2’, 3’, 4’-Trihydroxychalcone Changes Estrogen Receptor A Regulation of Genes and Breast Cancer Cell Proliferation by a Reprogramming Mechanism

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2', 3', 4'-Trihydroxychalcone changes estrogen receptor α regulation of genes and breast cancer cell proliferation by a reprogramming mechanism

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ABSTRACT

Background: Menopausal hormone therapy (MHT) is recommended for only five years to treat vasomotor symptoms and vulvovaginal atrophy because of safety issues with long-term treatment. We examined the ability of 2', 3', 4'-trihydroxychalcone (2', 3', 4'-THC) to modulate ERα-mediated responses on gene regulation and cell proliferation to identify drugs that can potentially overcome the adverse effects of estradiol (E2) in MHT so it can be used for long-term therapy to treat prolonged menopausal symptoms and prevent chronic diseases.

Methods: Transfection assays, quantitative real time-polymerase chain reaction, and microarrays were used to evaluate the effects of 2', 3', 4'-THC on gene regulation. Radioligand binding studies were used to determine if 2', 3', 4'-THC binds to ERα. The effects of 2', 3', 4'-THC on cell proliferation and the cell cycle were examined in MCF-7 breast cancer cells using growth curves and flow cytometry. Western blots were used to determine if 2', 3', 4'-THC alters the E2 activation of the MAPK pathway and degradation of ERα. Chromatin immunoprecipitation was used to measure ERα binding to genes.

Results: 2', 3', 4'-THC produced a synergistic response of E2 activation of reporter and endogenous genes in U2OS osteosarcoma cells. Microarrays identified 824 genes that we termed reprogrammed genes because they were not regulated in U2OS-ERα cells unless the cells were treated with 2', 3', 4'-THC and E2 together. 2', 3', 4'-THC blocked the proliferation of MCF-7 cells by preventing the E2-induced stimulation of MAPK activity and c-*MYC* transcription. The antiproliferative mechanism of 2', 3', 4'-THC differs from selective estrogen receptor modulators (SERMs) since 2', 3', 4'-THC does not bind to the E2 binding site in ERα like SERMs.

Conclusion: Our study identified 2', 3', 4'-THC as a reprogramming compound, because it changes the actions of E2 on gene regulation and cell proliferation without competing for the E2

binding site in ERα. The addition of a reprogramming compound such as 2', 3', 4'-THC to estrogens in MHT may offer a new strategy to overcome the adverse effects of estrogen in MHT through a reprogramming mechanism rather than an antagonist action.

Keywords: breast cancer, estradiol, estrogen, estrogen receptor, hormone, cell proliferation, menopause hormone therapy, selective estrogen receptor modulator, chalcone, MCF-7 cells

Background

In women of reproductive age, estrogen is mainly synthesized in the granulosa cells of ovarian follicles. Estrogen is secreted from the ovaries and then transported in the blood to target tissues containing one or both estrogen receptor subtypes (ERα and ERβ) (Dahlman-Wright et al. 2006). After binding to the ER in the cell, estrogen produces biological effects through genomic and non-genomic mechanisms (Nilsson et al. 2001; Levin and Hammes 2016). As women enter menopause, the follicles in the ovaries are depleted due to atresia during each menstrual cycle, and the amount of estrogen produced by the ovaries declines. When estrogen levels begin to drop and fluctuate, short-term symptoms such as hot flashes, night sweats, and mood changes frequently arise. As the years of menopause grow larger, the ovaries will eventually stop producing estrogen, and the duration of estrogen deficiency will increase, accelerating the risk of chronic diseases such as osteoporosis, cardiovascular disease, obesity, type 2 diabetes, and urogenital atrophy (Santen et al. 2010; El Khoudary et al. 2020).

Menopausal hormone therapy (MHT) has been used to prevent short-term menopausal symptoms and certain chronic conditions, such as osteoporosis, cardiovascular disease, and Alzheimer's disease (Santen et al. 2010; Pinkerton 2020). The original MHT regimen contained

only estrogen, which works through a single ligand-single receptor model in which an estrogen molecule binds to the ligand binding domain (LBD) of the estrogen receptor (ER) (Brzozowski et al. 1997; Shiau et al. 1998). Estrogen alone therapy was discontinued for women who have a uterus because it increased the risk of endometrial cancer (Ziel and Finkle 1975) by activating ERα (Shang 2006). Women with a uterus are prescribed a combination of estrogen and progesterone. This combination works through a dual ligand-dual receptor mechanism, where progesterone binds to the progesterone receptor (PR) and estrogen binds to the ER. The progesterone/PR complex effectively blocks the proliferative effects of ERα in the uterus. Although progesterone can prevent endometrial cancer, the Women's Health Initiative (WHI) trial found that the addition of progesterone to estrogen caused a greater risk of breast cancer, heart attack, venous thromboembolism (VTE), and probable dementia compared to estrogen alone (Rossouw et al. 2002; Anderson et al. 2004; Manson et al. 2013). The WHI results provide compelling evidence that progesterone exacerbates some adverse risks caused by estrogens, which is a main reason why the WHI trial was stopped prematurely. The risks of MHT have overshadowed its benefits that include a reduction in hot flashes, mood changes, vulvar and vaginal atrophy, osteoporosis, fractures, type 2 diabetes, and colon cancer (Manson et al. 2013, Mauvais-Jarvis et al. 2017).

After the publication of the WHI results, the number of women using MHT dropped sharply, and clinical recommendations have undergone major changes. MHT is recommended only for the treatment of moderate to severe vasomotor symptoms and vulvar and vaginal atrophy for only 5 years (Martin and Manson 2008; Pinkerton 2020) because a longer treatment time progressively increases the risk of breast cancer, stroke, VTE, and Alzheimer's disease (Rossouw et al. 2002; Savolainen-Peltonen et al. 2019; Collaborative Group on Hormonal Factors in Breast

Cancer 2019). Although considered safe for healthy menopausal women under 60 years of age, short-term therapy lessens the value of MHT because 5 years of treatment is often insufficient. The median duration of hot flashes and night sweats is 7.2 years, and many menopausal women suffer from these symptoms for more than 14 years (Avis et al. 2015). Furthermore, vulvar and vaginal atrophy is a chronic and progressive condition, so long-term estrogen treatment is often necessary. The recommendation that MHT should not be used for the primary prevention of chronic diseases associated with menopause (US Preventive Services Task Force 2017), such as diabetes, obesity, and cardiovascular disease also significantly reduces the impact of current MHT formulations. Menopausal women will only reap the full benefits of estrogen when a safer MHT formula is developed that maintains uterine safety, but does not increase the risk of breast cancer, blood clots, and other adverse effects.

The untoward risks from MHT in menopausal women introduces a pathophysiological conundrum. The risks of breast and uterine cancer, cardiovascular disease, VTE, dementia, Alzheimer's disease, type 2 diabetes (T2DM), and osteoporosis increase with age (D'Agostino et al. 2008; Leening et al. 2014; Cobin and Goodman. 2017; Reidel et al. 2016; Rojas and Stuckey 2016; Winters et al. 2017; Scheyer et al. 2018; Woodward 2019). It is not clear why the use of MHT to restore premenopausal sex hormone levels increases the risk of breast cancer, VTE, probable dementia, and Alzheimer's disease, while reducing the risk of osteoporosis and T2DM (Manson et al. 2013, Mauvais-Jarvis et al. 2017; Savolainen-Peltonen et al. 2019; Collaborative Group on Hormonal Factors in Breast Cancer 2019). In a clinical setting, it is impossible to calculate the actual risk-benefit ratio of MHT for individual women. Therefore, it is important to develop safer drugs to minimize the relative risk for each woman who take MHT.

A new approach to improve the safety of MHT is to combine estrogens with the selective estrogen receptor modulator (SERM) bazedoxifene, instead of progesterone (Komm et al. 2014, Archer et al. 2016). Bazedoxifene prevents the binding of estrogen to ERα, thereby preventing the proliferation of cells in the uterus. It is not clear whether bazedoxifene will prevent the proliferation of breast cells by estrogen to reduce the risk of breast cancer. This combination is approved only for short-term treatment of vasomotor symptoms so it does not broaden the clinical indications or extend the therapeutic window compared to the combination of estrogen and progesterone. Since the estrogen/progesterone and estrogen/SERM combinations are limited to short-term therapy for the treatment of vasomotor symptoms and vulvovaginal atrophy, there is a clear need to develop safer MHT formulas that can be used for long-term therapy to treat women with prolonged menopausal symptoms and prevent chronic diseases associated with menopause. In order to develop safer drugs for long-term MHT, alternative classes of drugs need to be discovered that do not operate through the dual ligand-dual receptor mechanism like estrogen and progesterone or by acting as an antagonist that blocks the binding of estrogens to ER as SERMs do. Our goal is to combine estrogens with a drug that has a different mechanism of action than progesterone and SERMs to overcome the adverse effects of estrogens. In this study, we investigated if 2', 3', 4'-trihydroxychalcone could be an alternative to progesterone and SERMs in MHT by changing the effects of E2 on ERα-mediated gene regulation and breast cancer cell proliferation by a reprogramming mechanism.

Materials and methods

Compounds

2', 3', 4'-trihydroxychalcone and the other chalcones were obtained from Indofine Chemical Company (Hillsborough Township, NJ). The structure of 2', 3', 4'-trihydroxychalcone (Lot No: 93033) was verified by the QB3 NMR Facility at the University of California, Berkeley. ICI 182,780 was obtained from Tocris Bioscience (Minneapolis, MN). All other compounds were obtained from Sigma-Aldrich (St. Louis, MO) or Thermo Fisher Scientific (Waltham, MA). The chalcones, steroids, ICI 182,780, tamoxifen, and raloxifene were dissolved in ethanol and used at a final concentration of 0.1%.

