3′ sequence and harbored in the *BRCA1* gene (17), and disrupts transcriptional activation by E2 (18). This repressive effect is coupled to the recruitment of DNA methyltransferase (DNMT) 1 and methyl binding protein (MBD) 2, loss of acetylated histone (AcH) 4 and AcH3K9 (19), and gain of trimethylated H3K9 (H3K9me3) and DNA CpG methylation (20). Recently, we reported that in rodent mammary tissue (21) and human breast tumors (22) with activated *AhR*, hypermethylation of *BRCA1* was associated with reduced BRCA-1 and ERα expression. These cumulative data raised the question of whether or not dietary compounds that possess DNMT and AhR inhibitory properties may protect against CpG hypermethylation of *BRCA1* and, ultimately, prevent breast tumorigenesis.

Genistein (GEN), a common dietary isoflavone, exerts antagonistic properties toward DNMT enzymes (23, 24). Evidence that it induces BRCA-1 expression in ERα-positive breast cancer cells suggests potential relevance for this isoflavone in cancer prevention (25). Rodent offspring exposed to GEN in utero, through weaning (26), and prepuberty (27, 28) showed reduced mammary tumorigenesis in adult life. Through the inhibition of DNMT activity, GEN was shown to reactivate the expression of various tumor suppressor genes (i.e., ataxia telangiectasia mutated, adenomatous polyposis coli, phosphatase and tensin homolog) in ERα-positive Michigan Cancer Foundation-7 (MCF-7) and ERα-negative M.D. Anderson Cancer Center-metastatic breast cancer-231 (MDA-MB-231) breast cancer cells (29). Here, we investigated the impact of GEN and (–)-epigallocatechin-3-gallate (EGCG) control on BRCA-1 methylation and expression in a human ERα-positive breast cancer cell line (MCF-7) with inducible AhR and hypomethylated *BRCA1*. We extended the BRCA-1 expression and DNA methylation studies with GEN and α-naphthoflavone (αNF; 7,8-benzoflavone), a synthetic flavone and AhR antagonist, to a human ERα-negative cell line model University of Arizona Cell Culture-3199 (UACC-3199) of sporadic breast cancer harboring constitutively activated AhR and hypermethylated *BRCA1* (4).

Methods

Cell culture

Human MCF-7 and UACC-3199 breast cancer cells were obtained from the American Type Culture Collection and maintained, respectively, in DMEM or Roswell Park Memorial Institute (RPMI) 1640 media (Mediatech) supplemented with 10% fetal calf serum (Hyclone Laboratories). The 2,3,7,8-tetrachlorodibenzo-p-dioxin

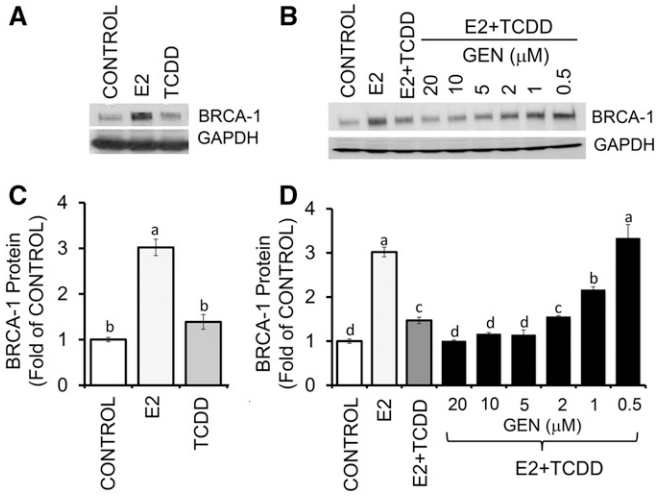

FIGURE 1 GEN prevents repression of BRCA-1 in breast cancer Michigan Cancer Foundation-7 cells with activated aromatic hydrocarbon receptor. Cells were cultured for 72 h in control medium containing E2 (10 nM) or TCDD (10 nM) (A) or E2 + TCDD and various concentrations of GEN (B) as described in Methods. Bands show immunocomplexes for BRCA-1 and the internal standard GAPDH. (C, D) Bars represent means ± SEMs of BRCA-1 expression (fold of control) from 4 (C) and 2 (D) separate experiments performed in duplicate. Labeled means without a common letter differ, $P < 0.05$. BRCA-1, breast cancer 1, early onset; E2, 17β-estradiol; GEN, genistein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

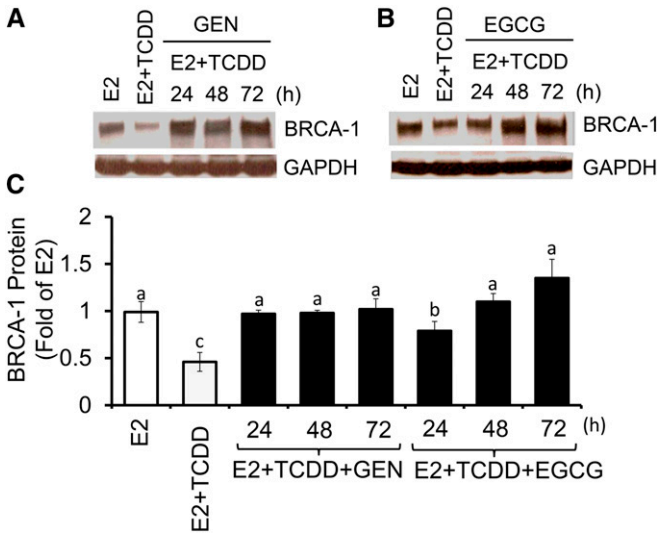

FIGURE 2 Time-dependent effects of GEN and EGCG against BRCA-1 downregulation in breast cancer Michigan Cancer Foundation-7 cells with activated aromatic hydrocarbon receptor. Cells were cultured for 24, 48, and 72 h in control medium containing E2 (10 nM), E2 + TCDD (10 nM), and E2 + TCDD and 1 μM GEN (A) or EGCG (B). Bands show immunocomplexes for BRCA-1 and the internal standard GAPDH. In panel C, bars represent means ± SEMs of BRCA-1 expression (fold of E2) from 2 separate experiments performed in duplicate. Labeled means without a common letter differ, $P < 0.05$. BRCA-1, breast cancer 1, early onset; EGCG, (–)-epigallocatechin-3-gallate; E2, 17β-estradiol; GEN, genistein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

