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# Antiestrogenic Activity of Flavonoid Phytochemicals Mediated via the c-Jun N-terminal Protein Kinase Pathway:

## Cell-type specific regulation of estrogen receptor alpha

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## Abstract

Flavonoid phytochemicals act as both agonists and antagonists of the human estrogen receptors (ERs). While a number of these compounds act by directly binding to the ER, certain phytochemicals, such as the flavonoid compounds chalcone and flavone, elicit antagonistic effects on estrogen signaling independent of direct receptor binding. Here we demonstrate both chalcone and flavone function as cell type-specific selective ER modulators. In MCF-7 breast carcinoma cells chalcone and flavone suppress ERα activity through stimulation of the stress-activated members of the mitogen-activated protein kinase (MAPK) family: c-Jun N-terminal kinase (JNK)1 and JNK2. The use of dominant-negative mutants of JNK1 or JNK2 in stable transfected



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cells established that the antiestrogenic effects of chalcone and flavone required intact JNK signaling. We further show that constitutive activation of the JNK pathway partially suppresses estrogen (E2)-mediated gene expression in breast, but not endometrial carcinoma cells. Our results demonstrate a role for stress-activated MAPKs in the cell type-specific regulation of ERα function.

**Keywords**

flavonoids; phytoestrogens; estrogen receptor; mitogen-activated protein kinase; antiestrogens; c-Jun N-terminal kinase (JNK)

## Introduction

Classic steroid hormone agonists and antagonists function through direct interaction with their cognate receptor, functioning as a ligand-inducible transcription factor, whereby they activate or inhibit transcriptional activity (1–2). Antiestrogens, such as tamoxifen, antagonize estrogen action by competing with estrogen for binding of the receptor. However, the complexity of tamoxifen's activity is revealed through studies describing both agonistic and antagonistic effects of this compound in a tissue specific manner (3–4). This mixed action is partially determined by the ability of the ER to activate transcription via its activation domains, AF-1 and AF-2, and to activate tissue specific factors such as cofactors and cellular signaling cascades. In ER-positive breast cancer cell systems, the generation of tamoxifen-refractory cell types can be established while ER status and binding remain unchanged (5–7). These studies suggest that the antiestrogen activity demonstrated by tamoxifen may also be determined by the alternate activation of non-steroid hormone receptor signaling pathways that converge upon the ER. For example, the peptide hormone epidermal growth factor (EGF) and insulin-like growth factor-I (IGF-I) enhance ER function in both ligand-dependent and -independent manners. This activity is regulated by the ability of these growth factors to activate intracellular signaling events such as the Ras-Raf-MEK-signaling pathway (8–10). EGF- and IGF- mediated enhancement of ER function depends in part on activation, which results in subsequent ER phosphorylation (11–12). Additionally, recent reports have demonstrated that other members of the MAPK family including JNK and p38 also can regulate ER function (13–15). Therefore, complexity of the activity of a steroid hormone mimetic is defined by both tissue-and-cell type specific factors as well as alternate signaling events. Added to this complexity is evidence that activation of members of the MAPK cascade occurs not only in response to peptide hormones, but also in response to estrogens and antiestrogens (16–21). We and others have also demonstrated that endocrine-active environmental agents such as organochlorine pesticides, PCBs and flavonoid phytochemicals can affect other cellular signaling cascades (such as MAPK cascades) (22–25).

Given the ability of peptide hormone signaling to enhance steroid hormone function, the possibility exists that certain endocrine disrupting chemicals may also act to enhance or suppress ER function by receptor-binding independent mechanisms. We have previously examined the ability of certain flavonoid phytochemicals to activate or inhibit ER function

in both yeast and mammalian cells (26–33). Our studies, and other reports, have identified certain phytochemicals that function as either agonists or antagonists through direct binding of the ER. In contrast, phytochemicals such as kaempferide, flavone, and apigenin can act as ER antagonists independent of direct ERα or ERβ binding (28, 34–37). This suggests that certain phytochemicals, as well as other environmental estrogens, might regulate ER function through mechanisms independent of direct receptor binding. Consistent with this evidence, certain flavonoids can stimulate MAPK-responsive transcription factors in hormone-independent ERnegative cell systems (34–38). These flavinoids, specifically flavone and chalcone, demonstrate anti-viability properties in breast and endometrial cells (XXX). Given the crosstalk between the MAPK cascade and the ER, we proposed that receptor-binding independent antiestrogenic flavonoids might function to regulate members of the MAPK cascade, specifically c-Jun N-terminal kinase (JNK), as a mechanism for their ER antagonistic activity. Here we describe the ability of certain flavonoid phytochemicals to activate the JNK signaling cascade and suppress ER transactivation in a JNK-dependent and cell type specific manner.