Cell culture

Human U2OS cells expressing a tetracycline-regulated ERα (U2OS-ERα) were prepared, characterized, and maintained as previously described (Tee et al. 2004). The cells were maintained in phenol red-free Gibco DMEM/F-12 (Thermo Fisher Scientific, Waltham, MA) supplemented with 5% charcoal-dextran stripped fetal bovine serum (FBS, Gemini Bio Products, West Sacramento, CA), 100 units/mL penicillin and streptomycin, 50 μg/mL fungizone, and 2 mM of glutamine. To maintain stable transfected cells, 50 μg/mL hygromycin B (Invitrogen) and 500 μg/mL of zeocin (Invitrogen) were included in culture media. MCF-7 breast cancer cells were maintained in phenol red-free DMEM/F-12 supplemented with 10% FBS, 100 units/mL of penicillin and streptomycin, 50 μg/mL fungizone and 2 mM of glutamine. For experiments, the culture medium was replaced with 5% stripped FBS in phenol red-free DMEM/F12.

Cell transfection and luciferase reporter assay

U2OS cells (wild type) were maintained in 5% charcoal-dextran stripped FBS (Gemini Bio Products, West Sacramento, CA). Cells were transfected with 3 μg of a plasmid containing the ERE upstream of the minimal thymidine kinase luciferase promoter (ERE-TK-Luc) or 3 copies of the ER regulatory element (Levy et al. 2007) in the NKG2E promoter (NKG2E-TK-Luc) and 1 μg of expression vectors for ERα by electroporation as previously described (An et al. 2001). MCF-7 cells were maintained for 3 days in 5% charcoal-dextran stripped FBS prior to transfection by electroporation with 5 μg ERE-TK-Luc. The transfected cells were treated with various compounds for 24 hours, and then lysed and assayed for luciferase activity using the Luciferase Assay System (Promega, Madison, WI) according to the manufacturer's protocol. Relative light units (RLU) were measured with a luminometer.

Estrogen receptor binding assay

MCF-7 cells were incubated with 5 nM [^{3}H]-E2 (specific activity 87.6 Ci/mmol; PerkinElmer, Waltham, MA) in the presence of increasing concentrations of 2', 3', 4'-THC at 37 °C for 1 hour as previous described (Cvoro et al. 2007). After washing the cells with 0.1% bovine serum albumin in phosphate buffered saline (PBS), 100% ethanol was added to the cells. Radioactivity was measure in the samples with a scintillation counter. Specific binding of [^{3}H]-E2 was calculated as the difference between total and nonspecific binding in counts per minute.

RNA isolation and quantitative real-time PCR

Total cellular RNA was extracted using the Aurum Total RNA Mini Kit (Bio-Rad Laboratories, Hercules, CA) following the manufacturer's protocol. Reverse transcription reactions were

performed using the iScript cDNA Synthesis Kit (Bio-Rad, Hercules, CA) with 1 μg of total RNA according to the manufacturer's protocol. Quantitative real-time reverse transcription polymerase chain reaction (qRT-PCR) was performed with the Bio-Rad CFX96 Thermal Cycler System using SsoFast EvaGreen Supermix (Bio-Rad, Hercules, CA). The results were analyzed by competitive Ct method (Schmittgen and Livak 2008). The Ct values of specific genes were adjusted by the reference gene glyceraldehyde-3-phosphate dehydrogenase running simultaneously to obtain ΔCt. The fold changes were computed by comparison of adjusted Ct values (ΔCt) from various treatments with control samples.

Microarray and data analysis

Total cellular RNA was isolated using the Aurum Total RNA Mini Kit (Bio-Rad, Hercules, CA) per the manufacturer's directions. RNA was first quantified with a nanodrop, and then qualitatively evaluated by the Bio-Rad Experion system per the manufacturer's instructions. Biotin-labeled complementary RNA samples were prepared using 750 ng of total RNA. Biotin-labeled samples were evaluated by both 260/280 absorbance spectrophotometry and capillary electrophoresis. Labeled complementary RNA samples were hybridized overnight to Human Genome HG U133A-2.0 Affymetrix GeneChip arrays (Thermo Fisher Scientific, Waltham, MA). All treatments were done in triplicate with the same batch of microarrays. The data was analyzed as previously described (Tee et al. 2004).

Western blot

Western blot for c-MYC and MAPK in MCF-7 cells was performed as previously described (Pan et al. 2016). In brief, the cells were grown in 6-well tissue culture dishes to reach 80% confluence.

The cultured medium was replaced with serum-free DMEM for 24 hours before the cells were treated with the compounds for 2 hours. The cells were lysed in radioimmunoprecipitation assay (RIPA) buffer containing the Roche cComplete proteinase inhibitor cocktail (Sigma-Aldrich, St. Louis, MO). Total protein concentration of the cell lysate was determined with the Coomassie Plus protein Assay Reagent (Thermo Fisher Scientific, Waltham, MA). 15 μg of cell lysates from each sample were then resolved by SDS-PAGE on a 4–12% Bis-Tris NuPage gel with MOPS running buffer (Thermo Fisher Scientific, Waltham, MA) and then transferred to an Immobilon-P polyvinylidene difluoride (PVDF) membrane (Sigma-Aldrich, St. Louis, MO). The membrane was blocked with 10% nonfat dry milk in tween-tris-buffered saline (TTBS) at room temperature for 1 hour. A mouse anti-c-MYC antibody (Takara Bio USA, Inc, Mountain View, CA) was used at 1 μg/ml in 1% nonfat dry milk-TTBS at 4 ºC overnight. After washing with TTBS three times, the membrane was incubated with a goat anti-mouse IgG horseradish peroxidase conjugated antibody (Santa Cruz Biotechnology, Dallas, TX) at 1:10,000 dilution in 1% nonfat dry milk-TTBS for 1 hour at room temperature. Immunocomplexes on the PVDF membrane were visualized using ECL Prime Western Blotting Detection Reagent (GE Healthcare, Chicago, IL). The membrane was then washed with Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA) and PBS followed by reprobing with a rabbit anti-actin IgG (Santa Cruz Biotechnology, Dallas, TX) and then a goat anti-IgG horseradish peroxidase conjugated antibody. β-actin was visualized with ECL prime. Active MAPK phospho-p44/42 MAPK (Erk 1/2) monoclonal antibody and inactive p44/42 MAPK (Erk 1/2) monoclonal antibody was purchased from Cell Signaling Technology (Danvers, MA) and used at the manufacturer's recommended concentrations for western blots.

Chromatin immunoprecipitation (ChIP) assays

ChIP assay was performed as previously described (Pan et al. 2016). U2OS-ERα cells were incubated for 24 hours with 1 μg/ml of doxycycline to induce ERα in serum-free DMEM/F12 when the cells reached 80% confluence. The cells were then treated with vehicle, E2, 2', 3', 4'-THC and the combination for 1 or 2 hours. For MCF-7 cells, the cultured media was switched to serum-free DMEM/F12 upon reaching 80% confluence and incubated for 24 hours. The cells were then treated with E2, 2', 3', 4'-THC or the combination for 1 hour. After treatment, 11X formaldehyde solution was added to the culture media and incubated for 15 minutes at room temperature with shaking. The reaction was quenched with 1.25 M glycine solution. The cell monolayer was then washed with PBS containing cOmplete Protease Inhibitor Cocktail (Sigma-Aldrich, St. Louis, MO), collected by scraping, and concentrated by centrifugation (2000 x g, 4 ºC for 5 minutes). The cell pellets were stored at -80 ºC. To perform the ChIP assay, the frozen pellets were lysed with buffer containing 0.5% of Triton X-100, 50 mM Tris (pH 7.4), 150 mM sodium chloride, 10 mM ethylenediaminetetraacetic acid, and protease inhibitor cocktail. Cell lysates were centrifuged and the pellets were resuspended in RIPA buffer. The suspensions were sonicated on ice using a Digital Sonifier and the supernatants were obtained by centrifugation at 14,000 rpm for 10 min at 4 ºC. The samples were then diluted with the appropriate amount of RIPA buffer without detergents. Approximately 10% of each diluted sample was used for the input and stored at 4 ºC. The samples were incubated with 4 μg/ml of rabbit anti-ERα IgG (sc-544, Santa Cruz Biotechnology, Dallas, TX) or the same concentration of normal rabbit IgG (sc-2025 Santa Cruz Biotechnology, Dallas, TX) at 4 ºC overnight with rotation. The immune complexes were then precipitated with Protein G Magnetic Sepharose beads (GE Healthcare, Chicago, IL) for 4 hours while rotating at 4 ºC. The DNA-protein complexes were then eluted from the magnetic beads with

a 1% SDS, 0.1 M sodium bicarbonate solution at 65 ºC for 10 minutes. The bound cross-linked DNA was reversed by incubation at 65 ºC overnight. Eluted DNA was purified and concentrated using the ChIP DNA Clean and Concentrator (Zymo Research, Irvine, CA). ERα antibody precipitated DNA was amplified by qRT-PCR with specific primers for the *c-MYC* enhancer region (Wang C et al. 2011) or ERE in *KRT19* (Choi et al. 2000). The Ct values from treatments were adjusted using the corresponding input Ct values. The fold changes were obtained by comparison of adjusted Ct values of treatments with control value.

Cell proliferation assay

Cells were plated at a density of 50,000 cells per well in 6-well tissue culture plates in DMEM/F12 supplemented with 5% stripped FBS. The next day the cells were treated with vehicle or E2 in the absence and presence of increasing doses of 2', 3', 4'-THC for 5 days. The cells were then detached with trypsin, neutralized with media containing 5% FBS, and resuspended. Appropriate amounts of cell suspension were placed in ISOTON II diluent (Thermo Fisher Scientific, Waltham, MA) and the cell numbers were then measured using a Coulter Counter.