(TCDD) was supplied by the National Cancer Institute, Division of Cancer Biology, Chemical and Physical Carcinogenesis Branch (NIH), and distributed by Midwest Research Institute (contracts 64 CFR 72090 and 64 CFR 28205). GEN, αNF, EGCG, and E2 were obtained from Sigma-Aldrich and solubilized in stock solutions with ethanol, which was added to DMEM or RPMI as the vehicle control. Cells (passages 3–15) were plated in 6-well plates at a density of 5×10^5 cells/well in Phenol-Red free DMEM (MCF-7) or RPMI (UACC-3199) supplemented with 10% charcoal-stripped fetal calf serum (22). For Western blotting, cells were washed with ice-cold PBS and scraped with cold lysis buffer containing protease inhibitor. For proliferation measurements, cells were washed with ice-cold PBS and counted by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) colorimetric assay (Promega), as described previously (30). This assay is based on the conversion of the yellow tretrazolium dye MTT to purple formazan crystals by metabolically active cells. Briefly, 2×10^4 cells were seeded in 96-well tissue culture plates and maintained overnight. Six replicates were assigned to each experimental treatment. After treatment, 15 μL MTT dye solution was added to each well, and the plate was incubated for 4 h at 37°C. Solubilization/stop solution (100 μL) was added for 1 h at room temperature, and the absorbance at 570/650 nm was recorded by using a Synergy HT plate reader (Bio-Tek Instruments).

Western blot analysis

Western blot analyses were performed as previously described (22). Immunoblotting was carried out with antibodies against human BRCA-1 (catalog no. 9010), DNMT-1 (catalog no. 5119), cyclin D1 (catalog no. 92G2), phospho-p53-(Ser20) (catalog no. 9287), and GAPDH (catalog no. 2118) obtained from Cell Signaling Technology, and ERα (catalog no. sc-542) obtained from Santa Cruz Biotechnology. Immunocomplexes were detected by using enhanced chemiluminescence (GE Healthcare Life Sciences). The GAPDH protein was used as an internal control for normalization of protein expression.

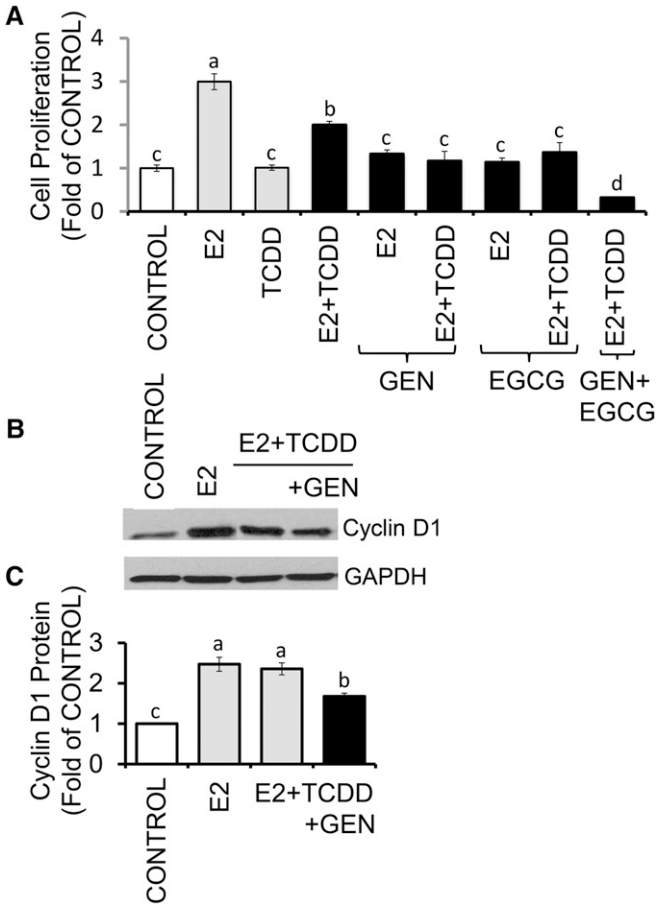

FIGURE 3 GEN and EGCG antagonize E2-induced proliferation of breast cancer Michigan Cancer Foundation-7 cells with activated aromatic hydrocarbon receptor. (A) Cells were cultured for 72 h in control medium or control plus E2 (10 nM), TCDD (10 nM), and E2 + TCDD in the absence or presence of 1 μM GEN or EGCG. Bars represent means ± SEMs of quantitation (fold of control) of proliferation determined by MTT assay from 2 separate experiments with 5 replicates. Panels B and C represent, respectively, immunocomplexes and quantitation (means ± SEMs) from 3 separate experiments performed in duplicate for cyclin D1 and the internal standard GAPDH. Labeled means without a common letter differ, $P < 0.05$. EGCG, (–)-epigallocatechin-3-gallate; E2, 17β-estradiol; GEN, genistein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

