A novel prenylflavone restricts breast cancer cell growth through AhR-mediated destabilization of ERα protein

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There is concern that ingestion of dietary phytoestrogens may increase risk of estrogen receptor alpha (ERα)-positive breast cancer. The prenylflavone icaritin, a phytoestrogen consumed in East Asian societies for its perceived beneficial effects on bone health, stimulated the growth of breast cancer (MCF-7) cells at low concentrations. Although acting like an estrogenic ligand, icaritin exerted an unexpected suppressive effect on estradiol-stimulated breast cancer cell proliferation and gene expression at higher concentrations. Like estradiol, icaritin could dose-dependently destabilize ERα protein. However, destabilization of ERα by the estradiol/icaritin combination was profound and greater than that observed for either compound alone. Microarray gene expression analyses implicated aryl hydrocarbon receptor (AhR) signaling for this suppressive effect of icaritin. Indeed, icaritin was an AhR agonist that competitively reduced specific binding of a potent AhR agonist and increased expression of the AhR-regulated gene CYPIA1. When AhR was knocked down by small interfering RNA, the suppressive effect of icaritin on estradiol-stimulated breast cancer cell growth and gene expression was abolished, and ERα protein stability was partially restored. Similarly in an athymic nude mouse model, icaritin restricted estradiol-stimulated breast cancer xenograft growth and strongly reduced ERα protein levels. Overall, our data support the feasibility for the development of dual agonists like icaritin, which are estrogenic but yet, through activating AhR-signaling, can destabilize ERα protein to restrict ERα-positive breast cancer cell growth.

Introduction

Estrogens are well known to have critical roles in development and progression of breast cancer (1). Notably, the majority of breast cancers express the estrogen receptor alpha (ERα), a member of the steroid/nuclear receptor superfamily of ligand-inducible transcription factors and thus respond to the mitogenic actions of estrogen (2). Upon binding to estradiol, ERα undergoes a major conformational change, recruits a multitude of coactivator and corepressor molecules to cognate DNA-response elements located at the promoter/enhancer elements of target genes, to initiate or inhibit the transcription of genes that regulate the growth of breast cancer cells (3). Most early-stage mammary tumors are ERα positive (4) and pharmacotherapy of these cases relies heavily on the ability to block the activity of ERα using specific antagonists or selective downregulators (5). Thus, selective ERα antagonists, such as tamoxifen, are used extensively for treating early-stage breast cancer and have great therapeutic relevance (6).

Besides endogenous estrogens, numerous compounds of botanical origin can exert estrogenic effects in vitro and in vivo. Despite their estrogenicity, consumption of phytoestrogens has been paradoxically associated with reduced risk of breast cancer (7–9). There is intriguing data that different classes of phytoestrogens can exert differential effects on the growth and development of estrogen-driven breast cancer in animal models. The intestinal phytoestrogen enterolactone inhibited the growth of estrogen-stimulated breast cancer xenografts, whereas the soy flavonoid genisteen did not display this suppressive effect (10). Interactions between ER-active compounds may also be dose dependent, as low doses of genistein promoted the development of breast cancer in tamoxifen-treated wild-type erbb-2/neu transgenic mice, whereas higher doses of genistein prolonged tumor latency (11). Among phytoestrogens, prenylflavone compounds originally isolated from beer are among the most ER active compounds (12,13). Prenylflavones are ubiquitous in fruits and vegetables, such as hops, apple, strawberry, persimmon, grape, onion and cucumber (14). In particular, plants of the genus Epimedium (Berberidaceae) are rich in prenylflavones and their extracts are traditionally used in East Asian countries to improve bone health (15,16) and other menopausal symptoms. The main prenylflavone in Epimedium is icarin whose active metabolite is icaritin (Figure 1A). Icaritin has been reported to have antitumor activity in several in vitro studies performed on hepatoma (17), gastric (18), prostate (19) and endometrial (20) cancer cells. Nevertheless, the effects of icaritin on breast cancer cell growth are uncertain. Although icaritin exert estrogenic effects, the prenylflavone has been reported to inhibit (21) or enhance (22) the growth of breast cancer cells. The ultimate effects of icaritin on breast cancer cell growth remain unresolved.

To investigate the hypothesis that icaritin may have significant antiproliferative properties, we studied its effects on breast cancer cell growth and gene expression, alone and when co-administered with estradiol. Counterintuitively we found that although icaritin and estradiol individually increased breast cancer cell proliferation, their joint effect was suppressive compared with the effect of either alone. Investigations indicated a unique mechanism whereby the estradiol and icaritin act jointly to profoundly degrade ERα protein through aryl hydrocarbon receptor (AhR)-mediated pathways leading to suppression of estradiol-stimulated breast cancer cell growth in vitro and in vivo. Thus, icaritin was a dual agonist that could exert estrogenic effects but which restricted excessive estrogenic stimulation through its ability to destabilize ERα protein.

Materials and methods

Cell cultures

Cell lines were obtained from American Type Culture Collection. All cell lines were grown in a 37°C incubator with 5% CO2.