Flow cytometry

Flow cytometry was performed based on a previously described method (Pan et al. 2016). Briefly, the cells were plated at a density of 500,000 cells per well in 6-well tissue culture dishes in DMEM/F-12 supplemented with 5% stripped FBS for 48 hours. The cultured medium was then replaced by serum-free DMEM/F12 for 24 hours. The cells were then treated with vehicle, E2 without or with the indicated amount of 2', 3', 4'-THC for 24 hours. The culture medium was then aspirated and the cells were washed with PBS, detached with trypsin and collected by

centrifugation at 1,700 rpm for 5 minutes. The cell pellets were washed with ice cold PBS followed by centrifugation at 1,700 rpm for 10 minutes at room temperature. The cell pellets were resuspended in 500 μl PBS containing 50 μg/ml propidium iodide, 0.1% of triton X-100, 0.1% of sodium citrate, and 10 μg/ml of RNase. The cell suspensions were then analyzed with a BD LSR II Flow Cytometer (BD Biosciences) in the Flow Cytometry facility at University of California, Berkeley and the percentage of cells in cell cycle phases were determined by using FlowJo 7.6.5 (FlowJo, LLC, Ashland, OR).

Statistical analysis

All data are presented as the mean ± SEM from at least biological triplicates. The statistical significance of the difference between two groups was assessed by Student's *t*-test. For the data sets consisting of more than two groups the statistical significance of differences among various groups (treatments) were analyzed by one-way analysis of variance (one-way ANOVA) tests or two-way ANOVA as specified in figure legends. All ANOVA tests were followed by Tukey's or Sidak's multiple comparisons *post hoc* tests to analyze the significance of differences between any two different treatment groups or control as indicated in figure legend. Statistical analysis and graph plotting were performed using GraphPad Prism version 6 (GraphPad Software Inc.; La Jolla, CA, USA). The statistical significance for the numbers of asterisks in the figures are * $p<0.05$; ** $p<0.01$,*** $p<0.001$, and **** $p<0.0001$.

Results

2', 3', 4'-THC acts synergistically with E2 in U2OS cells

Drugs that bind to ERs have two main pharmacological properties. They can be used as agonists to trigger biological responses or as antagonists to block the effects of agonists. E2 and conjugated equine estrogens are the main agonists used in MHT. SERMs are known to exhibit both agonist and antagonistic properties depending on the target tissue (Maximov et al. 2013). Since the agonist-antagonist model has failed to yield a MHT formula that is safe for long-term treatment, we screened compounds with a similar molecular size as E2 for their ability to produce a synergistic action when combined with E2. We reasoned that if a compound has a synergistic effect with E2 instead of acting as an agonist or antagonist, it may alter the biological actions of ERα to prevent its adverse effects, improving the safety of E2, as well as lower the pharmacological dose of E2 required to achieve clinical benefits. In a preliminary screening of compounds, we found that compounds known as chalcones, produced a synergistic effect when combined with E2. These screenings resulted in the discovery of 2', 3', 4'-THC (Fig. 1A), which is a small molecule that consists of two aromatic rings separated by a propanone group with a similar molecular weight (256 daltons) as E2 (272 daltons).

We used U2OS osteosarcoma cells to explore the synergistic action of 2', 3', 4'-THC in more detail since many studies have used these cells to study the mechanism of action of estrogens. U2OS cells were transfected with ERE-TK-Luc and an expression vector for ERα, and then treated for 24 hours with 2', 3', 4'-THC in the absence and presence of increasing concentrations of E2. No significant activation of ERE-TK-Luc occurred with 2', 3', 4'-THC alone, whereas E2 produced a maximal activation of ERE-TK-Luc at 10 nM (Fig. 1B). 2', 3', 4'-THC produced a

synergistic activation at all E2 concentrations tested, even at saturating levels (10 nM to 1 μM). We consider the effect of 2', 3', 4'-THC to be a synergistic response because the combination of 2', 3', 4'-THC and E2 produces a greater activation than the sum of two individual compounds since 2', 3', 4'-THC has no activation effect alone. In a previous study we showed that the NKG2E promoter contains a complex ER regulatory element (Levy et al. 2007). This element has the advantage over an ERE because it is activated by both E2 and SERMs in U2OS cells (Levy et al. 2007). Since our goal is to develop a drug that does not act as a SERM it is important to show that 2', 3', 4'-THC does not activate the NKG2E promoter like the SERMs. U2OS cells were transfected with NKG2E-TK-Luc and then treated with increasing concentrations of 2', 3', 4'-THC in the absence and presence of E2. Unlike the SERMs, tamoxifen and raloxifene (Levy et al. 2007), 2', 3', 4'-THC did not activate NKG2E-TK-Luc (Fig. 1C). A synergistic response with E2 was observed at 1 μM 2', 3', 4'-THC and the maximal response occurred at 5 μM. A synergistic effect by 2', 3', 4'-THC was observed with other estrogens, including equilin, ethinyl estradiol, estrone, and estriol and the E2 metabolites, 2-hydroxyestradiol and 4-hydroxyestradiol (Fig. 1D). A smaller activation was observed with 17α-estradiol and 2-methoxyestradiol. Structure activity relationships with different chalcones were performed with transfection assays using NKG2E-TK-Luc. The synergy was greatest with 2', 3', 4'-THC followed by 4, 2'-dihydroxychalcone (DHC) and 2', 4'-DHC (Fig. 1E). These studies indicate that the 2' and 4' OH groups of 2', 3', 4'-THC (Fig. 1A) are important for the synergistic effect. To determine the region of ERα that is required for the synergy, U2OS cells were transfected with five copies of the GAL-RE upstream of TK-Luc (GAL-TK-Luc) and a vector that expresses the full-length ERα or ERα LBD fused to the GAL4-DNA binding domain. A synergistic activation of GAL-TK-Luc by the E2/2', 3', 4'-THC combination occurred with both the full-length (Fig. 2A) and LBD (Fig. 2B), demonstrating that

the ERα LBD alone can produce synergy. A synergistic effect of 2', 3', 4'-THC was also observed with ERβ (Fig. 2C), but not with the human glucocorticoid (GR, Fig. 2D), androgen (AR, Fig. 2E), and progesterone-B (PR, Fig. 2F) receptors, demonstrating that the synergy is specific for ERs. We focused on the synergistic effect of 2', 3', 4'-THC on ERα instead of ERβ because ERα mediates the proliferative effects of estrogen on breast and uterine cells, which is a critical adverse action that needs to be overcome by new drugs for MHT.

The 2', 3', 4'-THC/E2 combination leads to the regulation of reprogrammed genes

In order to determine if 2', 3', 4'-THC alters E2 regulation of endogenous genes, we performed microarray analysis in U2OS-ERα cells. E2 alone regulated 756 genes, whereas 2', 3', 4'-THC alone regulated only 31 genes, of which 14 genes were also regulated by E2 and 25 genes were regulated by the 2', 3', 4'-THC/E2 combination (Table S1). The 2', 3', 4'-THC/E2 combination regulated 1,358 genes (Table S1). 534 genes were commonly regulated by E2 alone and the 2', 3', 4'-THC/E2 combination (Fig. 3A). 824 genes were termed reprogrammed genes because these genes were regulated by the 2', 3', 4'-THC/E2 combination, but not by 2', 3', 4'-THC or E2 alone (Table S1). Gene Ontology (GO) analysis shows that the molecular pathways regulated by E2 alone (Fig. S1) were markedly different than the genes regulated by the 2', 3', 4'-THC/E2 combination (Fig. S2). We verified the microarray results using multiple reprogrammed genes. Similar to the microarray data, *KCNK6* (Fig. 3B) and *KRT73* (Fig. 3C) were not appreciably regulated by E2 and 2', 3', 4'-THC alone. The 2', 3', 4'-THC/E2 combination produced a large activation of the *KCNK6* and *KRT73* genes at 10 nM E2 showing that these are reprogrammed genes because they are only regulated by the combination. The ER antagonist ICI 182,780 (ICI)

blocked the induction of the reprogrammed genes *K6iRS3* (Fig. 3D) and *FGR* (Fig. 3E), and the synergistic activation of the *NKG2E* gene (Fig. 3F) by the 2', 3', 4'-THC/E2 combination. These findings demonstrate that the regulation of reprogrammed genes and synergistic activation by the 2', 3', 4'-THC/E2 combination require ERα.

2',3',4'-THC does not act like a SERM

Crystal structures of ERα show that SERMs bind to the same binding pocket as E2 (Brzozowski et al. 1997; Shiau et al. 1998). Unlike the SERMs, 2', 3', 4'-THC did not exhibit antagonist activity on the ERE-TK-Luc or NKG2E-TK-Luc. To compare the activity of 2', 3', 4'-THC to SERMs on endogenous genes, we examined their effects on the expression of the *KRT19* and *NKG2E* genes. E2 induced *KRT19* mRNA expression in U2OS-ERα cells, whereas no effect was observed with 2', 3', 4'-THC, tamoxifen, or raloxifene (Fig. 4A). The 2', 3', 4'-THC/E2 combination produced a synergistic activation of *KRT19*, whereas tamoxifen and raloxifene act as antagonists by blocking the activation by E2. (Fig. 4A). E2, raloxifene, and tamoxifen activated the *NKG2E* gene, whereas no effect was observed with 2', 3', 4'-THC (Fig. 4B). A synergistic activation of the *NKG2E* gene occurred with the 2', 3', 4'-THC/E2 combination. No synergy or antagonism of the tamoxifen or raloxifene activation of the *NKG2E* gene was observed with 2', 3', 4'-THC. Similar results were observed in transfection assays. NKG2E-TK-Luc was activated by E2, tamoxifen, and raloxifene, but 2', 3', 4'-THC produced a synergistic activation only with E2 (Fig. 4C). ChIP shows that the 2', 3', 4'-THC/E2 combination increased the recruitment of ERα compared to E2 alone to a known ERE in the *KRT19* gene (Choi et al. 2000) suggesting that enhanced binding of ERα leads to the synergy (Fig. 4D). These observations demonstrate that 2', 3', 4'-THC does not function like an estrogen or SERM in U2OS cells since it does not act as an agonist or antagonist.