## MATERIALS & METHODS

### Materials

Flavone-(2-phenyl-4H-1-benzopyran-4-one) and chalcone-(1,3--diphenyl-2-propen-1-one) were purchased from INDOFINE Chemical Company Inc. (Somerville, NJ). 4-hydroxy tamoxifen and 17β-estradiol were purchased from Sigma Chemical Co. (St. Louis, MO) and ICI 182,780 was purchased from Tocris (Ellisville, MO). All chemicals were prepared in dimethylsulfoxide (DMSO) and added to the media so the final concentration of solvent did not exceed 0.1%. TNF-α was purchased from R&D Systems (Minneapolis, MN). Mammalian expression constructs containing dominant-negative JNK1 and JNK2 were generously provided by Dr. Roger Davis (University of Massachusetts).

### Cell Culture

The ERα-positive MCF-7 breast carcinoma cells (N variant) (39), ERα-positive Ishikawa endometrial carcinoma cells (Ishikawa(+))(38), ERα-negative Human Embryonic Kidney 293 (HEK293) and ERα-negative HepG2 cells were cultured in 150 $cm^2$ culture flasks in Dulbecco's Modified Eagle's Medium supplemented with 10% fetal bovine serum (FBS) (Gibco-BRL, Gaithersburg, MD), as well as BME and MEM amino acids, L-glutamine, sodium pyruvate, and penicillin-streptomycin (diluted in the medium to a 1× concentration from either 100× or 50× stocks for a final concentration of 1%), and porcine insulin ($10^{-8}$ M) (Sigma Chemical Co., St. Louis, MO). The culture flasks were maintained in a cell incubator at a humidified atmosphere of 5% $CO_2$ and 95% air at 37 °C. Cells were placed in phenol red-free Dulbecco's Modified Eagle's Medium (DMEM) supplemented with 5% dextran-coated charcoal-treated FBS for 48 hours prior to plating (5%CS-DMEM) (40–41).

Generation of stably transfected cell lines stable population expressing Vector, DN-JNK1 or DN-JNK2. MCF-7 cells were transfected with 3μg of vector or the dominant-negative JNK1 or JNK2 plasmid using 9μl of lipofectamine. After 5 hours, the transfection media was removed and replaced with 10% FCS-DMEM for 48 hours. Cells were split into T-75 flasks

in 10% FCS-DMEM and allowed to attach overnight. The next day the media was removed and replaced with 10% FCS-DMEM containing 750μg/ml of G418. Every three days the media was changed to fresh 10% FCS-DMEM +750μg/ml G418. Following three weeks of selection the MCF-7 cells were examined for expression.

### In Vitro JNK Kinase Assay

Cells were harvested in lysis buffer (20mM HEPES, 50mM β–glycerolphosphate, 2mM EGTA, 1mM DTT, 10mM NaF, 1mM $Na_2VO_4$, 1% Triton X-100, 10% glycerol, 1mM PMSF, .01mg/ml aprotinin, and .01mg/ml leupeptin) on ice and 300 μg of cell extract was immunoprecipitated for 60 min with antiJNK antibody (Santa Cruz) followed by a 60 min incubation with a 50% protein A Sepharose 4B slurry. The JNK bound beads were washed with a wash buffer (500mM LiCl, 100mM Tris-HCl pH 7.6, 0.1% Triton X-100, and 1mM DTT) and resuspended in 50μl of assay dilution buffer (20mM MOPS, pH 7.2, 25mM β-glycerolphosphate, 5mM EGTA, 1mM sodium orthovanadate, 1mM dithiothreitol). 1μg of c-Jun-GST protein (Upstate Biotechnology, Lake Placid, NY) and 10μl of ($\gamma$-$^{32}$P)ATP (100μCi/10μl) diluted into 90μl of Magnesium/ATP cocktail (500μM ATP and 75mM magnesium chloride in assay dilution buffer) were added to each experimental sample. The reaction was allowed to proceed at 30°C in a shaking incubator for 30 min and terminated with EDTA. 40μl of reaction mixture was placed on PC(P81) paper in a millipore apparatus and washed 5× with 180 mM phosphoric acid. Filters were allowed to air dry and then placed in scintillation fluid and counted using a Beckman LS6500 multi-purpose scintillation counter.

### Western Blot Analysis

Stable expression of DN-JNK1 and DN-JNK2 were examined by western blot analysis as described previously (42–43). Briefly, cells were harvested in sonicating buffer (62.5 mM Tris-HCl, pH 6.8, 4% (w/v) SDS, 10% glycerol. 1 mM phenylmethylsulfonyl fluoride (PMSF), 25 mg/ml leupeptin, 25 mg/ml aprotinin) and sonicated for 30 seconds. Following centrifugation at 1,000 × g for 20 minutes, 50 μg of protein was resuspended in sample loading buffer (62.5 M Tris-HCl, pH 6.8, 2% (w/v) SDS, 10% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue), boiled for 3 minutes and electrophoresed on a 10% polyacrylamide gel. The proteins were transferred electrophoretically to a nitrocellulose membrane. The membrane was blocked with PBS-Tween (0.05%) - 5% lowfat dry milk solution at 4°C overnight. The membrane was subsequently incubated with mouse anti-human JNK1,2 (Pharmingen, San Diego, CA) specific monoclonal antibody 1.5μg/μl and incubated for 2 hours at room temperature. Blots were washed in PBS-Tween solution and incubated with goat anti-mouse secondary antibodies conjugated to horseradish peroxidase (1:5000 dilution Oxford, Oxford, MI) for 30 minutes at room temperature. Following four washes with PBS-Tween solution, immunoreactive proteins were detected using the ECL chemiluminescence system (Amersham, Arlington Heights, IL) and recorded by fluorography on Hyperfilm, according to the manufacturer's instructions. Fluorograms were quantitated by image densitometry using the Molecular Analyst program for data acquisition and analysis (BioRad).