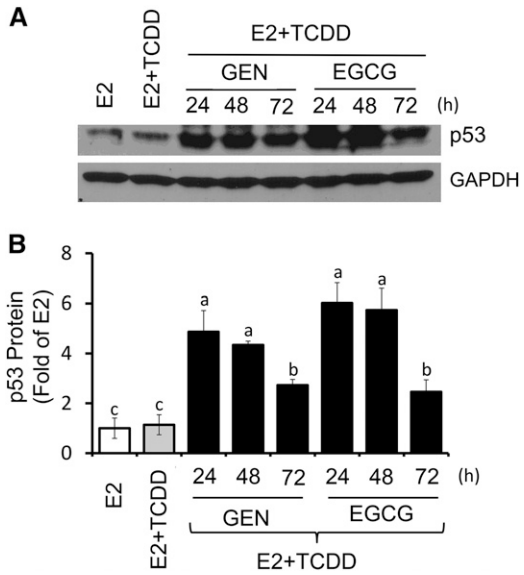

FIGURE 4 GEN and EGCG induce p53 expression in breast cancer Michigan Cancer Foundation-7 cells with activated aromatic hydrocarbon receptor. Cells were cultured for 24, 48, and 72 h in control medium containing E2 (10 nM), E2 + TCDD (10 nM), and E2 + TCDD and 1 μM GEN or EGCG. (A) Bands show immunocomplexes for p53 and the internal standard GAPDH. (B) Bars represent means ± SEMs of p53 expression (fold of E2) from 2 separate experiments performed in duplicate. Labeled means without a common letter differ, $P < 0.05$. EGCG, (–)-epigallocatechin-3-gallate; E2, 17β-estradiol; GEN, genistein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

Promoter CpG methylation

qPCR analysis of human *BRCA1* and *ESR1* promoter CpG methylation was performed as described previously (20) with bisulfonated genomic DNA with the use of the following unmethylated (U)- and methylated (M)-specific primers (Sigma-Aldrich):

BRCA1: U-sense: 5′-TTGGTTTTTGTGGTAATGGAAAAGTGT-3′; and U-antisense: 5′-CAAAAAATCTCAACAAACTCACACCA-3′; M-sense: 5′-TGGTAACGGAAAAGCG-3′; and M-antisense 5′-ATCTCAACGAACTCACGC-3′

ESR1: U-sense: 5′-GGATATGGTTTGTATTTTGTTTGT-3′; and U-antisense: 5′-ACAAACAATTCAAAAACTCCAACT-3′; M-sense: 5′-GGTTTTTGAGTTTTTTGTTTTG-3′; and M-antisense: 5′-AACTTACTACTATCCAAATACACCTC-3′

The qPCR was carried out in a volume of 10 μL consisting of the following master mix: 5 μL SYBER Green mix (Life Technologies), 1 μL each of forward and reverse primers, 2 μL nuclease-free water, and 1 μL bisulfonated genomic DNA. Data from qPCR of bisulfonated DNA were presented as ratios of CpG M:U.

mRNA analyses

Total RNA was purified by using an RNeasy Mini Kit as per the manufacturer's instructions (Qiagen) (22). Concentrations and quality of RNA were verified by using the Nanodrop1000 Spectrophotometer (Thermo Scientific). Equal amounts of total RNA (500 ng) were transcribed into cDNA by using the ISCRIPT supermix kit (Bio-Rad Laboratories). Next, cDNA aliquots were analyzed by qPCR with the use of the SYBR Green PCR Reagents kit (Life Technologies). Briefly, reactions were assayed at a final volume of 25 μL consisting of the following master mix: 12.5 μL SYBR Green mix, 1 μL each of forward and reverse primers, 9.5 μL nuclease-free water, and 1 μL cDNA. Amplification of *GAPDH* mRNA was used for normalization of transcript levels. The primer (Sigma-Aldrich) sequences were as follows—*BRCA1*: sense, 5′-AGCTCGCTGAGACTTCCTGGA-3′; antisense, 5′-CAATTCAATGTAGACAGACGT-3′; cytochrome P450 (*CYP*)-1A1 (*CYP1A1*): sense, 5′-TAACATCGTCTTGGACCTCTTTG-3′; antisense, 5′-GTCGATAGCACCATCAGGGGT-3′; *CYP1B1*: sense,

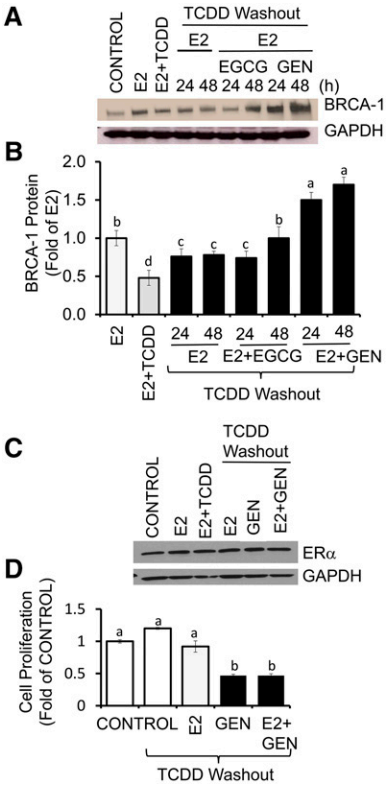

FIGURE 5 Short-term reversal effects of GEN and EGCG on BRCA-1 expression in breast cancer Michigan Cancer Foundation-7 cells with activated aromatic hydrocarbon receptor. Cells were precultured for 48 h in control medium plus E2 (10 nM) or E2 + TCDD (10 nM). Then, media were washed out and cells were cultured for an additional 24 and 48 h in the presence of E2 alone or E2 + 1 μM GEN or EGCG. Panels A and B show, respectively, bands and quantitation (fold of E2; means ± SEMs) of immunocomplexes for BRCA-1 from 3 independent experiments performed in duplicate. (C) Bands show immunocomplexes for ERα and the internal standard GAPDH. (D) Bars represent means ± SEMs of cell proliferation determined by MTT assay (fold of control) from 2 separate experiments with 6 replicates. Labeled means without a common letter differ, $P < 0.05$. BRCA-1, breast cancer 1, early onset; EGCG, (–)-epigallocatechin-3-gallate; ERα, estrogen receptor α; E2, 17β-estradiol; GEN, genistein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