2', 3', 4'-THC reprograms the effect of E2 on MCF-7 breast cancer cell proliferation

Estrogens are known to increase breast cancer risk by activating ERα. Thus, we assessed the effects of 2', 3', 4'-THC alone and in combination with E2 using MCF-7 breast cancer cells. Similar to U2OS cells, 2', 3', 4'-THC produced a dose-dependent synergistic activation of E2 stimulation of ERE-TK-Luc in MCF-7 cells (Fig. 5A). E2 expectedly increased MCF-7 cell proliferation whereas 2', 3', 4'-THC did not cause proliferation at concentrations from 1-10 μM (Fig. 5B). 2', 3', 4'-THC blocked the proliferation induced by E2 in a dose-dependent manner (Fig. 5B). It is known that estrogen causes the proliferation of breast cancer cells by stimulating the progression of cells from the G_1 phase to the S phase (Foster et al. 2001). 2', 3', 4'-THC dose-dependently blocked the E2-induced entry of S phase cells from G_1, as measured by flow cytometry (Fig. 5C). This finding is consistent with a G_1 arrest. 2', 3', 4'-THC also inhibited the percentage of cells entering the S phase when stimulated by 10 nM estrone, estriol and equilin (IC_{50} = 5 μM) similar to 1 nM E2 (Fig. 5D). Tamoxifen caused a G_1 cell cycle arrest at doses only 3- to 8-fold lower than 2', 3', 4'-THC (G_1 phase, EC_{50} = 0.32 μM vs 2.9 μM; S phase IC_{50} = 0.98 μM vs 2.6 μM) (Fig. 5E).

Even though 2', 3', 4'-THC did not show antagonistic activity in U2OS cells, the most straightforward explanation whereby 2', 3', 4'-THC inhibits cell proliferation and causes a G_1 cell cycle arrest in MCF-7 cells is that it blocks the binding of E2 to ERα similar to SERMs. Since no radiolabeled 2', 3', 4'-THC is available, we performed competition binding studies to determine if 2', 3', 4'-THC competes for [^{3}H]-E2 binding sites in MCF-7 cells. No competition of [^{3}H]-E2 binding occurred until 50 μM 2', 3', 4'-THC (Fig. 6A). This concentration is 50 times higher than the concentration required for synergy (Figs. 1C) and the antiproliferative effect (Fig. 5B). These

findings demonstrate that 2', 3', 4'-THC does not compete with [^{3}H]-E2 at active concentrations. To further evaluate the binding of 2', 3', 4'-THC to ERα, we examined the effects of 2', 3', 4'-THC on ERα degradation, since E2 enhances ERα degradation (Nawaz et al. 1999). We speculated that if 2', 3', 4'-THC binds to the same binding site as E2 it would be expected to cause degradation alone or block the degradation of ERα by E2. As expected, E2 produced a time-dependent loss of ERα by 6 h (Fig. 6B). No change in ERα levels were observed with 2', 3', 4'-THC alone, and it did not block the degradation induced by E2. These findings provide additional evidence that 2', 3', 4'-THC does not bind to the E2 binding site in ERα.

2', 3', 4'-THC inhibits nongenomic and genomic effects of E2 in MCF-7 cells

We next investigated the mechanism for the antiproliferative effect of 2', 3', 4'-THC. In addition to its well characterized genomic effects, estrogenic compounds are also known to have nongenomic effects that promote cell proliferation. E2 activates the MAPK/ERK 1/2 pathway leading to downstream signaling that regulates cell proliferation (Improta-Brears et al. 1999; (Zhang and Liu 2002; Levin 2005). To determine the effect of 2', 3', 4'-THC on the MAPK pathway, MCF-7 cells were treated with E2 in the absence and presence of 2', 3', 4'-THC and the levels of active, phosphorylated MAPK were determined by western blotting. A time-dependent increase in phosphorylated MAPK by E2 was blocked by 2', 3', 4'-THC (Fig. 6C). In contrast, 2', 3', 4'-THC had no effect on the phosphorylation of MAPK induced by epidermal growth factor (EGF) (Fig. 6D), demonstrating that 2', 3', 4'-THC acts selectively on the ER pathway. The induction of the *c-MYC* gene by E2 is essential for breast cancer cell proliferation (Foster et al. 2001). 2', 3', 4'-THC inhibited the E2 increase of *c-MYC* mRNA (Fig. 6E) and protein levels (Fig. 6F) at doses similar to tamoxifen. ChIP was performed to determine the effect of 2', 3', 4'-THC

on ERα recruitment to the *c-MYC* enhancer, which contains an ERα binding site (Shang and Brown, 2002; Wang C et al. 2011). E2 caused a significant recruitment of ERα to the *c-MYC* enhancer by 1 hour (Fig. 6G), which was inhibited by 2', 3', 4'-THC. This finding suggest that 2', 3', 4'-THC blocks the induction of the c-*MYC* gene by preventing the binding of ERα to the enhancer.

Discussion

We demonstrated that 2', 3', 4'-THC behaves differently than both E2 and SERMs. It does not act as an agonist or antagonist in U2OS cells. In transfection studies 2', 3', 4'-THC did not activate reporter genes like E2 or block the effects of E2 as SERMs. Instead, 2', 3', 4'-THC produces a synergistic activation of ERE-TK-Luc and NKG2E-TK-Luc in the presence of E2. Similar synergistic activation was observed on the endogenous *KRT19* and *NKG2E* genes. Microarray analysis in U2OS-ERα cells demonstrated that 2', 3', 4'-THC regulated a total of 31 genes compared to 756 genes by E2. These data demonstrate that 2', 3', 4'-THC has little or no agonist activity alone in U2OS cells. The combination of E2 and 2', 3', 4'-THC regulated 1,358 genes, and E2 alone regulated 534 of these genes. We refer to the other 824 genes as reprogrammed genes since they are not regulated by ERα unless 2', 3', 4'-THC is added to physiological concentrations of E2. Although it is likely that many of these reprogrammed genes are direct targets of ERα since the ERα downregulator ICI prevented the induction of the reprogrammed genes tested, other genes could be regulated by gene products induced by the 2', 3', 4'-THC/E2 combination.

Since ERα promotes the proliferation of breast cancer cells in response to E2 (Ali and Coombes 2000) it would appear that the synergistic effect, the lack of antagonism, and potentially the induction of some reprogrammed genes observed in U2OS cells could be deleterious. However,

we found that 2', 3', 4'-THC blocks the proliferative response of E2 at doses similar to tamoxifen in MCF-7 cells. While tamoxifen and 2', 3', 4'-THC both cause a G_1 cell cycle arrest, several observations suggest that the mechanism of 2', 3', 4'-THC is not the same as SERMs. First, unlike SERMs, 2', 3', 4'-THC does not bind to the E2 binding site in ERα since it did not compete with [^{3}H]-E2 at active concentrations. Second, tamoxifen increases the binding of ERα to the *c-MYC* gene in MCF-7 cells (Shang and Brown 2002), whereas 2', 3', 4'-THC inhibits E2 recruitment of ERα as demonstrated by the ChIP data. These results suggest that 2', 3', 4'-THC does not block MCF-7 cell proliferation by the classic antagonistic activity of SERMs that involves binding to the E2 pocket (Brzozowski et al. 1997; Shiau et al. 1998). The antiproliferative effect of 2', 3', 4'-THC is not mediated by ERβ since our MCF-7 cells do not express ERβ (Paruthiyil et al. 2004). Our studies suggest that the antiproliferative effect in MCF-7 cells is due to a reprogramming mechanism whereby 2', 3', 4'-THC switches a stimulatory action of E2 on MAPK activity and *c-MYC* transcription to an inhibitory action. Based on these findings, we refer to 2', 3', 4'-THC as an ERα reprogramming compound.

A major question raised by our findings is how does 2', 3', 4'-THC reprogram the action of E2 on gene regulation and cell proliferation. One possibility is that E2 and 2', 3', 4'-THC form a heteroligand with E2 binding to one subunit and 2', 3', 4'-THC binding to the E2 binding site on the other subunit of ERα, as described with cotreatment with E2 and SERMs using mutant and chimera ERs (Liu et al. 2013). A heteroligand model is unlikely because 2', 3', 4'-THC did not compete with [^{3}H]-E2 binding at concentrations that were biologically active. 2', 3', 4'-THC also did not cause ERα degradation or block the degradation by E2. The competitive binding and ERα degradation studies provide compelling evidence that 2', 3', 4'-THC does not bind to the same site as E2 on ERα. Another possibility is that 2', 3', 4'-THC reprograms the actions of E2 by binding

to factors other than ERα, such as coregulatory proteins or transcription factors that interact with ERα and other nuclear receptors (Lonard et al. 2007). A variety of small molecules have been identified that bind to and modulate the activity of coregulatory proteins (Szwarc et al. 2015). The natural polyphenol gossypol directly binds to the steroid receptor coactivators SRC-1 and SRC-3 to promote their degradation (Wang Y et al. 2011). While these findings raise the possibility that coregulatory proteins could be a target for 2', 3', 4'-THC, it seems unlikely because we found that 2', 3', 4'-THC did not cause synergy with other nuclear receptors, including GR, AR, and PR-B. It is conceivable that the reprogramming action occurs from the binding of 2', 3', 4'-THC to a secondary site in ERα as a coligand. In a coligand model when E2 binds to its binding pocket, it creates a secondary site that 2', 3', 4'-THC binds to forming a ternary complex consisting of E2, 2', 3', 4'-THC, and ERα. Since the structure of the ternary complex should be different from the binary E2 and ERα complex, it might recognize different regulatory elements that are present in the reprogrammed genes. The formation of the ternary complex and change in ERα conformation could be a potential mechanism whereby 2', 3', 4'-THC inhibits the binding of ERα to the c-MYC gene. The observation that the size of the ERα binding cavity is nearly two times larger than the molecular volume for E2 (Brzozowski et al. 1997), suggests that two small ligands, such as E2 and 2', 3', 4'-THC can occupy the pocket at the same time. It is also possible that a secondary site is located outside of the E2 binding pocket. A coligand model is consistent with the reporter gene data that showed that even when cells are treated with saturating levels of E2 that should occupy all the E2 binding sites, 2', 3', 4'-THC still produces a synergistic effect, possibly by binding to a secondary site on ERα. Future x-ray crystallography studies will need to be done to determine if the reprogramming action of 2', 3', 4'-THC results from its binding to a secondary site on ERα concurrently with E2.