### Luciferase Assays

Transient transfections: The cells were plated in 6-well plates at $1 \times 10^6$ cells/well in 5%CS-DMEM media and allowed to attach overnight. The next day the cells were transfected for 5 hours in serum/supplement-free DMEM with 2 μg of ERE2-luciferase plasmid which contains two copies of the vitellogenin ERE linked to the luciferase gene. For ERE-luciferase experiments in ERα-negative HEK293 or HepG2 cells, co-transfection with pcDNA3- ERα (500 ng) was used (26, 44–45). For experiments in stable Vec, DN-JNK1 or DN-JNK2 expressing MCF-7 cells were transfected with either pERE2-luciferase using Lipofectamine as above or with an AP-1(7×)-luciferase plasmid (Stratagene, La Jolla, CA) using FuGENE6 (Roche, Indianapolis, IN) according to manufacturer's instructions. Similarly experiments using stable cells transfected with Gal4-luciferase in combination with Gal4-cJun or Gal4-Elk were performed with FuGENE6 as described previously (46). After 6 hours, the transfection media was removed and replaced with 5%CS-DMEM containing vehicle, 17β-estradiol (1 nM), flavone (25 uM), chalcone (10 uM), or 17β-estradiol plus flavone or chalcone and incubated for 18 hours at 37 °C. After the 18 hours the treatment containing media was removed and 250 μl of 1× lysis buffer (Analytical Luminescence Laboratory, Ann Arbor, MI) was added per well and incubated for 15 min at RT. The cell debris was then pelleted by centrifugation at 15000g for 5 min. The cell extracts were normalized for protein concentration using the Bio-Rad Reagent following the supplied protocol (Bio-Rad Laboratories, Hercules, CA). Luciferase activity for the cell extracts were determined using Luciferase Substrate (Promega, Madison, WI) in a Monolight 2010 luminometer (Analytical Luminescence Laboratory, Ann Arbor, MI).

### Statistical Analysis

Data was analyzed using Student's unpaired t-test with $p<0.05$ as the limit of statistical Significance (47–49). Experiments comparing multiple concentrations to the control were tested with one way ANOVA with Bonferroni post-test to compare individual concentrations. All statistical analyses were done using GraphPad Prism 5.0 (GraphPad Software).

## RESULTS

### Cell specific antiestrogenic activity of chalcone and flavone

We sought to determine the cell-type specific estrogenic and antiestrogenic activity of the flavonoid phytochemicals flavone and chalcone (Fig 1). Using MCF-7 breast and Ishikawa(+) endometrial carcinoma cell lines which endogenously express ERα as the predominant estrogen receptor subtype (38) we examined the effects of flavone and chalcone on estrogen-stimulated ERE-luciferase activity (Fig 2A). 17β-estradiol (1nM) stimulated a 38.74±19.37 and 12.63±4.27 fold induction of ERE transcriptional activity in MCF-7 and Ishikwa cells, respectively, over control (data not shown). Flavone displayed an estrogenic trend, though not statistically significant, in MCF-7 (19.2±3.4%) and Ishikawa(+) (14.1±3.2%) cells as compared to the reference activity of the major endogenous estrogen, 17b-estradiol, alone (100%) (Fig 2A). In combination with 17β-estradiol, chalcone exhibited antiestrogenic activity in both cell types reducing ERE activity to 28.3± 11.2% in MCF-7 and 65.4±7.1% in Ishikawa(+) cells as compared to estradiol alone (100%). Interestingly

flavone acted as an antiestrogen only in the MCF-7 cell line, suppressing ERE-activity to 51.3±13.8% as compared to E2 alone while actually increasing ERE-activity in conjunction with E2 in the Ishikawa(+) cells (120.9±13.6%). To further explore the cell type-specific estrogenic/antiestrogenic activities of chalcone and flavone we used ERα-negative HepG2 and HEK293 cell lines co-transfected with ERE-luciferase and an ERα expression construct (Fig 2B). We demonstrated that again flavone, but not chalcone, exerted a weak agonistic activity in the HepG2 line (20.2±8.6%), an effect that was not observed in the HEK293 cells. Both chemicals exerted antiestrogenic effects in the HepG2 cell line with flavone and chalcone reducing ERE-activity to (59.2±20.9%) and (16.5±7.4%) respectively as compared to E2 alone (100%). Interestingly, the HEK293 cells were refractory to the antiestrogenic activity of both flavone and chalcone. The antiestrogenic effects of these compounds did not correlate with direct competition for receptor binding, suggesting a binding independent mechanism for regulation of ER activity (28).