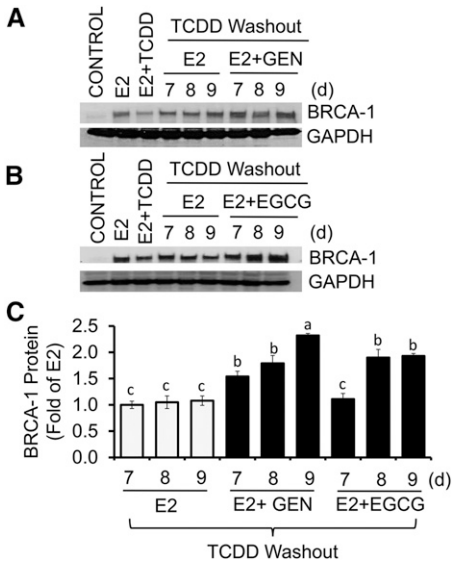

FIGURE 6 Long-term reversal effects of GEN and EGCG on BRCA-1 expression in breast cancer Michigan Cancer Foundation-7 cells with activated aromatic hydrocarbon receptor. Cells were precultured for 48 h in control medium plus E2 (10 nM) or E2 + TCDD (10 nM). Then, media were washed out and cells were cultured for an additional 7, 8, and 9 d in the presence of E2 alone or E2 + 1 μM GEN (A) or EGCG (B). Bands show immunocomplexes for BRCA-1 and the internal standard GAPDH. (C) Bars represent means ± SEMs of BRCA-1 expression (fold of E2) from 3 separate experiments with 3 replicates. Labeled means without a common letter differ, $P < 0.05$. BRCA-1, breast cancer 1, early onset; EGCG, (–)-epigallocatechin-3-gallate; E2, 17β-estradiol; GEN, genistein; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

5′-AACGTCATGAGTGCCGTGTGT-3′; antisense, 5′-GGCCGGTACGTTCTCCAAATC-3′; *AhR*: sense, 5′-GAAGCCGGTGCAGAAAACAG-3′; antisense: 5′-GCCGCTTGGAAGGATTTGAC-3′; and control *GAPDH*: sense, 5′-ACCCACTCCTCCACCTTT-3′; antisense, 5′-CTCTTGTGCTCTTGCTGGG-3′.

Statistical analysis

Densitometry after Western blotting was performed by using Kodak ID Image Analysis Software (Eastman Kodak Company). Statistical analyses were performed by 1-factor ANOVA after assessing data normality by using a Shapiro-Wilk test and variance homogeneity by using Bartlett's test. Post hoc multiple comparisons among all means were conducted by using Tukey's test after main effects and interactions were found to be significant at $P \leq 0.05$. Data are presented as means ± SEMs. When ≥3 means were compared, significant differences are highlighted with different letters (a > b > c).

Results

GEN prevents AhR-dependent downregulation of BRCA-1

In control experiments, we first confirmed that at 24-h post-treatment, E2 (10 nM) induced an increase of ~2.0-fold in BRCA-1 protein expression compared with the vehicle control (**Figure 1**A, B). This dose of E2 was used throughout this study and was similar to that used previously to investigate regulation of BRCA-1 in human breast cancer cells (16) and detected in women around the human menstrual phase and in patients receiving E2 replacement therapy (31, 32). In contrast, as documented previously (19, 20), an equimolar dose (10 nM) of TCDD did not change basal BRCA-1 concentrations (Figure 1B), but it reduced (~50%) E2-induced BRCA-1 expression (Figure 1C, D). This dose of TCDD was used throughout this study and approached the concentration found in blood (33, 34) and lipid tissue (35) of women exposed to environmental AhR agonists. Compared with E2 plus TCDD, doses of 0.5 and 1.0 μM GEN counteracted the repressive effects of TCDD on BRCA-1 expression (Figure 1C, D), whereas 2.0 μM GEN had no protective effects. Conversely, GEN at 5, 10, and 20 μM synergized with TCDD to lower BRCA-1 expression to control levels.

In follow-up experiments, we examined the time-dependent effects of GEN at the 1-μM concentration, which approaches the serum concentration of GEN measured in persons with habitual soy intake and is known to induce BRCA-1 expression in breast cancer cells (25). The cotreatment with GEN protected against

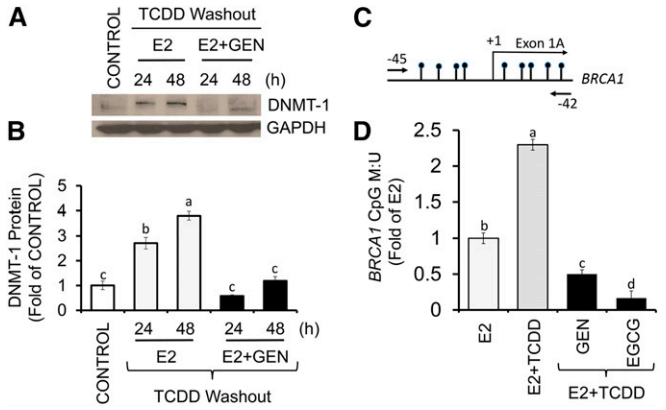

FIGURE 7 GEN antagonizes DNMT-1 expression and *BRCA1* CpG methylation in Michigan Cancer Foundation-7 cells with activated aromatic hydrocarbon receptor. Cells were cultured in control medium for 48 h and after washout of TCDD (10 nM) in the presence of E2 (10 nM) alone or E2 + GEN (1 μM) for 24 and 48 h. (A) Bands show representative immunocomplexes for DNMT-1 and the internal standard GAPDH. (B) Bars represent means ± SEMs of quantitation (fold of control) from 3 independent experiments performed in duplicate of DNMT-1 expression. (C) Location of CpG island flanking exon 1A of the *BRCA1* gene and position (arrows) of oligonucleotides for methylation-specific PCR. (D) *BRCA1* CpG methylation after pretreatment for 12 h with TCDD (10 nM) and post-treatment for 72 h with E2 (10 nM) + TCDD in the presence or absence of 1 μM GEN or EGCG. Bars represent means ± SEMs (fold of E2) from 3 independent experiments performed in quadruplicate. Labeled means without a common letter differ, $P < 0.05$. *BRCA1*, breast cancer 1, early onset; CpG, cytosine-phosphate guanine; DNMT-1, DNA methyltransferase 1; EGCG, (–)-epigallocatechin-3-gallate; E2, 17β-estradiol; GEN, genistein; M:U, methylated/unmethylated; TCDD, 2,3,7,8-tetrachlorodibenzo-p-dioxin.