Chemicals

Icaritin (purity >98%) was provided by Dr Wilmar, Schwabe Pharmaceuticals (Karlsruhe, Germany). Dimethylsulfoxide (DMSO), estradiol, 4-hydroxytamoxifen, 3-methylcholanthrene, genistein and MG-132 were purchased from Sigma (Sigma–Aldrich). [3H]-2,3,7,8-Tetrachlorodibenzo-p-dioxin (TCDD, dioxin) (14.5 Ci/mmol) and 2,3,7,8-tetrachlorodibenzo-p-dioxin were obtained from Dr Safe (Texas A&M University).

Cell proliferation assay

The MCF-7 human breast cancer cells were grown in Eagle’s minimum essential medium supplemented with 10% charcoal-treated fetal bovine serum, for 6 days and then plated in 96-well plates at a density of 1 × 104 cells per well. Cells were allowed to attach overnight, followed by addition of 100 μl fresh...
culture media containing test sample(s) at desired concentrations in DMSO. The cells were incubated with treatment media for 6 days with media renewal at 48 h intervals. MDA-MB-231 cells were treated under identical conditions as MCF-7 cells to serve as controls. All experiments were performed in four replicates. Cell proliferation was determined using Cyquant™ Cell Proliferation Assay (Invitrogen) and CellTiter96® Aqueous Non-Radioactive Cell Proliferation Assay (Promega) or counted under an inverted microscope with a hemocytometer. For cell counting, the cells were trypsinized with 100 µl trypsin and 10 µl of the cell suspension was counted on a hemocytometer. Cell proliferations were expressed as fold increase over DMSO-treated cells.

Luciferase reporter assay
pERE-luciferase reporter assay. Stably transfected HeLa-ERE4-Luc cells (23) were treated with the chemicals for 24 h, and the luminescence was measured with Luciferase Assay System (Promega) in a GloMax™ 20/20 Lumimeter (Promega). Transcriptional activity was expressed relative to that obtained with 1 nM estradiol (100%) and values were presented as mean ± standard error of the mean of triplicates.

pXRE-luciferase reporter assay. MCF-7 cells were transfected with pXRE2-luc containing two consensus copies of the xenobiotic response element using Amaxa® Cell Line Nucleofector® Kit V (Amaxa Inc.). At 24 h post transfection, cells were incubated with treatment media for 24 h and the luminescence measured. The results were representative of at least three independent experiments.

Western immunoblotting
MCF-7 cells were seeded in 24-well plates at density of 1 x 10⁵ cells per well. The cells were treated with ligands for 24 h. Whole-cell extracts were harvested using MPER Mammalian Protein Extraction Reagent (Thermo Scientific) and protein concentration determined using the Bio-Rad protein assay (Bio-Rad Laboratories, Hercules, CA). Proteins (10 µg/sample) was resolved on 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis gel and transferred to nitrocellulose membrane using iBLOT (Invitrogen). Blots were probed with anti-ERα antibody and AhR antibodies (Santa Cruz Biotechnology). Anti-β-actin antibody (Sigma–Aldrich) was used as loading control. Densitometry analysis was performed by quantifying the band intensities on the immunoblot using ImageJ (National Institutes of Health).

Knockdown analysis using siRNAs
Validated small interfering RNA (siRNA) directed against a control nontargeting sequence (siScrambled) or AhR (siAhR) were obtained from Qiagen. MCF-7 cells were transfected with siRNA using Amaxa® Cell Line Nucleofector® Kit V following manufacturer’s instructions. Cells were plated in 12-well plates at density of 2 x 10⁵ cells per well after transfection. After 24 h, the cells were incubated with ligands for indicated time points. The knockdown efficiency was verified by real-time PCR quantification of AhR messenger RNA (mRNA) levels or western blotting for protein expression.

ERα ligand binding assay
ERα stable cell line was plated in 24-well plate at a density of 1 x 10⁵ cells per well overnight. The cells were incubated for 19 h with 0.2 nM [3H]estradiol in culture media and the indicated concentrations of icaritin or unlabeled estradiol. The culture media was removed and the cells were rinsed three times with 400 µl ice-cold phosphate-buffered saline. Aliquots (150 µl) of MPER (Mammalian Protein Extraction Reagent; Thermo Scientific, Waltham, MA) were added to lyse cells and the plates shaken for 5 min. Total cell lysates (130 µl) were mixed with 2.5 ml of liquid scintillation counting cocktail and bound [3H]estradiol was measured using a scintillation counter (Wallac, Finland). Protein concentrations of cell lysates were measured. Non-specific binding was assessed by exposing cells to [3H]estradiol in the presence of a 250-fold excess of unlabeled estradiol. Specific binding of icaritin was expressed as disintegrations per minute/mg protein. Each data point represents the mean of three replicates.