## Flavonoid-induced JNK-activation

The ability of estrogens and antiestrogens to activate MAPKs suggests a mechanism by which endocrine-active agents such as flavonoids may exert biological effects. Additionally, environmental agents such as flavonoids and organochlorines also regulate gene expression in part through the activation of specific members of the MAPK signaling cascade. Given that the basal or stimulated activation of members of the MAPK cascade regulate ER-function in both a ligand-dependent and -independent manner, we initially examined the activation of JNK signaling in response to flavone and chalcone.

TNF-α rapidly and potently activates JNK in MCF-7 cells (50) and represses ER function in certain systems (51–53). Here, we demonstrate that TNF-α (10 ng/ml) activates JNK in MCF-7 cells in a time dependent biphasic manner with a maximal fold activation of 7.8±1.78 and 7.8±2.03 occurring at 5 and 15 minutes respectively with activity returning to near control levels (2.25±0.25 fold) at 45 minutes (Fig 3A). Flavone and chalcone (Fig 3B) displayed a time dependent activation of JNK similar to TNF-α. At 5 min, chalcone and flavone activated JNK 9.86±0.06 fold and 9.41±0.64 fold, with subsequent increases in JNK activity up to 45min compared to control. These results demonstrate the ability of both chalcone and flavone to potently stimulate JNK activity with a similar duration and magnitude as TNF.

## Stable inhibition of JNK-1 and JNK-2 in MCF-7 cells blocks flavonoid-induced activation of AP-1, Elk, and c-Jun

To determine if JNK activation by phytochemicals mediated antagonistic effects of the receptor-binding independent antiestrogenic flavonoids, MCF-7 cells were transfected with either empty vector (Vec) or mammalian expression vector containing either a dominant-negative JNK-1 (DN-JNK1) or a dominant-negative JNK-2 (DN-JNK2) gene and pooled, stable-expressing cell lines were selected. Western blot analysis confirmed stable expression of either DN-JNK1 or DN-JNK-2 (Fig 4A). For comparison to the stable cell lines, MCF-7 cells were transiently-transfected with Vec, DN-JNK1, or DN-JNK2 and expression was confirmed (Fig 4B). Exclusive overexpression of individual isoforms of JNK1 (p46) or JNK2 (p54) can be seen in both stable pools and transiently-transfected cells.

To confirm that stable expression of either DN-JNK1 or DN-JNK2 suppressed signaling through the JNK cascade, reporter-gene assays responsive to MAPK stimulation were utilized. Stable MCF-7/Vec, MCF-7/DN-JNK1, or MCF-7/DN-JNK2 cells were transfected with an AP-1 responsive luciferase construct and treated with a known activator of the MAPKs, 12-phorbol-myristate-13-acetate (PMA), or with selected JNK-activating flavonoids (Fig 5A). PMA stimulated AP-1-reporter activation in MCF-7/Vec (26.8±8.5 fold), MCF-7/DN-JNK1 (23.7±11.9 fold) or MCF-7/DN-JNK2 (24.3±5.8 fold) cells to a similar extent. The inability of either DN-JNK1 or DN-JNK2 to suppress AP-1 stimulation by PMA is consistent with PMA's predominant activation of Erk as compared to JNK in these cells (data not shown). In MCF-7/Vec cells, AP-1 stimulation by both flavone (4.0±0.3 fold) and chalcone (92±31.3 fold) was reduced in MCF-7/DN-JNK1 (1.9±0.5 and 12.2±8.2 fold) or MCF-7/DN-JNK2 (2.8±0.8 and 37.5±11.1 fold) cells.

Increased AP-1 activity is induced by MAPK phosphorylation of the transcription factor Elk, resulting in increased expression of *c-fos* as well as by direct phosphorylation of c-Jun by JNK. Using Gal4 fusions of either Elk or c-Jun, flavone stimulated activity 4.3 and 1.9 fold respectively; while chalcone stimulated 5.8 and 2.9 fold respectively in MCF-7/Vec cells above baseline (Fig 5B). Activation of Gal4-c-Jun by flavone and chalcone was reduced in MCF-7/DN-JNK1 (0.53 and 1.3 fold) and MCF-7/DN-JNK2 (0.8 and 0.3 fold) cells. Similarly, activation of Gal4-Elk by flavone and chalcone was reduced in both MCF-7/DN-JNK1 (1.0 and 0.4 fold) and MCF-7/DN-JNK2 (0.5 and 0.1 fold) cells. The ability of both chalcone and flavone to stimulate AP-1 transcription through c-Jun or Elk activation is correlated with increased JNK activation, and dependent upon an intact JNK 1/2 cascade.