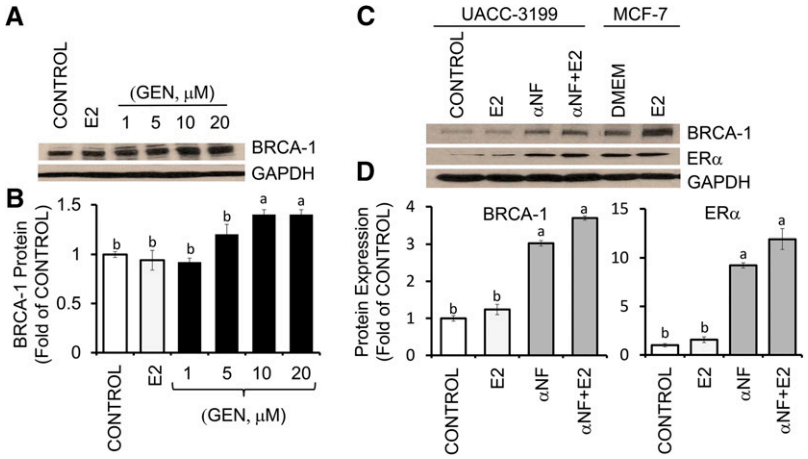

FIGURE 8 Reversal effects of GEN and αNF on BRCA-1 silencing in UACC-3199 breast cancer cells with constitutively active aromatic hydrocarbon receptor. (A) ERα-negative UACC-3199 cells were cultured for 72 h in control medium and in the presence of E2 (10 nM) or various concentrations of GEN. Bands show representative immunocomplexes for BRCA-1 and the internal standard GAPDH. (B) Bars represent means ± SEMs (fold of control) of BRCA-1 expression from 3 independent experiments (n = 3) performed in duplicate. (C) Bands show immunocomplexes for BRCA-1, ERα, and GAPDH protein in UACC-3199 cells cultured for 72 h in control medium and after treatment with E2, αNF (2 μM), or αNF + E2 and in MCF-7 cells cultured in control medium or in the presence of E2. (D) Bars represent means ± SEMs (fold of control) of BRCA-1 and ERα expression from 3 independent experiments performed in duplicate. Labeled means without a common letter differ, $P < 0.05$. BRCA-1, breast cancer 1, early onset; ERα, estrogen receptor α; E2, 17β-estradiol; GEN, genistein; MCF-7, Michigan Cancer Foundation-7; UACC, University of Arizona Cell Culture; αNF, α-naphthoflavone.

TCDD-mediated repression of BRCA-1 at 24 h, an effect that persisted at 48 and 72 h (**Figure 2**A, C). As a positive control for GEN, we used EGCG, which in previous studies was shown to reactivate methylation-silenced genes in cancer cells (36). At equimolar concentrations (1 μM), EGCG counteracted the repressive effects of TCDD and restored BRCA-1 expression to E2 levels by 48 and 72 h (Figure 2B, C).

Growth of MCF-7 cells was induced (~2.0 fold) by E2 within 72 h. The treatment with TCDD had no effects on cell proliferation, whereas the combination of TCDD plus E2 reduced cell growth by ~30% (**Figure 3**A) compared with E2 alone. The cotreatment with GEN plus E2 repressed E2-induced cell growth by ~50%, irrespective of the presence or absence of TCDD. Inhibitory effects (~50%) on E2-induced cell growth were also seen for EGCG, whereas the combination of GEN plus EGCG reduced cell growth by ~80% compared with E2 treatment. As a control for cell proliferation, we examined by Western blotting changes in cyclin D1, whose expression in MCF-7 cotreated with E2 plus TCDD was reduced (~30%) by GEN (Figure 3B, C). In contrast, GEN and EGCG increased the expression of the tumor suppressor p53 (**Figure 4**).

GEN reverses AhR-mediated BRCA-1 downregulation

We next asked whether or not GEN could exert reversal effects on BRCA-1 after the removal of AhR agonist. MCF-7 cells were cultured in the presence of E2 or E2 plus TCDD for 24 h. Then, cells were cultured for an additional 24 and 48 h in fresh control medium containing E2 alone or E2 plus GEN or EGCG. The post-treatment with EGCG at 48 h, but not E2 alone or E2 plus EGCG at 24 h, restored BRCA-1 expression to E2 levels (**Figure 5**A, B). Similarly, the post-treatment with GEN for 24 and 48 h induced (~0.5–0.7 fold) BRCA-1 expression compared with the E2 control. As previously shown (19), the expression of ERα was not influenced by cotreatment with TCDD plus E2 or GEN plus E2 after washout of TCDD (Figure 5C). Compared with control, the treatment with E2 did not induce cell growth (Figure 5D). We did, however, observe a significant reduction (~60%) in cell proliferation with GEN (1 μM), irrespective of the presence or absence of E2. We extended the washout studies to longer time periods and found that the post-treatment for 7, 8, and 9 d with E2 plus GEN rescued BRCA-1 expression above E2 alone (**Figure 6**A, C). Reversal effects on BRCA-1 were also seen for EGCG at days 8 and 9 compared with the E2 control (Figure 6B, C). Overall, these results suggested that post-treatment with GEN and EGCG reversed AhR-dependent downregulation of BRCA-1, albeit with different efficacy (GEN > EGCG).