AhR competitive ligand-binding assay
Male C57BL/6 mice (20 g) were purchased from Charles River Laboratories (Wilmington, NC) and maintained in a 12 h light: 12 h dark cycle with free access to food and water. Hepatic cytosol was prepared in HEDG buffer [25 mM N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid, pH 7.5, 1 mM ethylene-diaminetetraacetic acid, 1 mM dithiothreitol, 10% (vol/vol) glycerol] as described previously (24). Aliquots of mouse hepatic cytosol (2 mg/ml) were incubated with 2 nM [3H]-TCDD in the presence of DMSO [1% (vol/vol)], 200 nM 2,3,7,8-tetrachlorodibenzofuran or increasing concentrations of icaritin for 2 h in a room temperature water bath. [3H]-TCDD binding in aliquots of incubation (200 µl) was determined by the hydroxyapatite-binding assay as described previously (24). The total amount of [3H]-TCDD-specific binding was obtained by subtracting the non-specific binding from the total binding.

RNA isolation and reverse transcription–PCR
MCF-7 cells were seeded in 24-well plates at density of 1 x 10⁵ cells per well. The cells were incubated with ligands for 6 or 24 h. Total RNA was extracted from cultured cells using RNeasy mini kits (Qiagen). First strand complementary DNA (cDNA) was synthesized from 500 ng of total RNA using SuperScript™ III Reverse Transcriptase (Invitrogen).

Quantitative real-time PCR
The synthesized cDNA was diluted to 1:100, 9 µl of the diluted mixture was used. Quantitative real-time PCR was performed using Taqman Gene Expression Assay and Taqman Fast Universal PCR Mix using StepOnePlus Real-Time PCR System (Applied Biosystems). The expression levels of GREB1,
Fig. 2. Effects of estradiol and icaritin on ER\(\alpha\)-regulated transcription. (A, B, C) Effect of estradiol (E2) and icaritin (ICT) on GREB1 mRNA expression. MCF-7 cells were incubated with indicated ligands alone (A) or in combination with estradiol for (B) 6 h or (C) 24 h. Messenger RNA was extracted and GREB1 mRNA levels were measured using quantitative reverse transcription–PCR. Concentrations used: E2, 100 pM; 4-hydroxytamoxifen (OHT), ICT, 1 \(\mu\)M. * P < 0.05, n = 3. (D) Competitive binding of icaritin to ER\(\alpha\). Hela cells stably expressing ER\(\alpha\) were exposed to 0.2 nM [\(3^H\)]E2 and increasing concentrations of unlabeled E2 or ICT for 19 h at 37°C. Unbound ligand was washed off and ligand-bound [\(3^H\)]E2 was measured using a scintillation counter. Specific counts in disintegrations per minute were obtained by subtracting non-specific binding from total counts and normalized against total protein concentration. (E, F) ER\(\alpha\) reporter gene assay. Hela cells with estrogen-driven reporter (ERE4-Luc) in its genome and stably expressing ER\(\alpha\) were exposed to (E) ICT or E2 or (F) E2 with/without ICT for 24 h. Transcriptional activity was expressed as a percentage of the response induced by E2 (1 nM) and are represented as mean ± standard error of the mean for triplicate readings from one representative experiment.

CYP1A1 and AhR were normalized to that of 18S ribosomal RNA. The results were representative of at least three independent experiments.

Microarray experiment

MCF-7 cells were first grown in phenol red-free Eagle’s minimum essential medium containing 10% charcoal-treated fetal bovine serum in a monolayer culture for a week, with medium change performed on every alternate day. After 7 days, the cells were trypsinized and seeded onto 12-well plates at a density of 1 \(\times\) 10⁵ cells per well. Cells were incubated with test compounds dissolved in phenol red-free cell culture medium containing 5% dextran-coated charcoal-stripped fetal bovine serum for 6 h. Total RNA was extracted using the RNeasy Minikit as described by the manufacturer. Total RNA was amplified to yield biotinylated cDNA and then purified using the Illumina RNA amplification kit (Ambion Inc.). Seven hundred and fifty nanograms of amplified biotinylated cDNA were used. Gene expression analysis was executed using Illumina Sentrix HumanRef-8 V2 BeadChip Arrays containing ~23,000 transcripts. Fluorescence detection was carried out by confocal laser scanning with the Illumina BeadArray Reader at 532 nm and 0.8 μm resolution.

Analysis of global gene expression

GeneSifter software (Geospiza) was used to identify differential gene expression due to ER-dependent regulation. Microarray data from triplicate experiments were subjected to analysis of variance. ER-regulated genes were defined to be those that were changed by ≥2-fold and statistically different from control cells treated with vehicle where the adjusted \(P\) value using the Benjamini and Hochberg procedure (BH-adjusted \(P\) value) is <0.0001.

Xenograft models

Athymic and ovariectomized female nude mice (Balb/c nu/nu), 5- to 6-week-old, were obtained from Harlan (Harlan). After 1 week of acclimatization, an estradiol pellet (0.72 mg, 90 day release, inducing 3-4 nM estradiol in blood, Innovative Research of America) was implanted subcutaneously under the neck using a trochar. After 1 week, an MCF-7 cell suspension containing 2 \(\times\) 10⁶ cells in Matrigel (BD Biosciences) was subcutaneously injected into the two mammary fat pads of each animal. The tumor volume was calculated using the formula \((\text{length}/2) \times (\text{width}/2) \times (\text{width}/2) \times \pi\). Four weeks after estradiol implantation, when tumor volume reached 100 mm³, the mice were randomly divided into four groups such that the tumor size and body weight were similar within each group (eight mice per group). Estradiol pellets were removed from Groups 1 and 2. Group 1 (placebo) was fed with soy-free mouse feed (Altromin, Germany). Group 2 (icaritin) was fed with soy-free mouse feed and injected intraperitoneally with 10 μmol/kg icaritin every 2 days. Groups 3 (estradiol pellet) and 4 (icaritin + estradiol pellet) were treated in the same way as Groups 1 and 2, except estradiol pellets were not removed. Tumor volume and body weight were monitored weekly. Mice were killed after 9 weeks of treatment. Tumors were excised, weighed and processed for western blot analysis. Ethical approval for the study was granted by the Institutional Animal Care and Use Committee, Biological Resource Center, Singapore.