### JNK-mediated antiestrogenic effects of flavonoid phytochemicals

The effects of stable expression of Vec, DN-JNK1, or DN-JNK2 on ERE-activity were found to be similar with 17β-estradiol (1nM) stimulating a 14.4±1.6, 14.9±2.1 and 14.3±1.7 fold induction respectively over control (data not shown). In the MCF-7/Vec cells, 4-OH tamoxifen, chalcone, and flavone reduced ER-mediated gene transcription by 93±1.1%, 84±4.7%, and 68.4±2.5% respectively (Figure 6). Stable inhibition of the JNK1 signaling pathway in the MCF-7/DN-JNK1 cells did not significantly ($P>0.05$) affect chalcone's inhibition of E2-stimulated transcription which decreased from an 84±4.7% reduction in MCF-7/VEC cells to 72.8±5.6% in the MCF-7/DN-JNK1 cells (Figure 6). In the case of flavone, a significant role ($p<0.05$) for this signaling pathway is demonstrated with a reduction in its antagonistic activity from a 68.4±2.3% reduction in MCF-7/VEC cells to a 38.7±5.5% reduction in the stable MCF-7/DN-JNK1 cells, suggesting a role for the signaling pathway in the mediation of ER-mediated gene transcription. Similar results were observed in the stable DN-JNK2 expressing MCF-7 cells with chalcone's inhibition of ER-mediated gene transcription significantly ($p< 0.05$) decreased from a 84±4.7% reduction in the MCF-7/VEC cells to a 37.9±7.1% reduction in the MCF-7/DN-JNK2 cells. Likewise, a potential role for the JNK2 signaling pathway for flavone is suggested by the statistically significant reduction in flavone's inhibition of ER-mediated transcription from a 68±2.3% reduction in MCF-7/VEC to a 49.2±13.8% reduction in the MCF-7/DN-JNK2 cells. Inhibition of the JNK1 signaling pathway did not significantly affect the ability of 4-OH

tamoxifen to function as an antiestrogen which still inhibited E2-stimulated ERE-activity by 88.3±3.3% in the MCF-7/DN-JNK1 cells. In the MCF-7/DN-JNK2 cells, 4-OH tamoxifen's antagonism of E2-mediated gene expression was significantly abrogated from a 93±1.1% reduction to a 77.1±1.8% reduction ($p<0.05$). Interestingly however, stable expression of DN-JNK2 enhanced the agonistic activity with 4-OH tamoxifen alone yielding a statistically significant 9.0±3.7% activation of ERE in MCF-7/DN-JNK2 cells as compared to MCF-7/VEC cells.

### Constitutive activation of MKK7-JNK signaling suppresses E2-mediated gene expression in a cell-type specific manner

To confirm the role of JNK activation in suppression of ER activation, a constitutively-active mitogen-activated protein kinase kinase 7 expression construct (MKK7-CA) was used in MCF-7 (Fig 7A) and Ishikawa(+) cells (Fig 7B). In MCF-7 cells, MKK7-CA predominantly activates the downstream JNK1,2 isoforms, and when transfected along with ERE-luc suppressed a dose dependent E2-stimulated transcriptional activation of ERE-Luc as compared to vector transfected cells (Fig 7A). Interestingly, in Ishikawa(+) cells expression of MKK7-CA potentiated estrogen activation of ERE-luciferase. Thus activation of the MKK7-JNK pathway leads to decreased ER activation in the MCF-7 cells, confirming a role for JNK in suppression of ER function in this system while enhancing ER-activity in the Ishikawa(+) cells. These results demonstrate that similar to the JNK dependent cell type specific effects of flavone, direct activation of MKK7-JNK functions to differentially regulate ER activity in a cell type specific manner.

## DISCUSSION

We have previously demonstrated that certain flavonoid phytochemicals including flavone, chalcone, apigenin and kaempferide can negatively regulate ER function independent of direct binding to the ER (28) and are thus classified as binding-independent antiestrogens. Here we demonstrate that flavone and chalcone negatively regulate ER function in ER-α-positive MCF-7 breast and ERα transfected HepG2 liver cancer cells. In contrast, ERα-positive Ishikawa(+) endometrial carcinoma and ERα transfected HEK 293 cells are refractory to the antiestrogenic effects of flavone. Therefore, these cell type specific effects of flavone and chalcone demonstrate the ability of naturally occurring flavonoid phytochemicals to function as selective estrogen receptor modulators or phyto-SERMs. The ability of flavone and chalcone to function as phyto-SERMs through an ER-binding independent manner suggests they regulate cell signaling pathways that converge upon ER function. Recent reports have implicated specific phytochemicals, such as the soy isoflavone genistein and flavonoid apigenin, in the selective activation of MAPK pathways (54–59). In this report we demonstrate that the flavonoid phytochemicals chalcone and flavone are also capable of activation of JNK signaling cascades as measured by both JNK *in vitro* kinase assay and targeted activation of a Gal4-cJun mediated transcription.