GEN counteracts AhR-inducible *BRCA1* CpG methylation

DNMT-1 is a maintenance DNA methylation enzyme (7). Therefore, we tested whether or not the BRCA-1 responses to AhR activation and GEN were linked to changes in DNMT-1 expression. We found that after washout of TCDD, the post-treatments of MCF-7 cells with E2 for 24 and 48 h were associated with a 1.8- and 2.8-fold accumulation of DNMT-1, respectively (**Figure 7**A, B). In contrast, the post-treatment with E2 plus GEN reduced DNMT-1 to control levels. These DNMT-1 changes correlated with induction (1.3-fold) in methylation of a CpG island flanking the *BRCA1* transcription start site of exon 1A (Figure 7C, D). Conversely, the post-treatment with GEN lowered *BRCA1* CpG methylation compared with E2 control (~50%) and E2 plus TCDD (~80%). Similarly, the post-treatment with EGCG reduced *BRCA1* methylation compared with the E2

control (~70%) and E2 plus TCDD (~90%). These DNA demethylation results were consistent with earlier reports documenting reactivation of tumor suppressor genes by GEN (23, 37) and EGCG (36).

GEN reverses *BRCA1* silencing associated with constitutively active AhR

ERα-negative UACC-3199 cells harbor constitutive hypermethylated *BRCA1* (4) and active AhR (22). Compared with control, E2 (10 μM) and GEN at 1 and 5 μM did not influence BRCA-1 expression, which, however, was induced ~0.4-fold by 10 and 20 μM GEN (**Figure 8**A, B). As a positive control for AhR inhibition, we used the synthetic flavone αNF (2 μM) (14), which in UACC-3199 cells induced BRCA-1 and ERα expression irrespective of the presence or absence of E2 (Figure 8C, D). Western blots of cell lysates from MCF-7 cells (Figure 8C) provided a positive control for the detection of BRCA-1 and ERα immunocomplexes.

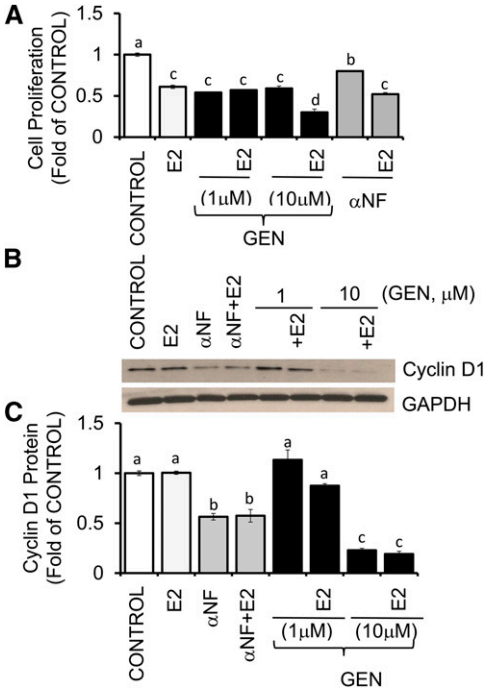

FIGURE 9 GEN and αNF inhibit cell proliferation in UACC-3199 cells with constitutively active aromatic hydrocarbon receptor. (A) UACC-3199 cells were cultured for 72 h in control medium and in the presence of E2 (10 nM), GEN (1 and 10 μM), and E2 + GEN or αNF (2 μM) and E2 + αNF. Bars represent means ± SEMs of quantitation (fold of control) of proliferation determined by MTT assay from 2 separate experiments with 5 replicates. (B) Bands show representative immunocomplexes for cyclin D1 and the internal standard GAPDH in UACC-3199 cells cultured for 72 h in control medium and after treatment with E2, αNF, E2 + αNF, or E2 + GEN. (C) Bars represent means ± SEMs of cyclin D1 quantitation (fold of control) from 2 independent experiments performed in duplicate. Labeled means without a common letter differ, $P < 0.05$. E2, 17β-estradiol; GEN, genistein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; UACC, University of Arizona Cell Culture; αNF, α-naphthoflavone.

The treatment of UACC-3199 cells for 72 h with E2 and GEN (1 and 10 μM) or αNF (2 μM) (**Figure 9**A) reduced cell proliferation by ~50% and 20%, respectively. The antiproliferative effects of GEN (10 μM) and αNF (2 μM) increased to ~70% and 50%, respectively, in combination with E2. The expression of cyclin D1 (Figure 9B, C) was reduced by αNF (~40%) and to a larger degree by 10 μM GEN (~70%), regardless of the absence or presence of E2. Interestingly, although E2 alone reduced growth of UCAA-3199 cells (Figure 9A), it did not elicit measurable changes in cyclin D1 expression compared with the control (Figure 9C).

The upregulation of BRCA-1 protein by GEN in UACC-3199 cells was paralleled by demethylation of *BRCA1* and *ESR1* (ERα), as determined by qPCR amplification of bisulfonated DNA (**Figure 10**A). The treatment with αNF reduced by ~60% *BRCA1* CpG methylation, thus providing a positive control for methylation changes related to AhR. GEN and αNF stimulated *BRCA1* mRNA by ~1.0- and 6.0-fold, respectively, compared with E2 treatment (Figure 10B).