Statistical analyses

Mixed effects linear regression was performed to determine the relationship between treatment and the rate of tumor growth over time. The tumor volume determined in the animal studies was log-transformed and used as the dependent variable. The best-fit coefficient [and confidence interval (CI)] derived was used to calculate average tumor growth per week in each treatment group, and the difference between the estradiol + icaritin and estradiol alone groups was compared. Analyses were performed using Stata version 10.1 (College Station, TX) using the xtmixed command. Values were expressed as mean ± standard error of the mean. Statistical differences among treatment and control groups were evaluated by analysis of variance followed by Bonferroni test for multiple comparisons, using SPSS® (SPSS Inc.). \(P < 0.05\) was considered significant.

Results

Differential effects of icaritin and estradiol/ICaritin on cell proliferation

Breast cancer cells (MCF-7) were exposed to estradiol and icaritin, alone and in combination for 6 days, and their effects on cell growth were measured. Icaritin alone stimulated MCF-7 cell proliferation reaching maximal growth at 5 μM, followed by a decline at 10 μM (Figure 1B). Maximal proliferative effects of icaritin were comparable with those observed with estradiol but with potency that was several orders of magnitude lower. In comparison, 4-hydroxytamoxifen, the active metabolite of tamoxifen, did not increase MCF-7 proliferation. To explore the effects of icaritin in combination with estradiol, MCF-7 cells were exposed to increasing concentrations of icaritin in the presence of a high concentration of estradiol (100 pM) (Figure 1C). In
Icaritin binds AhR to regulate AhR-mediated transcriptional activity and cell proliferation

To identify the mechanism(s) that mediate suppressive effects of icaritin on estradiol-stimulated signaling pathways, we performed gene expression microarray analyses in breast cancer cells exposed to these ligands. The heat map generated using stringent criteria (adjusted \( P < 0.0001 \)) suggested that icaritin induced a pattern of gene expression that was dissimilar with respect to estradiol, genistein and 4-hydroxytamoxifen. GeneSifter software was used to identify differential gene expression. Microarray data from triplicate experiments were subjected to analysis of variance. A differentially regulated gene was defined as one that was activated or inhibited \( \geq 2 \)-fold and statistically different from the control cells treated with vehicle. Adjusted \( P \) value \( <0.0001 \). Refer to Supplementary Table 1, available at Carcinogenesis Online for list of genes that are differentially expressed. CYP1A1 signal was induced by icaritin but not other ligands.

Contrast to the stimulatory effects of estradiol or icaritin alone, the presence of icaritin at concentrations \( \geq 1 \mu M \) suppressed estradiol-stimulated cell growth, measured using two different methods (Figure 1C and D). When cells were exposed to \( 1 \mu M \) icaritin and varying concentrations of estradiol, estradiol even at large concentrations was unable to restore cell growth (Figure 1E). Maximal cell proliferation with the icaritin/estradiol combination was also about one-third lower than that observed with estradiol alone (Figure 1E). Repeated experiments indicated that combination of icaritin at its EC\(_{50}\) or \( C_{\text{max}} \) concentration with 100 \( \mu M \) estradiol can reduce the breast cancer cell growth by 21 and 42\%, respectively, compared with the effects observed with estradiol alone (Supplementary Figure 1 is available at Carcinogenesis Online). Suppressive effects of icaritin on estradiol-stimulated growth was also confirmed by direct cell counting, with icaritin + estradiol treatment resulting in 37.5\% lower cell counts compared with either ligand alone (Figure 1F).

Differential effects of icaritin and estradiol/icaritin on ER-dependent gene expression