The JNK and p38 signaling pathways have been described as stress response pathways in many cell types and are activated by peptide hormones (TNF-α receptor, Fas ligand receptor, IL-1 receptor, and EGFR) or non-receptor mediated stresses (ex. heat shock,

oxidative stress, ionizing UV radiation, tumor promoters, protein synthesis inhibitors, and chemotherapeutic drugs) (60– 64). These pathways have been shown to exert both positive and negative regulatory effects on gene transcription. Our results here suggest that stress-activated MAP-kinases, JNK1 and 2, are critical in regulating the antiestrogenic effects of certain phytochemicals. The suppression of JNK signaling by expression of dominant-negative mutants of JNK-1 or JNK-2 results in an inhibition of the antiestrogenic effects of selected phytochemicals on ERE transcription. Chalcone and flavone both demonstrate the ability to initiate a JNK signaling pathway although with different magnitudes and time courses. Flavone and chalcone require JNK-signaling for their antiestrogenic effects in MCF-7 cells.

Our results demonstrate as a whole, that stress-activated MAP-kinases, JNK1 and 2, are important in regulating the estrogenic and antiestrogenic responses of the ER. We demonstrate the direct involvement of the JNK pathway in the negative regulation of estrogen receptor-mediated gene transactivation. While JNK-activation by Src and MEKK1 has been shown to positively regulate ER function in Hela and Ishikawa(+) cells (15, 65)], other studies have demonstrated negative regulation of ER by members of the Rho GTPase family and p21-activated kinase family which are known activators of JNK signaling (66–67)]. In particular the reported differences in the ability of RAC1, upstream of JNK activation, to either stimulate or repress ER function emphasizes the potential methodologic or cell-type specific factors that influence ER-signaling (15, 66)]. Additionally, work by Bodine et. al. further hints at a potential cell type specific regulation of ER by stress-mediated signaling in which TNFα, a known activator of JNK, suppresses ER activity in MCF-7 cells but not in ERα overexpressing CHO cells even though both cell lines responded to the cytokine with regards to NF-κB activation (52)].

Our results investigating JNK, along with the published reports demonstrating differential effects of p38 on ER function, highlight the complicated task of understanding the MAPK-ER cross-talk and the importance of cellular context. Our data here, using two endogenous ERα-expressing (MCF-7, Ishi(+)) and two artificial ERα expressing (HepG2, HEK293) cell lines, helps better elucidate the cell-type specific nature of estrogen receptor regulation by both specific ligands and signaling pathways. MCF-7 and HepG2 cells respond to flavone and chalcone as ER antagonists while HEK293 and Ishikawa(+) cells are refractory to the effects of flavone and chalcone. The ability of a constitutively active MKK7 to alternately activate or inhibit ER function in Ishikawa(+) and MCF-7 cells respectively further emphasizes the cell-specific nature of the MAPKs in ER regulation. Both Lee et al. and Feng et al. demonstrated an enhancement of ER-activity in a JNK dependent manner which is consistent with our direct MKK7-JNK activation of ERE in Ishikawa(+) cells (15, 65)]. In contrast, Su et al. obtained results like ours, demonstrating a negative regulation of ER by known JNK–activating pathways in MCF-7 cells (66)]. This suggests that JNK or some other signaling pathway(s) are intact and functionally repress ER-activity in the MCF-7 and HepG2 systems but not in HEK293 or Ishikawa(+) cells. Mechanistically the question remains as to how individual MAPKs, and in particular JNK, regulate ER in a cell type specific manner.

Overall, our results here implicate JNK as a component of the antiestrogenic action of the receptor binding-independent phytochemicals flavone and chalcone in a cell specific manner. JNK stress-signaling pathway functions as a cell-type specific regulatory signaling cascade of ER mediated transcription. These results therefore broaden the spectrum of selective estrogen receptor modulators (SERMs) to include both cell-signaling selective ER modulation and phytoestrogen selective ER modulation.

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## Highlights

- Chalcone and flavone have cell-type specific antiestrogenic activity
- Chalcone and flavone suppress ERα activity through stimulation of MAPKs
- Constitutive activation of the JNK pathway suppresses ER signaling in breast cancer
- Constitutive activation of the JNK pathway does not suppresse ER signaling in endometrial cancer