GEN and αNF preferentially induce *CYP1A1* in breast cancer cells with constitutively active AhR

CYP1A1 and *CYP1B1* genes are transcriptional targets for AhR, which is constitutively active in subsets of preclinical and human breast tumors (38, 39). The treatment with GEN alone or in combination with E2 induced (1.2-fold) *CYP1A1* mRNA (**Figure 11**A) but did not affect the expression of *CYP1B1* (Figure 11B) or *AhR* (Figure 10C). As previously shown (22), we found that αNF induced a large increase (~40-fold) in *CYP1A1* (Figure 10A) and a smaller accumulation (1.0- to 1.7-fold) in *CYP1B1* (Figure 11B) while lowering (~50%) *AhR* (Figure 11C) mRNA. Overall, these cumulative data indicated that in ERα-negative breast epithelial cells with constitutively active AhR, both GEN and αNF stimulated BRCA-1 via CpG demethylation, an effect that was associated with reactivation of *ESR1* and preferential activation of *CYP1A1* over *CYP1B1*.

Discussion

Historically, AhR has been investigated for its role in the transcriptional regulation of genes encoding phase I enzymes (e.g., CYP1A1, CYP1B1). However, studies also proposed a causative role for AhR in the etiology of breast tumorigenesis (39). Our published findings obtained from human breast cell lines (17–20) and rodent mammary tissue (21) indicated that the activation of AhR induced a pattern of *BRCA1* methylation around exon 1a that overlapped with that observed in human sporadic breast tumors with reduced BRCA-1 expression (10) and overexpressing AhR (22). Therefore, the first objective of this study was to examine in ERα-positive breast cancer cells with wild-type *BRCA1* (4) and a functional AhR pathway (40) the regulation by GEN of *BRCA1* expression and CpG methylation. To activate AhR, we used the agonist TCDD because of its long half-life (~8 y) (33). Therefore, changes in BRCA-1 expression could be analyzed without the confounding effects due to reactive metabolites. We focused on GEN as a cancer preventative because it is the major isoflavone in soy, and its consumption during early life has been linked to reduced breast cancer risk in Asian (41) and North

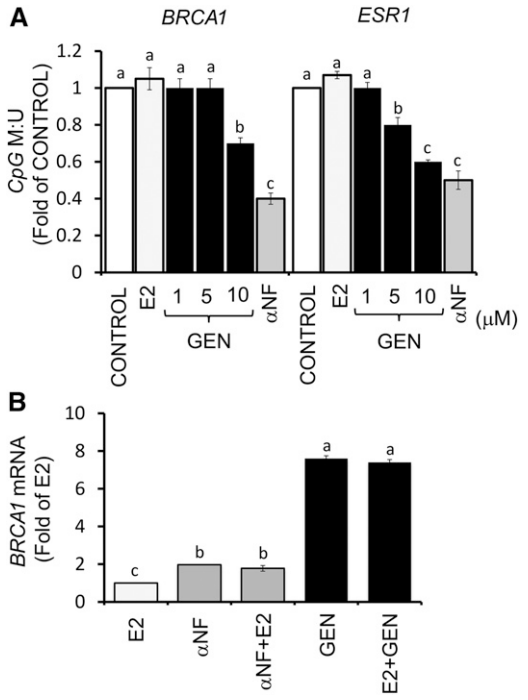

FIGURE 10 Reversal effects of GEN and αNF on *BRCA1* CpG methylation in UACC-3199 cells with constitutively active aromatic hydrocarbon receptor. (A) Bars represent means ± SEMs of quantitation from 2 independent experiments performed in triplicate of *BRCA1* and *ESR1* promoter CpG methylation (fold of control) in UACC-3199 cells after treatment for 72 h with E2 (10 nM), GEN (1, 5, and 10 μM), or αNF (2 μM). (B) Bars represent means ± SEMs of quantitation of *BRCA1* mRNA (fold of E2) by qPCR from 2 independent experiments performed in quadruplicate with GEN (1 μM) and αNF (2 μM) and corrected for *GAPDH* mRNA as the internal standard. Labeled means without a common letter differ, $P < 0.05$. *BRCA1*, breast cancer 1, early onset; CpG, cytosine-phosphate-guanine; *ESR1*, estrogen receptor-1; E2, 17β-estradiol; GEN, genistein; M:U, methylated/unmethylated; UACC, University of Arizona Cell Culture; αNF, α-naphthoflavone.

American (42) women. We found that GEN exerted bimodal effects on MCF-7 cells with activated AhR. Doses ranging from 5 to 20 μM amplified the repressive effects of TCDD on BRCA-1 expression. The latter results were supportive of the tumor-promoting effects previously observed for GEN in ERα-dependent (43) and AhR-dependent (44) mammary tumor models. In contrast, the treatment of MCF-7 cells with lower doses of GEN (0.5 and 1.0 μM) counteracted the effects of TCDD and restored BRCA-1 expression to E2 levels. In previous studies, similar concentrations (0.5–1.0 μM) of GEN were shown to stimulate BRCA-1 expression in breast cancer cells (25). We also found that 1.0 μM GEN antagonized cell proliferation, an effect associated with downregulation of cyclin D1 and upregulation of p53. These results were in accord with earlier reports that highlighted the requirement for cyclin D1 in cell proliferation (45) and with other studies that showed that GEN induced G1 arrest in ERα-positive breast cancer cells (46) and protected against AhR-induced mammary tumorigenesis (27, 28).

The presence of putative binding elements for AhR and ERα in the *DNMT1* gene (47, 48) may explain, at least in part, the increased DNMT-1 expression observed in MCF-7 cells treated with TCDD and E2. Conversely, GEN reduced DNMT-1 expression and *BRCA1* CpG methylation. Repression of DNMT-1 by GEN has been described previously in human breast cancer cells (MCF-7, MDA-MB-231) (29). EGCG provided a positive control for *BRCA1* methylation experiments with GEN in MCF-7 cells. It has been shown to reactivate the expression of methylated-silenced tumor suppressor genes (36).