Different ligands bind to the same binding pocket of ER to create unique conformations, which leads to the recruitment of distinct coregulatory proteins to alter gene expression profiles (Paige et al. 1999; Nettles et al. 2007; McDonnell and Wardell 2010). A major pharmaceutical strategy to overcome drug side effects is to design and synthesize selective nuclear receptor modulators that bind to the same binding pocket as the cognate ligand to produce distinct conformations and different clinical responses. Another possible approach for drug discovery is to explore ER and other nuclear receptors for secondary binding sites for coligands. A secondary negative allosteric ligand binding site for 27-hydroxycholesterol has been reported for ERβ based on the observation that it antagonized the activation of E2 on an ERE in reporter assays, but only partially competed with [^{3}H]-E2 binding (Starkey et al. 2018). It has been suggested that the antagonism is due to the conformational changes caused by the binding of 27-hydroxycholesterol to a secondary site in the ERβ, thereby reducing the binding affinity of E2. Compounds have been identified that bind to a secondary site on membrane receptors that produce positive or negative allosteric effects (Sieghart 2015; Dopart et al. 2018). If nuclear receptors have secondary allosteric ligand binding sites, they may become potential targets for drugs. One potential clinical use of reprogramming compounds such as 2', 3', 4'-THC that we are exploring is combining it with estradiol to create a safer MHT that can be used for long-term therapy to treat prolonged menopausal symptoms and prevent chronic diseases during menopause. All current estrogen/progestin MHT formulations on the market increase the risk of breast cancer (Collaborative Group on Hormonal Factors in Breast Cancer 2019). Our study shows that 2', 3', 4'-THC blocks the proliferative effects of E2 on breast cancer cells by a mechanism distinct from SERMs. This response is a key first step for clinical development of a safer MHT. The next steps will be to evaluate the safety and efficacy of the E2/2', 3', 4'-THC combination in multiple

preclinical animal models and women to determine if this combination is a safe and effective alternative to current MHT formulas.

The pharmacological effects of drugs vary depending on the individual who uses them (McLean and Le Couteur 2004). At the prescribed therapeutic doses, clinical benefits, toxicity, and side effects are rarely universally manifested. Differences in benefits and risks of drugs are often elucidated in clinical trials or in post-marketing pharmacovigilance observations. Genetic and metabolic studies have been used to investigate the different individual responses to pharmacological interventions by detecting mutations in specific target genes or metabolic enzymes in order to identify individuals or groups who have such differences (Spear et al. 2001; Mirsadeghi and Larijani 2017). Similarly, the risks and benefits of combinations of two or more pharmacological interventions, commonly prescribed in clinical practice, are studied through preclinical and clinical drug-drug interaction studies, aimed at defining clashes in metabolic enzymes that result in hyperactivation or inhibition of one of the drugs (Benet et al. 2019). Following the clinical observation that estrogen replacement therapy increased the risk for uterine cancer in menopausal women with an intact uterus, this risk was mitigated by employing physiological means, as in the case of progestogens, or a mechanistic approach with SERMs. In most other clinical situations, multiple drug intervention is based on the type of symptoms, but research studies are rarely conducted to determine the reasons behind the benefits and risks. 2', 3', 4'-THC is a member of a diverse naturally occurring compounds, flavonoids/chalcones that are found in many plant species commonly consumed by humans in the diet. We showed that there is a significant alteration of the ligand receptor transcriptional outcome of 2', 3', 4'-THC/E2 compared to E2 alone and the combination inhibits MCF-7 breast cancer cell proliferation. The discovery of reprogramming/coligand compounds can open up a new field of research, which may

lead to a greater understanding of the different pharmacological outcomes that are commonly observed in patients. For example, it is not clear why some women develop breast cancer after MHT, but it is conceivable that the presence of potential reprogramming/coligand compounds in the diet may affect the breast cancer outcome of MHT. Reprogramming/coligand compounds can also be used as chemical scaffolds for drug development. Since E2 interacts with over 300 tissues and is responsible for major physiological functions in female growth, development, reproduction, and health, the alteration of the actions of E2 by a reprogramming/coligand drug could have profound implications to other areas of research in women's health.

Conclusions

Our study shows that 2', 3', 4'-THC can modulate the activity of ERα without acting as an agonist or antagonist. We termed 2', 3', 4'-THC a reprogramming compound because it changes the genes regulated by E2 and the proliferation of breast cancer cells without binding to the E2 and SERM binding site in ERα. Combining a reprogramming drug, such as 2', 3', 4'-THC to estrogen instead of progesterone or a SERM may offer an alternative strategy to improve the drug safety of MHT.

Abbreviations

ANOVA: Analysis of variance

AR: Androgen receptor

ChIP: Chromatin immunoprecipitation

Ct: Cycle threshold

DBD: DNA binding domain

Dex: Dexamethasone

DHC: Dihydroxychalcone

DHT: Dihydrotestosterone

DMEM: Dulbecco's Modified Eagle Medium

E2: 17β-estradiol

ECL: Enhanced chemiluminescence

EGF: Epidermal growth factor

ER: Estrogen receptor

ERE: Estrogen response element

ERK: Extracellular signal-regulated kinase

FBS: Fetal bovine serum

GAL-RE: GAL-response element

GR: Glucocorticoid receptor

ICI: Imperial Chemical Industries 182,780

KRT: Keratin

LBD: Ligand binding domain

Luc: Luciferase

MAPK: Mitogen-activated protein kinase

MHT: Menopausal hormone therapy

PBS: Phosphate-buffered saline

PCR: Polymerase chain reaction

PR: Progesterone receptor

Prog: Progesterone

PVDF: Polyvinylidene fluoride

RIPA: Radioimmunoprecipitation assay

RLU: Relative light units

qRT-PCR: Quantitative real-time reverse transcription polymerase chain reaction

SDS-PAGE: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis

SERM: Selective estrogen receptor modulator

Tam: Tamoxifen

TAT3: tyrosine aminotransferase 3

THC: Trihydroxychalcone

Tk: Thymidine kinase

TTBS: Tween tris-buffered saline

T2DM: Type 2 diabetes

VTE: Venous thromboembolism

Ethics approval and consent to participate

Not Applicable

Consent for publication

All the authors read and approved the submission of this manuscript.

Availability of data and material

The analyzed microarray data generated are available in the supplementary material. Other data are available from the corresponding author on request.

Competing interests

CY, IC, and DCL are employees of Iaterion Inc., which is developing reprogramming compounds as drugs. The other authors declare no competing interests.

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Authors' contributions

Study design: CBH, CY, JCW, IC and DCL. Performed experiments: CBH, CY, AC, and DCL. Performed analyses of data: CBH, CY, JCW, IC, and DCL. Wrote the paper: CBH, CY, IC, and DCL.

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Figure Legends

Fig. 1 2', 3', 4'-THC synergizes with E2 to induce transcription with ERα. (**A**) Chemical structure of 2', 3', 4'-THC. (**B**) U2OS cells cotransfected with ERE-TK-Luc and ERα were treated with increasing concentrations of E2 in the absence and presence of 5 μM 2', 3', 4'-THC for 24 hours. (**C**) U2OS cells cotransfected with NKG2E-TK-Luc and ERα were treated with increasing concentrations of 2', 3', 4'-THC in the absence and presence of 10 nM E2 for 24 hours. (**D**) U2OS cells cotransfected with NKG2E-TK-Luc and ERα were treated with 10 nM E2 or 100 nM of the other estrogens in the absence and presence of 5 μM 2', 3', 4'-THC for 24 hours. (**E**) U2OS cells cotransfected with NKG2E-TK-Luc and ERα were treated with 5 μM of each chalcone in the absence or presence of 10 nM E2 for 24 hours. Luciferase activity was measured in cellular lysates with a luminometer. RLU is relative light units. Each point in the figures represent the mean of triplicate samples ± SEM. The asterisks indicate statistical significance between two groups analyzed by t-test.

Fig. 2 The synergistic effect of 2', 3', 4'-THC is selective for ERs. U2OS cells were cotransfected with GAL-TK-Luc and an expression vector for (**A**) GAL4-DBD-full-length ERα or (**B**) GAL4-DBD-ERα LBD. Cells were treated with 10 nM E2 and 5 μM 2', 3', 4'-THC alone or in combination for 24 hours. (**C**) U2OS cells cotransfected with ERE-TK-Luc and an expression vector for ERβ were treated with 10 nM E2 and 5 μM 2', 3', 4'-THC alone or the E2/2', 3', 4'-THC combination for 24 hours. (**D**) U2OS cells cotransfected with GRE-TK-Luc and human GR were treated with 10 nM dexamethasone (Dex) and 5 μM 2',3',4'-THC alone or in combination for 24 hours. (**E**) U2OS cells cotransfected with human tyrosine aminotransferase 3 (TAT3)-Luc reporter gene and human AR were treated with 10 nM dihydrotestosterone (DHT) and 5 μM 2',3',4'-THC alone or in combination for 24

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Fig. 3 The effects of 2', 3', 4'-THC on gene expression in U2OS-ERα cells. The cells were treated with 10 nM E2 and 5 μM 2', 3', 4'-THC alone or in combination for 24 hours and then the gene expression profiles were determined with microarrays. (**A**) Venn diagram shows the total number of genes regulated in the cells. Genes expression was assessed by up or down regulation of 3-fold or more and a *p*-value ≤ 0.05 determined by Student *t*-test. Microarray verification of the reprogrammed genes for (**B**) *KCNK6* and (**C**) *KRT73* mRNA. U2OS-ERα cells were treated with 10 nM E2 and 5 μM 2', 3', 4'-THC alone or in combination for 24 hours and the levels of mRNA were measured by qRT-PCR. ICI blocks the E2/2', 3', 4'-THC combination induction of the reprogrammed genes (**D**) *K6iRS3*, (**E**) *FGR*, and the synergistic activation of the (**F**) *NKG2E* gene. U2OS-ERα cells were treated with 10 nM E2, 5 μM 2', 3', 4'-THC, and 1 μM ICI alone or in combination for 24 hours and mRNA levels were measured by qRT-PCR. The data are mean ± SEM. The statistical significances were determined by one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test.