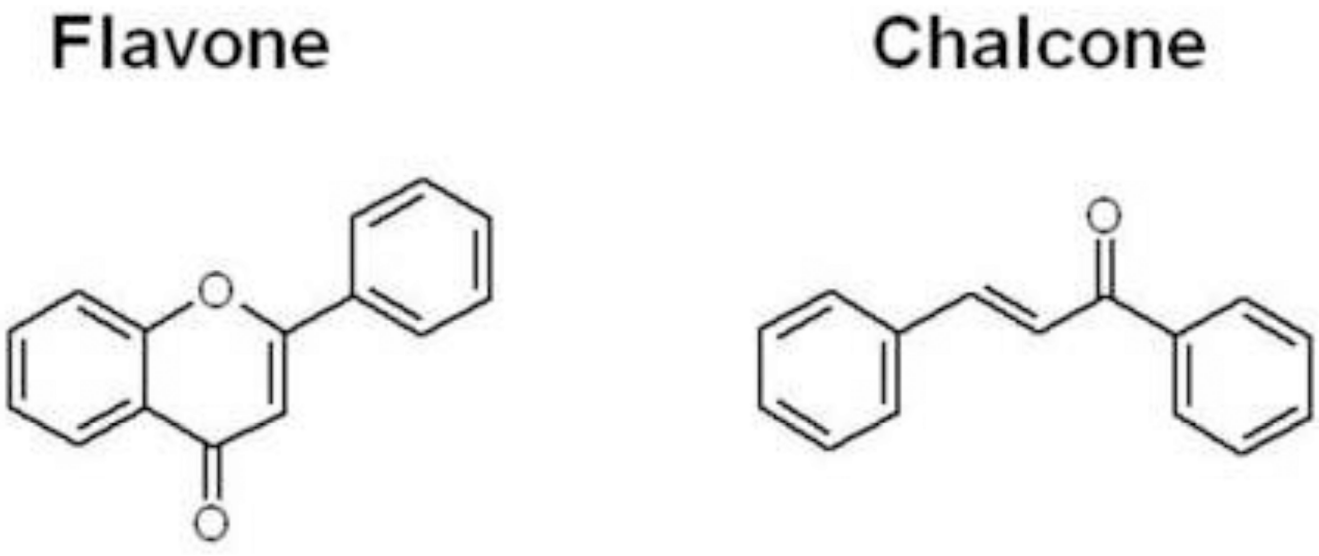


**Figure 1.**
Chemical structures of the receptor-binding independent antiestrogenic flavonoids chalcone and flavone.

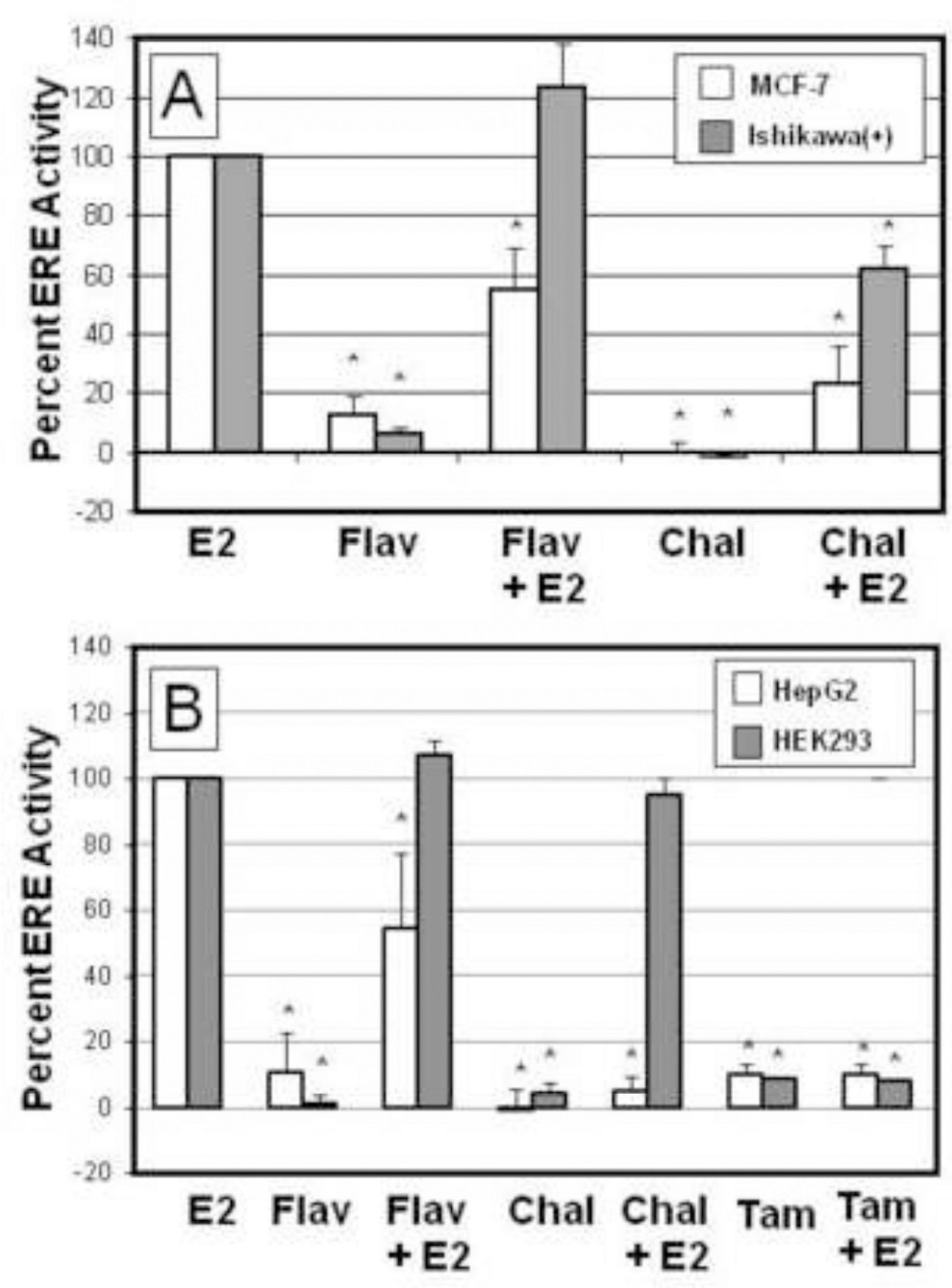


**Figure 2.**

Cell specific antiestrogen activity of chalcone and flavone. (**A**), MCF-7 or Ishikawa(+) cells transfected with ERE-luciferase for 6 hours were treated with vehicle (DMSO), flavones (25 μM), or chalcone (25 μM) alone or in combination with 17β-estradiol for 24 hours and harvested for luciferase assay. (**B**), ER-α-negative HEK293 or HepG2 cells were transfected with both ERE-luciferase and ER-α followed by treatment with with vehicle (DMSO), Tamoxifen (Tam, 100 nM), flavone (25 μM), or chalcone (25 μM), alone or in combination with 17β-estradiol for 24 hours and harvested for luciferase assay. Data are represented as