The second objective of this study was to test if GEN could reactivate *BRCA1* under conditions of constitutive expression and activation of AhR. For this purpose, we turned to the ERα-negative

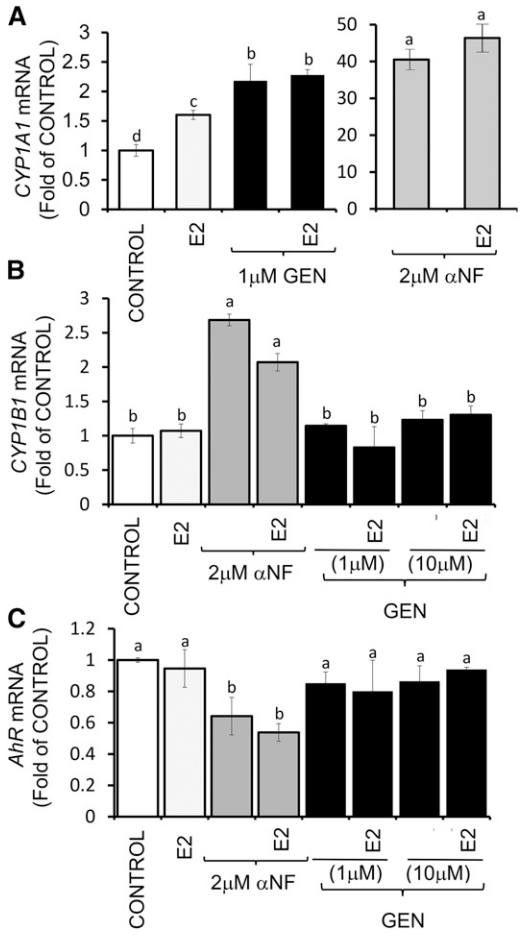

FIGURE 11 GEN and αNF differentially regulate expression of *CYP*s and *AhR* in UACC-3199 cells with constitutively active AhR. UACC-3199 cells were cultured for 72 h in control medium or in the presence of E2 (10 nM) + GEN or αNF. Bars represent means ± SEMs of quantitation (fold of control) of *CYP1A1* (A), *CYP1B1* (B), and *AhR* (C) mRNA from 2 independent experiments performed in quadruplicate and corrected for *GAPDH* mRNA as the internal standard. Labeled means without a common letter differ, $P < 0.05$. *AhR*, aromatic hydrocarbon receptor; *CYP*, cytochrome P450; *CYP1A1*, cytochrome P450-1A1; *CYP1B1*, cytochrome P450-1B1; E2, 17β-estradiol; GEN, genistein; UACC, University of Arizona Cell Culture; αNF, α-naphthoflavone.

UACC-3199 cell line, which was derived from a sporadic human breast tumor harboring wild-type, but hypermethylated, *BRCA1* (4), and constitutively high levels of AhR (22). GEN doses of 10 and 20 μM induced BRCA-1 expression, whereas lower concentrations (1 and 5 μM) had no effects. These data suggest that higher amounts of GEN may be needed to trigger a BRCA-1 response in ERα-negative breast tumors overexpressing AhR. The CpG demethylation of *BRCA1* and *ESR1* observed in UACC-3199 cells with 10 μM GEN may be clinically relevant because comparable serum concentrations have been measured in animals (49, 50) and humans (51, 52) for GEN (~4.5 μM) and for total isoflavones (~3.0–7.0 μM). Previous studies of isoflavones and catechins showed weak affinity for AhR with half maximal inhibitory concentration (IC_{50}) >50–200 μM (53). Therefore, it is unlikely that GEN and EGCG induced demethylation of *BRCA1* through physical interference with AhR. Possibly, the CpG demethylating effects of GEN and EGCG could be due to reduced DNMT-1 expression (29) and activity on *BRCA1* (20) and *ESR1*, as recently reported in ERα-negative breast cancer cells (54).

Previous studies documented the antiproliferative effects of E2 and GEN in ERα-negative breast cancer cells (55). GEN was shown to have greater affinity for ERβ than for ERα, whereas the binding affinities of E2 for ERα and ERβ were equivalent (56). Therefore, E2 and GEN may inhibit the growth of ERα-negative cells by targeting ERβ (57). In support of this idea, agonism and overexpression of ERβ have been shown to attenuate the proliferation of triple-negative breast cancers through cell cycle arrest in the G1 phase (58) and to reduce tumor formation by causing G2 phase arrest (59). Furthermore, CpG demethylation of *BRCA1* and *ESR1* (ERα) by GEN and αNF in UACC-3199 cells suggested that regimens based on dietary flavonoids may have clinical relevance for therapy of tumors with BRCAness. In support of this inference, we noted that GEN and αNF hampered the expression of cyclin D1 and the growth of UACC-3199 cells. αNF is an AhR antagonist at the *BRCA1* gene (60), with IC_{50} approaching ~0.4 μM (53), and a potent aromatase inhibitor (61). It shares structural similarity with GEN and the flavonol galangin (3,5,7-trihydroxyflavone; IC_{50} ~0.2 μM) (53), which was found to block the proliferation of ERα-negative breast cancer cells overexpressing AhR (62).

Finally, we reported that treatment of UACC-3199 cells with GEN and αNF activated preferentially *CYP1A1* with only modest (αNF) or no (GEN) effects on *CYP1B1*. This selective activation of CYP1A1 may have therapeutic relevance because increased CYP1A1 expression associates with reduced basal AhR activity (63) and increased apoptosis (64). In addition, a reduction in 4-hydroxylation of E2 by αNF, a reaction catalyzed by CYP1B1, was shown to reduce the production of the highly carcinogenic metabolite 4-hydroxy-E2 and mammary tumorigenesis (65). In summary, the results of this study suggest preventative effects for GEN and EGCG against proliferation and AhR-mediated *BRCA1* CpG methylation in ERα-positive breast cancer cells. We also presented evidence that GEN and αNF, selected respectively, as a prototype flavone and AhR antagonist, may hold promise for reactivation of *BRCA1* in ERα-negative sporadic breast tumors with constitutively activate AhR.

Acknowledgments

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