Fig. 4 2', 3', 4'-THC acts different than SERMs on gene transcription**.** (**A**) *KRT19* and (**B**) *NKG2E* gene expression in U2OS-ERα cells. The cells were treated with 10 nM E2 or 5 μM 2', 3', 4'-THC in the absence and presence of 1 μM tamoxifen and 1 μM raloxifene alone or in combination for 24 hours. The mRNA levels were determined by qRT-PCR. (**C**) U2OS cells cotransfected with NKG2E-

TK-Luc and ERα were treated with 10 nM E2, 5 μM 2', 3', 4'-THC, 1 μM tamoxifen, and 1 μM raloxifene alone or in combination for 24 hours. Luciferase activities were measured with a luminometer. (**D**) ERα recruitment to the *KRT19* ERE in U2OS-ERα cells. The cells were treated with 10 nM E2 and 5 μM 2', 3', 4'-THC alone or in combination for 2 hours and then ERα binding was determined by ChIP. The *KRT19* gene was used because the ERE was previously characterized and the ERE in the reprogrammed genes are not known. The data shown are the mean of triplicate samples ± SEM. The statistical significance was determined by one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test.

Fig. 5 E2-induced MCF-7 breast cancer cell proliferation is blocked by 2', 3', 4'-THC. (**A**) MCF-7 cells transfected with ERE-TK-Luc were treated with 5 μM 2', 3', 4'-THC and 10 nM E2 or in combination for 24 hours and then luciferase activity was determined. (**B**) Growth curve in MCF-7 breast cancer cells. The cell numbers were counted with a Coulter counter after treatment with increasing concentrations of 2', 3', 4'-THC without or with 1 nM E2 for 7 days. (**C**) Cell cycle in MCF-7 breast cancer cells. The percentage of S phase cells was determined by flow cytometry after the cells were treated with increasing amounts of 2', 3', 4'-THC without or with 0.1 nM E2 for 24 hours. (**D**) Percentage of S phase cells were determined by flow cytometry in cells treated with increasing concentrations of 2', 3', 4'-THC in the presence of 0.1 nM E2 or 1 nM estrone, estriol or equilin for 24 hours. (**E**) Percentages of S and G1 phase cells in the MCF-7 cells treated with increasing amount of tamoxifen (Tam) or 2', 3', 4'-THC in the presence of 0.1 nM E2 for 24 hours were determined by flow cytometry. The error bars are means ± SEM. The differences among various treatments (**A**, **B** and **C**) were analyzed with two-way ANOVA followed by Tukey's multiple

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Fig. 6 Effects of 2', 3', 4'-THC on MAPK activity and *c-MYC* expression. (**A**) 2', 3', 4'-THC does not compete for [^{3}H]-E2 binding in MCF-7 breast cancer cells. Competitive binding of 2', 3', 4'-THC in the cells treated with 5 nM [^{3}H]-E2 and increasing doses of 2', 3', 4'-THC for 1 hour at 37 ºC was measured with a scintillation counter. Specific [^{3}H]-E2 binding was calculated by subtracting non-specific binding from total binding. The error bars are mean ± SEM. (**B**) 2', 3', 4'-THC does not alter ERα degradation in MCF-7 cells. The cells were treated with 10 nM E2 and 5 μM 2', 3', 4'-THC alone or the combination and then ERα was measured by Western blotting. (**C** and **D**) 2', 3', 4'-THC inhibits E2 activation of MAPK. Active phospho-p44/42 MAPK (Top panel) and inactive p44/42 MAPK (Bottom panel) were measured by western blotting after the cells were treated with E2 or EGF for various times. (**E**) 2', 3', 4'-THC inhibits E2 activation of the *c-MYC* gene. MCF-7 cells were treated with 10 nM E2, 5 μM 2', 3', 4'-THC, and 5 μM Tam alone or in combination for 2 hours and the levels of *c-MYC* mRNA were measured by qRT-PCR. The error bars are means ± SEM. (**F**) 2', 3', 4'-THC inhibits E2 stimulation of c-MYC protein levels. The cells were treated with 10 nM E2 alone or in combination with increasing doses of 2', 3', 4'-THC (THC) or Tam for 3 hours and c-MYC was determined by Western blotting. (**G**) 2', 3', 4'-THC inhibits E2 inhibits ERα recruitment to *c-MYC* enhancer region. The cells were treated with 10 nM E2 without or with 5 μM 2', 3', 4'-THC for 1 hour and ERα binding was determined by ChIP assay. Each bar represents the mean of triplicate samples± SEM. Asterisks over bars show the significance between E2 alone and various treatments determined by one-way ANOVA followed by Tukey's multiple comparisons *post hoc* test.

Fig. S1 Gene Ontology (GO) analysis of the molecular function of genes. GO analysis shows that the molecular pathways regulated by E2 alone compared to control cells.

Fig. S2 Gene Ontology (GO) analysis of the molecular function of genes. GO analysis shows that the molecular pathways regulated by the 2', 3', 4'-THC/E2 combination compared to E2 alone treated cells. The genes in green (cellular component) and blue (molecular function) bars were new since they were regulated only by the combination.

Figures

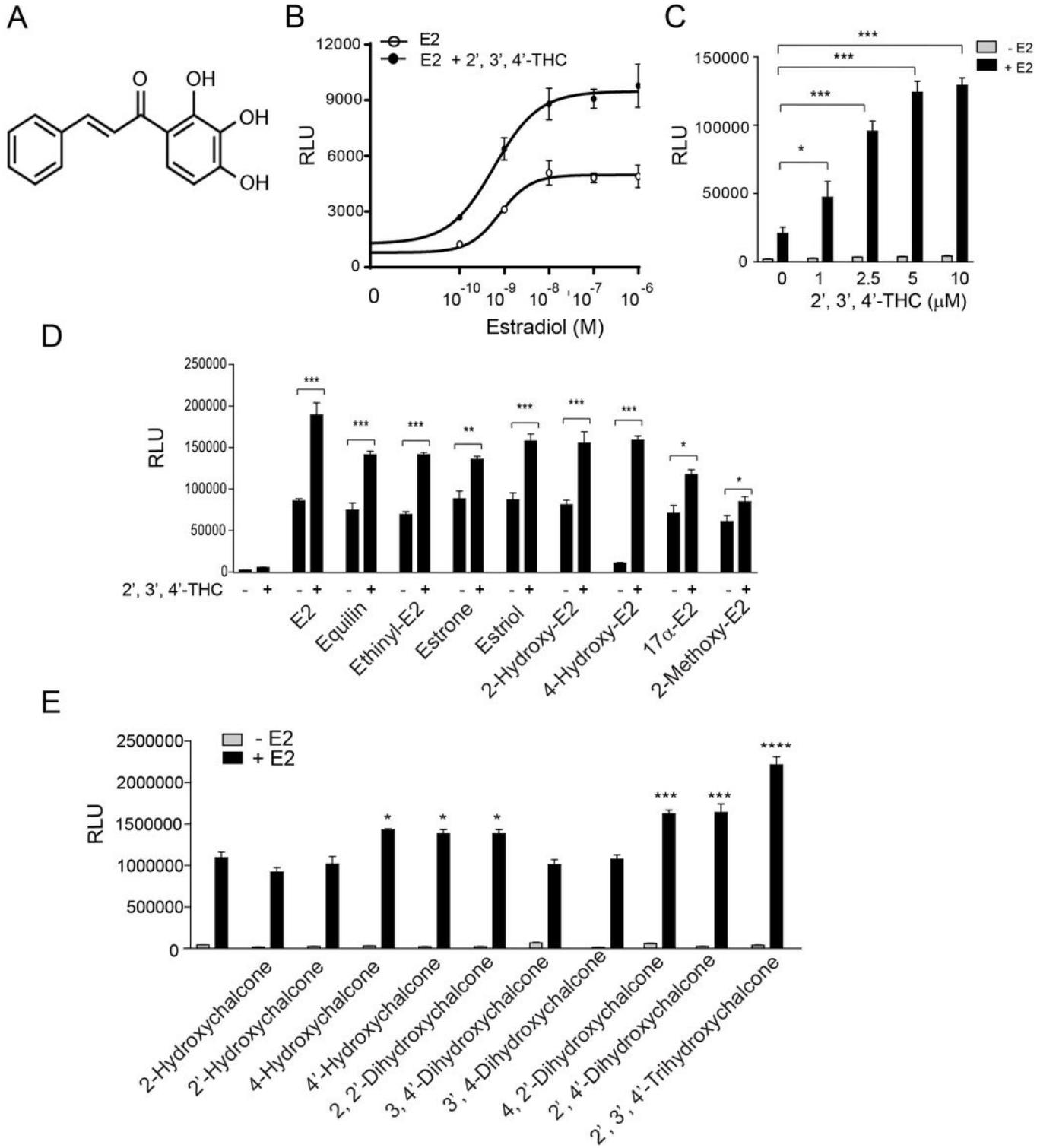

Figure 1

2', 3', 4'-THC synergizes with E2 to induce transcription with ERα. (A) Chemical structure of 2', 3', 4'-THC. (B) U2OS cells cotransfected with ERE-TK-Luc and ERα were treated with increasing concentrations of E2 in the absence and presence of 5 μM 2', 3', 4'-THC for 24 hours. (C) U2OS cells cotransfected with NKG2E-