To investigate whether inhibition of growth was associated with changes in estrogen-regulated gene expression, we measured the effects of the icaritin/estradiol combination on expression of GREB1, a critical hormone-dependent regulator of breast cancer growth (25,26). As expected, the presence of 4-hydroxytamoxifen reduced GREB1 mRNA levels (Figure 2A). After 6 h of treatment with estradiol, a concentration-dependent increase in GREB1 mRNA level was observed with icaritin treatment (0.01–1 \( \mu M \)). Remarkably, the combination of estradiol/icaritin resulted in a significant reduction in GREB1 mRNA compared with the individual effects of estradiol and icaritin, 65 and 52\%, respectively (Figure 2B). This suppressive effect was lower than that observed for the 4-hydroxytamoxifen/estradiol combination. At 24 h, the suppressive effect was even more pronounced, with GREB1 mRNA levels being 97 and 89\% lower (Figure 2C) than \( E_2 \) or icaritin treatment alone, respectively. Reduced GREB1 expression in the presence of icaritin/estradiol mimicked their suppressive effects on breast cancer cell proliferation as shown in Figure 1B-F. To determine whether icaritin can act directly on the estrogen signaling pathways, competitive ligand-binding assays were performed in cell lines stably expressing ER\( \alpha \). In whole-cell binding experiments, icaritin competitively displaced \([\text{H}]\)estradiol bound to ER\( \alpha \), with an IC\(_{50}\) of 7.3 \( \mu M \) (Figure 2D). In HeLa cells stably transfected with ER\( \alpha \) and estrogen-driven reporter genes, estradiol or icaritin alone increased estrogen-regulated promoter activity (Figure 2E). However, the joint effect of estradiol and icaritin on transcription was unexpected, in that the presence of icaritin (1 \( \mu M \)) suppressed estrogen-stimulated transcriptional activity by about a third and this effect could not be reversed by high concentrations of estradiol (Figure 2F). Unexpected modulatory effect on estrogen-stimulated ER\( \alpha \) promoter activity of the icaritin/estradiol combination was not due to changes in cell number (data not shown).

Icaritin binds AhR to regulate AhR-mediated transcriptional activity and cell proliferation

To identify the mechanism(s) that mediate suppressive effects of icaritin on estradiol-stimulated signaling pathways, we performed gene expression microarray analyses in breast cancer cells exposed to these ligands. The heat map generated using stringent criteria (adjusted \( P < 0.0001 \)) suggested that icaritin induced a pattern of gene expression that was dissimilar with respect to estradiol, genistein and 4-hydroxytamoxifen (Figure 3). In particular, hierarchical clustering placed CYP1A1 in an isolated node, indicating that the expression of CYP1A1 was significantly different from other genes in this panel. Treatment with icaritin increased CYP1A1 mRNA level by 5.5-fold compared with estradiol (Supplementary Table 1 is available at Carcinogenesis Online). Interestingly, CYP1A1 is a key enzyme in xenobiotic metabolism and its expression is activated by the AhR in a ligand-dependent manner through its ability to bind xenobiotic response elements (XREs) in its promoter (24). To investigate the relationship of icaritin and AhR signaling, we performed quantitative reverse transcription–PCR on CYP1A1 mRNA using 3-methylcholanthrene, a known AhR agonist, as a positive control (27). As expected, 3-methylcholanthrene increased CYP1A1 mRNA level by more than 10-fold (Figure 4A). Consistent with the microarray data, icaritin, but not estradiol or 4-hydroxytamoxifen, increased CYP1A1 expression in a concentration-dependent manner. To determine whether icaritin is a cognate ligand for AhR, the ability of ligand to competitively reduce the specific binding of the potent AhR agonist \([\text{H}]\)TCDD to AhR in mouse hepatic cytosol was measured (24). Icaritin dose-dependently displaced \([\text{H}]\)TCDD-specific binding to AhR, with IC\(_{50}\) of 1.28 \( \mu M \) (Figure 4B). Icaritin was also able to stimulate AhR-driven XRE-dependent luciferase reporter gene expression (Supplementary Figure 2 is available at Carcinogenesis Online). Thus, concentrations of icaritin, which inhibited estradiol-simulated breast cancer cell proliferation (Figure 1), were similar to those required for AhR ligand binding and activation of AhR-dependent expression.

To investigate the effects of icaritin-activated AhR activity on breast cancer cell growth, we performed experiments whereby AhR was silenced using RNA interference. Transfection of siAhR reduced the expression of AhR mRNA by \( \sim 85\% \) at 24–48 h time points compared with cells expressing a scrambled siRNA sequence (Supplementary Figure 3 is available at Carcinogenesis Online). As expected, icaritin
suppressed estradiol-stimulated growth in cells expressing scrambled siRNA (Figure 4C). While siRNA suppression of AhR restored the growth of estradiol-stimulated breast cancer cells, it blocked the suppressive effect of high concentrations of icaritin alone on cell growth (Figure 4D), an effect not observed with estradiol (Figure 4E). Silencing of AhR also resulted in reversal of the icaritin-dependent inhibition of estradiol-stimulated GREB1 mRNA expression (Figure 4F). Overall, our data indicated that suppressive effect of high concentrations of icaritin on estradiol-stimulated cell growth and gene expression may be mediated, at least partially, through AhR signaling pathways.

**Estradiol/icaritin destabilized ERα protein through AhR-mediated proteasome pathways**

While the AhR has been shown to repress estrogen- and ER-dependent signaling by multiple mechanisms (28), a major mechanism is the ability of the AhR to promote proteolysis of ERα through assembly of an ERα-specific ubiquitin ligase complex (29,30). To explore the hypothesis that suppressive effects of icaritin on estradiol-stimulated cell proliferation may be related to AhR-directed proteolysis of ERα, we examined the effect of icaritin on ERα protein stability using immunoblotting. In breast cancer cells, icaritin dose-dependently destabilized ERα protein (Figure 5A). Unlike 4-hydroxytamoxifen that stabilized ERα protein, icaritin and estradiol independently reduced ERα protein levels (Figure 5B). However, the most striking effect on ERα stability was observed with the estradiol/icaritin combination. The presence of icaritin/estradiol in combination resulted in 83% and 68% lower ERα protein levels compared with the individual effects of estradiol and icaritin, respectively (P < 0.001) (Figure 5B).