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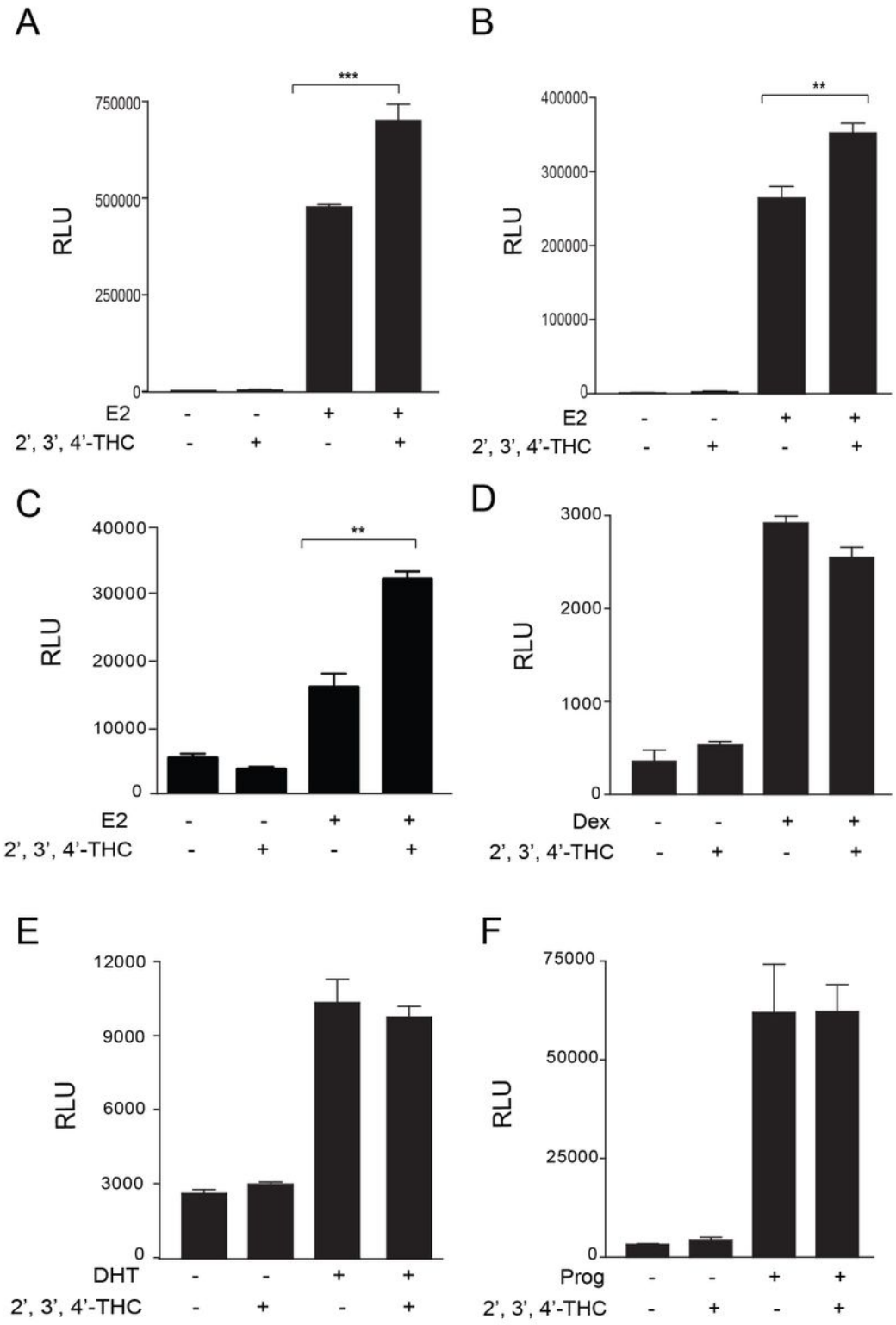

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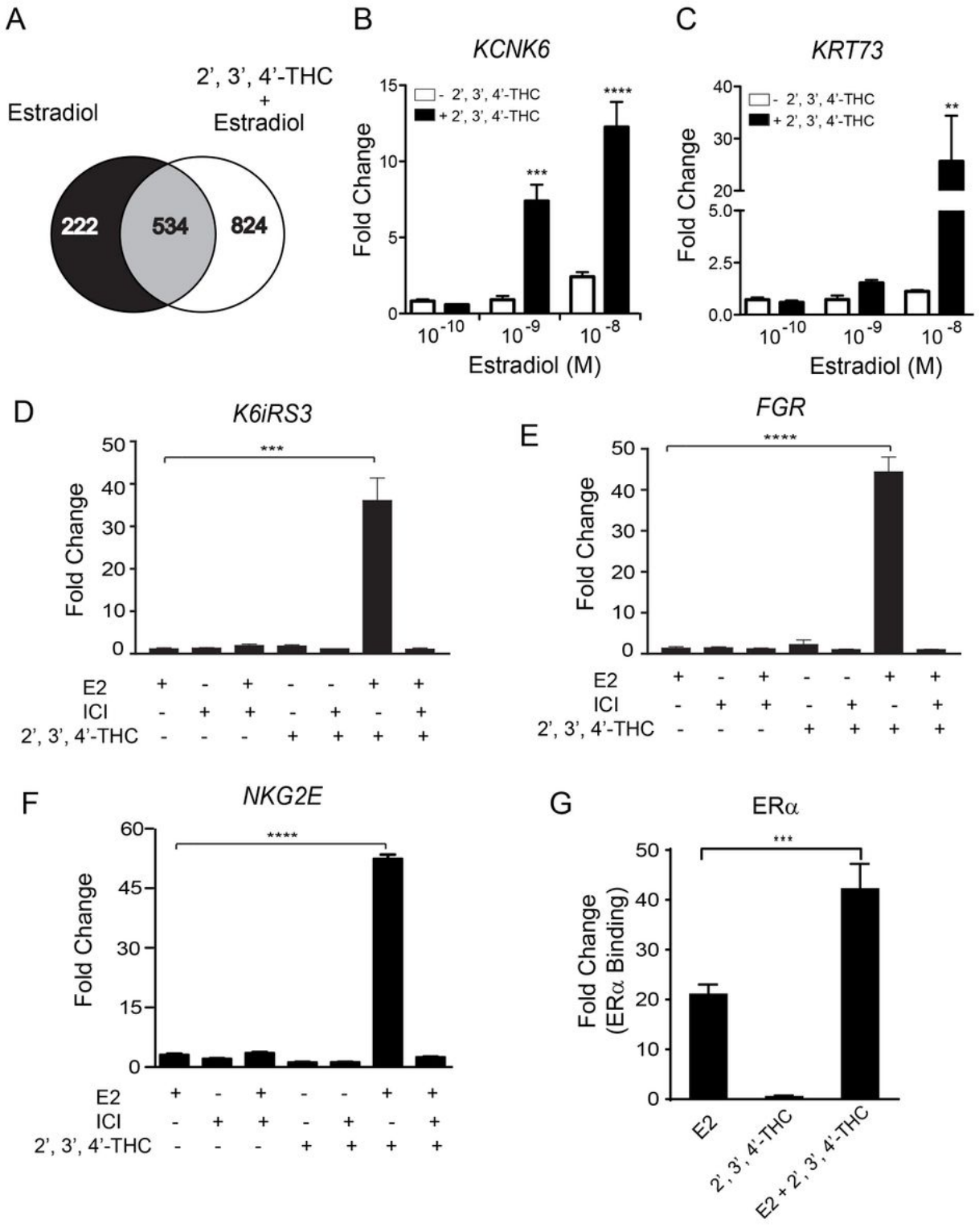