Destabilization of ERα in the presence of icaritin and estradiol was partially reversed by addition of proteasome inhibitor MG132, suggesting that these changes were partially mediated through the proteasomal pathway. These effects could not be attributed to changes in ERα mRNA levels (Supplementary Figure 4 is available at Carcinogenesis Online). The additional effect of icaritin on ERα stability in the presence of estradiol was dose dependent (Figure 5C), consistent with its suppressive effects on the estradiol-stimulated growth of these breast cancer cells.

To test the role of the AhR in ERα stability, we performed experiments whereby AhR was silenced using RNA interference. MCF-7 cells were transfected with siAhR and treated with icaritin and estradiol. Knockdown of AhR gene induced a partial rebound in ERα protein (Figure 5D), congruent with the observation that AhR mediates this action of icaritin. In contrast, knockdown of AhR gene did not restore estradiol-mediated degradation of ERα. Most strikingly, destabilization of ERα protein by icaritin/estradiol was blocked by AhR knockdown (Figure 5D).

**Estradiol/icaritin reduced ERα content to inhibit mammary tumor growth in a xenograft model**

To evaluate the role of estradiol, icaritin and icaritin/estradiol combination on tumor proliferation in vivo, we examined the effects of these treatments on the growth of MCF-7-derived breast tumors in a xenograft mouse model. Growth of tumors in mice injected with icaritin, with or without estradiol implanted, was compared with animals fed a soy-free placebo diet. Animals fed placebo or injected with icaritin alone did not show any growth of xenografts (Figure 6A, upper panel).

**Fig. 4.** Effect of icaritin on AhR-mediated gene expression and cell proliferation. (A) Effect on CYP1A1 gene expression. MCF-7 cells were treated with the AhR agonist 3-methylcholanthrene (3MC) (1 μM), estradiol (E2) (100 pM), 4-hydroxytamoxifen (OHT) (1 μM) or icaritin (ICT) (1 μM) and analyzed using quantitative reverse transcription–PCR (RT–PCR) and normalized against time-matched DMSO and to ribosomal 18S RNA levels, n = 3. (B) Competitive binding of icaritin to AhR. Hepatic cytosol containing AhR was incubated with [3H]TCDD in the presence of DMSO (1%), 2,3,7,8-tetrachlorodibenzofuran (200 nM) or increasing concentrations of icaritin. [3H]TCDD binding was determined by HAP binding. Data was plotted using one-site competitive inhibition model. IC50: 1.28 μM; R² value: 0.9398. (C, D, E) Effect of AhR-silencing on breast cancer cell proliferation. Growth effects of ICT and estradiol, in combination (C) or alone (D, E), on cells whose AhR was silenced (siAhR) was compared with cells transfected with a random siRNA sequence (siScrambled). Cell proliferation was measured using Cyquant cell proliferation assay. (*P < 0.05, comparing cells with/without siAhR). Data points represented the fold increase in cell number over vehicle control (mean + standard error of the mean) of four replicates. (F) Effect of AhR-silencing on GREB1 mRNA expression. Cells were transiently transfected for 24 h with indicated siRNA and incubated with ICT (1 μM) and E2 (100 pM). GREB1 mRNA level was measured using quantitative RT–PCR. *P < 0.05, n = 3.
As expected, tumor xenografts exposed to estradiol grew at a rate of 19.4% (95% CI 16.8–22.1%) per week. In comparison, the increase in tumor size in the estradiol + icaritin group was significantly lower at 11.3% per week (95% CI 8.6–14.1%) (Figure 6A, lower panel). Using the mixed-effect model, reduction in growth with estradiol + icaritin versus estradiol alone was 6.8% (95% CI 3.6–9.8%) (P, 0.001). The excised tumors from the icaritin + estradiol group in week 9 had an average size of 402.9 mm³, whereas average tumor size from estradiol group was 478.4 mm³. There was no apparent change in body weight in animals in each group (data not shown). Xenograft tumors were processed for immunoblot analysis to study the effect of icaritin in ERα stability in vivo. As expected, ERα protein levels were high in tumors from placebo-fed animals, comparable with that observed in MCF-7 cells cultured in vitro (Figure 6B, last lane). Either estradiol or icaritin alone reduced ERα protein levels in tumors, consistent with their in vitro effects observed earlier in Figure 5. Strikingly, the estradiol/icaritin in combination was associated with a nearly complete destabilization of ERα protein, such that ERα protein was almost undetectable (Figure 6B, lane 4), indicating that slower xenograft breast cancer growth in the presence of estradiol/icaritin likely resulted from ERα degradation.

Discussion
In this study, we described an unexpected mechanism whereby the prenyllavone icaritin restricted estradiol-stimulated breast cancer cell growth by activating AhR signaling. Complementing estradiol-mediated degradation pathways, activation of AhR resulted in a profound destabilization of ERα protein, removing a critical driver for the proliferation of breast cancer cells. Icaritin was an estrogenic ligand that stimulated ER-regulated gene expression and could induce the growth of breast cancer cells on its own. However, the effects of icaritin in combination with estradiol were unexpected. The estradiol/icaritin combination induced cell proliferation that was lower than that observed with either icaritin or estradiol alone. Although icaritin and estradiol individually stimulated expression of the ERα-regulated gene GREB1, the estradiol/icaritin combination unexpectedly suppressed GREB1 mRNA expression and was associated with reduced ERE-regulated reporter gene activity. This suggests a role for an additional pathway(s) in mediating the action of icaritin. In microarray studies, CYP1A1 was identified, using stringent criteria, as the gene whose expression was most differentially induced by icaritin. Since CYP1A1 is mainly regulated by AhR/aryl hydrocarbon receptor nuclear translocator acting through multiple consensus XRE sequences in its promoter (31), we deduced that the suppressive effects of icaritin on estradiol-stimulated growth might be mediated through AhR signaling.

Our data indicate that icaritin was an AhR agonist. Icaritin competitively reduced specific binding of the potent AhR agonist TCDD, stimulated XRE-reporter gene activity and increased CYP1A1 mRNA levels. Recently, liganded AhR has been reported to be an ERα-specific E3 ligase, promoting proteosomal degradation of ERα through targeted assembly of the ubiquitin ligase complex, CUL4-BAhR (32).
Icaritin restricts breast cancer cell growth

Since icaritin was able to activate AhR signaling, we examined the hypothesis that ERα protein could be degraded through AhR-mediated proteasomal pathways. Indeed, icaritin could destabilize ERα protein in a concentration-dependent manner. The almost complete degradation of ERα by high concentrations of icaritin provides one possible rationale for the biphasic action of icaritin (Figure 1B and C) on breast cancer cell proliferation, a phenomenon which has also been observed in other laboratories (21). At low concentrations, icaritin being estrogenic was pro-growth. However, higher concentrations of icaritin activated AhR and destabilized ERα protein (Figure 5A), resulting in suppression of cell growth, an effect which can be reversed by silencing AhR (Figure 4D).

Intriguingly, destabilization of ERα by the estradiol/icaritin combination was greater than that observed for either compound alone. ERα protein stability was partially restored when AhR was knocked down by siRNA. While reduction of AhR abolished the restrictive effect of icaritin on estradiol-dependent breast cancer cell growth and gene expression, transfection with siAhR did not reduce estradiol-induced cell proliferation or gene expression. In an athymic nude mouse model, icaritin restricted estradiol-stimulated mammary tumor xenograft growth. It is noteworthy to mention that based on our previous studies, icaritin can be detected at concentrations ranging between 0.5 and 3 μM in the sera of animal models after oral ingestion of the Epimedium standard extract despite constituting only 10% of the total Epimedium standard extract (16,33). Therefore, the concentrations of icaritin used in this study are physiologically achievable and sufficient to bind ERα and AhR as shown in Figures 2D and 4B, respectively, to exert pharmacological action against breast cancer. Overall, our data support the concept that icaritin, through AhR-directed proteasomal degradation, leads to profound degradation of ERα protein thereby restricting estradiol-stimulated breast cancer cell growth and gene expression.

Our study suggests the critical role of ERα stability in the regulation of breast cancer cell proliferation. In common with many short-lived transcription factors that affect cell proliferation, ERα expression is tightly linked to its degradation. Basal ERα ubiquitination and proteasomal degradation are achieved through interaction with Hsc70-interacting protein acting as an E3-ubiquitin ligase (34). In the presence of estradiol, ERα degradation is accelerated, reducing the half-life of ERα protein from 5 days to 3–4 hours (35). Estradiol-mediated turnover of ERα has been linked with activation of extracellular signal-regulated kinase (ERK) 7, the loss of which is associated with breast cancer progression (36). There is also evidence that estradiol-ERα attracts sequentially the coactivator SRC3 and then the 20S proteasome subunit LMP2 (37,38) leading to ubiquitination and 26S proteasome degradation. Our data suggest another distinct pathway whereby an estrogenic ligand can affect ERα stability. In this model, icaritin was also an AhR agonist inducing AhR-mediated mechanisms to target ERα protein for accelerated proteasome degradation.