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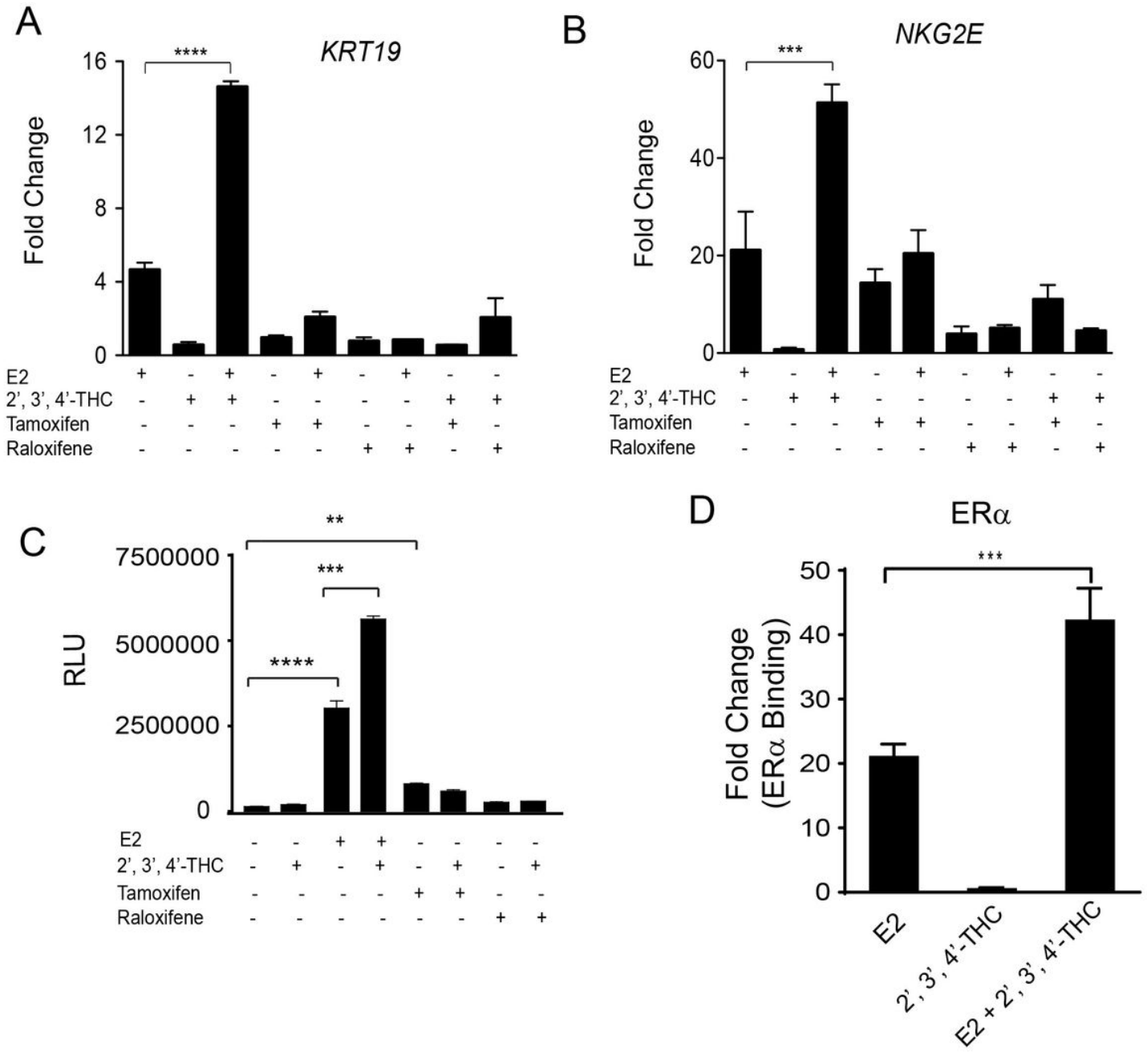

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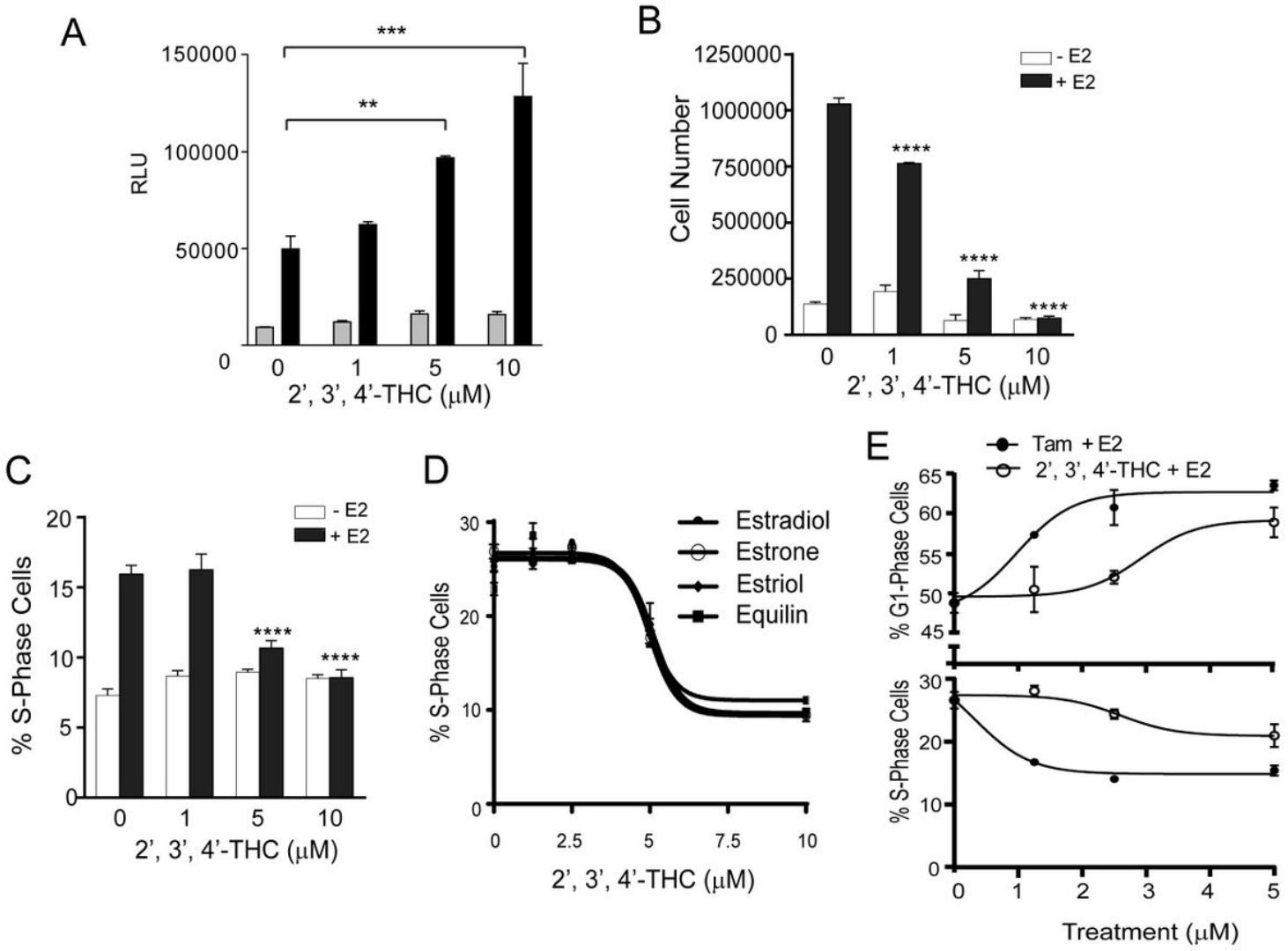

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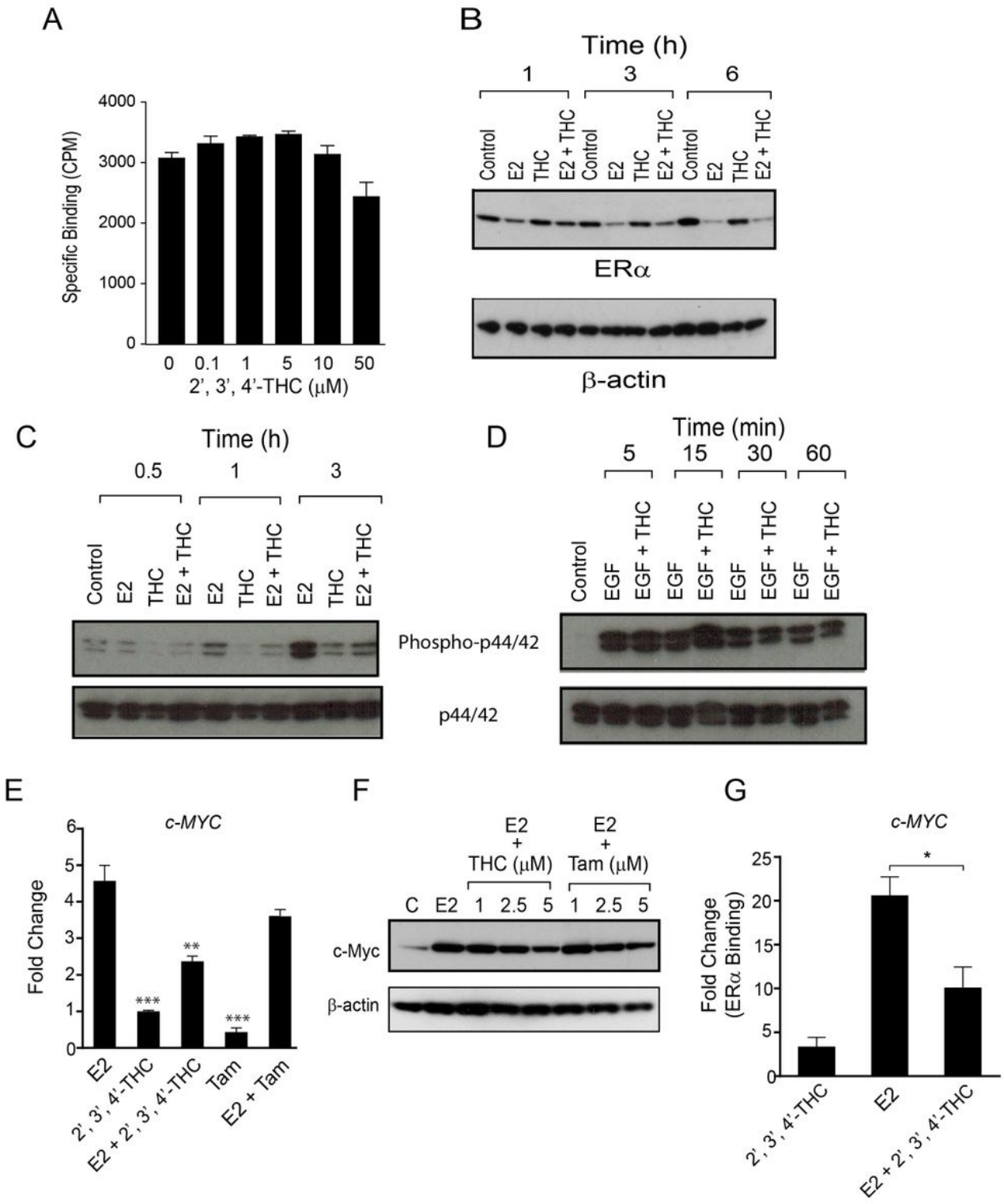

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Supplementary Files

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- TableS1.xls