AhR is expressed in tissues from normal and cancerous breasts (39) and its detoxification functions through induction of the metabolizing enzyme CYP1A1 are widely known (40). Although evolutionary and structurally dissimilar to the steroid hormone receptor family, AhR is the only known ligand-activated member of the basic helix-loop-helix/Per-Arnt-Sim (PAS) family of transcription factors. There is increasing evidence that AhR signaling intersects with ER function and a variety of AhR-ER interactions (28). Moreover, targeted disruption of AhR in the mouse results for degradation, inhibitory ERE elements, squelching mechanisms, ER:AhR and ER:aryl hydrocarbon receptor nuclear translocator interaction (28). Therefore, targeted disruption of AhR in the mouse results in altered ovarian follicle and mammary development (40,41). Recent studies of an in vitro model of human breast cancer indicate that ligand-activated AhR can inhibit cellular motility and can promote expression of markers for cell differentiation, and knockdown of AhR reversed this effect (39). The AhR ligand dioxin is able to reverse the proliferative effects of estrogens in vitro and in vivo (42). Although antiproliferative effects of AhR may be mediated through non-estrogen-dependent pathways by stimulating p27kip1 expression and interfering with E2F-RB (43,44), our study indicates that its ability to degrade ERα protein may be critical. This concept is reminiscent of the finding that AhR, activated by natural ligands in the diet, increases β-catenin degradation to reduce intestinal cancer in the APC mutant mice (45). Further studies are required to determine if consumption of dietary flavonoids that activate AhR may ultimately reduce the risk of estradiol-stimulated breast cancer.

Interestingly, it has also been reported that the combination of estradiol and the AhR agonist TCDD reduced ERα protein more than either estradiol or TCDD alone (28). Administration of TCDD can result in suppression of estradiol-dependent growth of xenotransplanted human breast cancer xenografts. Mice bearing breast cancer (MCF-7) xenografts were fed soy-free feed and randomized to placebo, estradiol (E2) pellet implantation, icaritin (ICT) injected intraperitoneally or receiving both estradiol pellets and ICT injections (E2 + ICT). (A) Tumor volume was measured weekly until mice were killed at week 9. Mixed effects linear regression was performed to determine the relationship between treatment and the rate of tumor growth over time (lower panel). (B) Effect of treatments on ERα protein levels. Tumors were excised and protein extracted. Immunoblot analyses were performed on 20 μg of protein extracts from each treatment group. The intensity of ERα signal was measured, normalized to β-actin and compared with ERα-positive (MCF-7) and -negative (MDA-MB-231) cell extracts. ICT plus E2 induced greater ERα degradation compared with E2 and ICT alone. Results are the means ± standard error of the mean, *, P < 0.05, n = 4. Representative western blot is shown.

**Fig. 6.** Effect of icaritin treatment on human breast cancer cell xenografts. Mice bearing breast cancer (MCF-7) xenografts were fed soy-free feed and randomized to placebo, estradiol (E2) pellet implantation, icaritin (ICT) injected intraperitoneally or receiving both estradiol pellets and ICT injections (E2 + ICT). (A) Tumor volume was measured weekly until mice were killed at week 9. Mixed effects linear regression was performed to determine the relationship between treatment and the rate of tumor growth over time (lower panel). (B) Effect of treatments on ERα protein levels. Tumors were excised and protein extracted. Immunoblot analyses were performed on 20 μg of protein extracts from each treatment group. The intensity of ERα signal was measured, normalized to β-actin and compared with ERα-positive (MCF-7) and -negative (MDA-MB-231) cell extracts. ICT plus E2 induced greater ERα degradation compared with E2 and ICT alone. Results are the means ± standard error of the mean, *, P < 0.05, n = 4. Representative western blot is shown.
MCF-7 breast cancer (46). However, polyhalogenated hydrocarbons such as TCDD and other dioxins are unsuitable as anticancer agents because of their inherent carcinogenic properties (47), although less toxic forms are being examined for such purposes (28). However, it has to be noted that icaritin may inhibit estrogen-responsive breast cancer cell growth through non-genomic mechanisms. Such non-nuclear membrane-initiated mechanisms have been reported to underlie the apoptotic effect of estrogens in bone cells (48). A membrane-associated splice variant of ERα (ERα-36) can exert sustained activation of mitogen-activated protein kinase/extracellular signal-regulated kinase (MAPK/ERK) to cause apoptosis in osteoclasts (49). The MAPK/ERK pathways may even be independent of ERα signaling since icaritin at high doses has been reported to activate MAPK/ERK signaling pathways to cause cell cycle arrest and to inhibit the growth of ERα-negative breast cancer cells (21). Whether icaritin can activate ERα-36 and/or MAPK/ERK signaling to regulate breast cancer cell proliferation will require future studies.

This study presents the novel concept that estrogen prenylflavones like icaritin may activate AhRs signaling to enhance proteasomal degradation of ERα, thus reducing maximal estrogen responsiveness. When icaritin was co-administered with estradiol, the net effect was counterintuitive, in that icaritin/estradiol combination was unable to induce maximal breast cancer cell proliferation due to destabilization of ERα. Our studies predict that the consequence of icaritin administration or its glycosylated precursors to women may not stimulate breast cancer growth but may in fact be suppressive in the presence of endogenous estradiol. Being a dual agonist for both ER and AhR signaling, icaritin may serve as a framework for the development of compounds for estrogen replacement therapy but with reduced risk of undesirable adverse effects due to excessive estrogen stimulation. Given the current challenges in treating aggressive metastatic breast cancer, the clinical development of compounds with enhanced selective AhR activity might provide an effective adjuvant therapy for ERα-positive breast cancers.

Supplementary material
Supplementary Figures 1–4 and Table 1 can be found at http://carcin.oxfordjournals.org/.

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References

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