percent activation as compared to 1nM 17β-estradiol (100%) ± SEM from four independent experiments each in duplicate (*, $p < 0.05$).

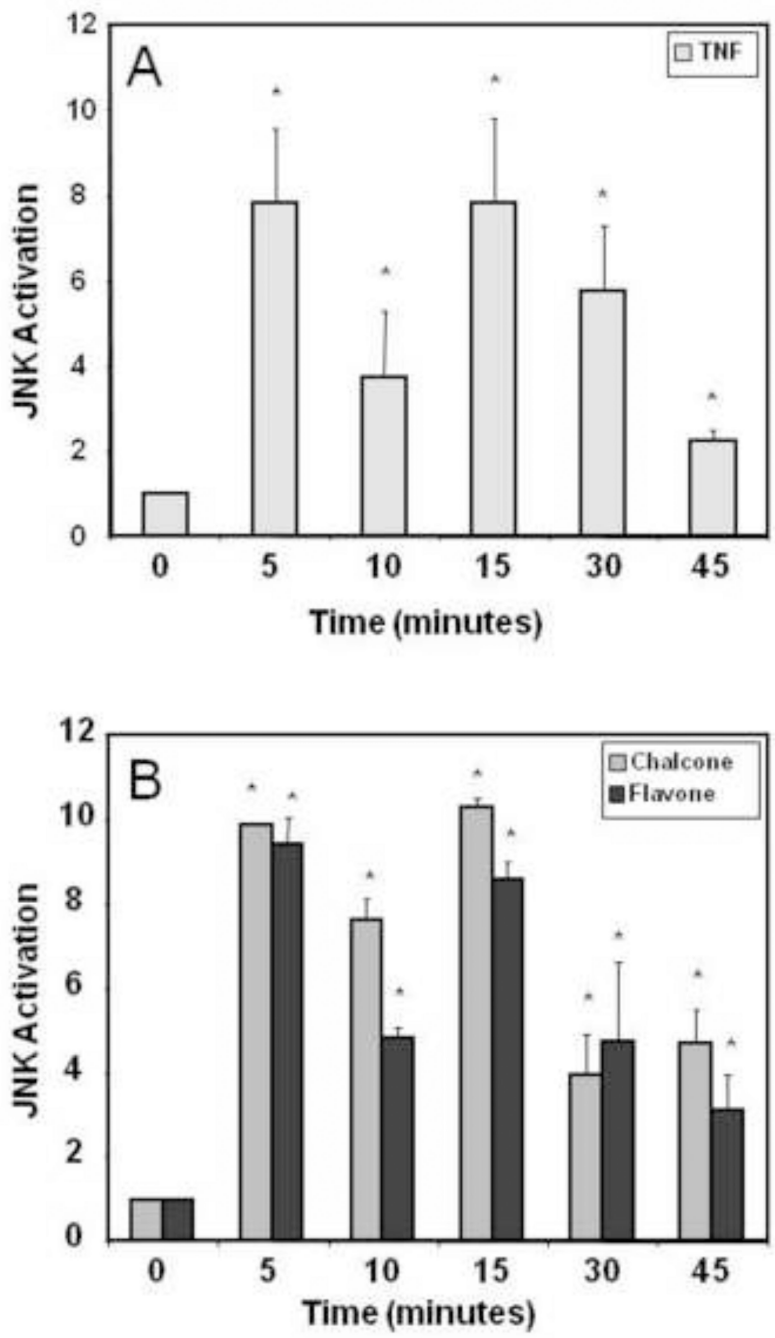


**Figure 3.**

Activation of JNK by TNF-α and the flavonoid phytochemicals chalcone and flavone. MCF-7 cells were treated with either (**A**) TNF-α (10 ng/ml), (**B**) chalcone (25 μM), or flavone (25 μM), for times indicated from 0 to 45 minutes. Cells were harvested and assayed for JNK activity as described in Material & Methods using an in vitro kinase assay. Data are represented as fold activation from control (0 minutes) ± SEM from three independent experiments each in duplicate.

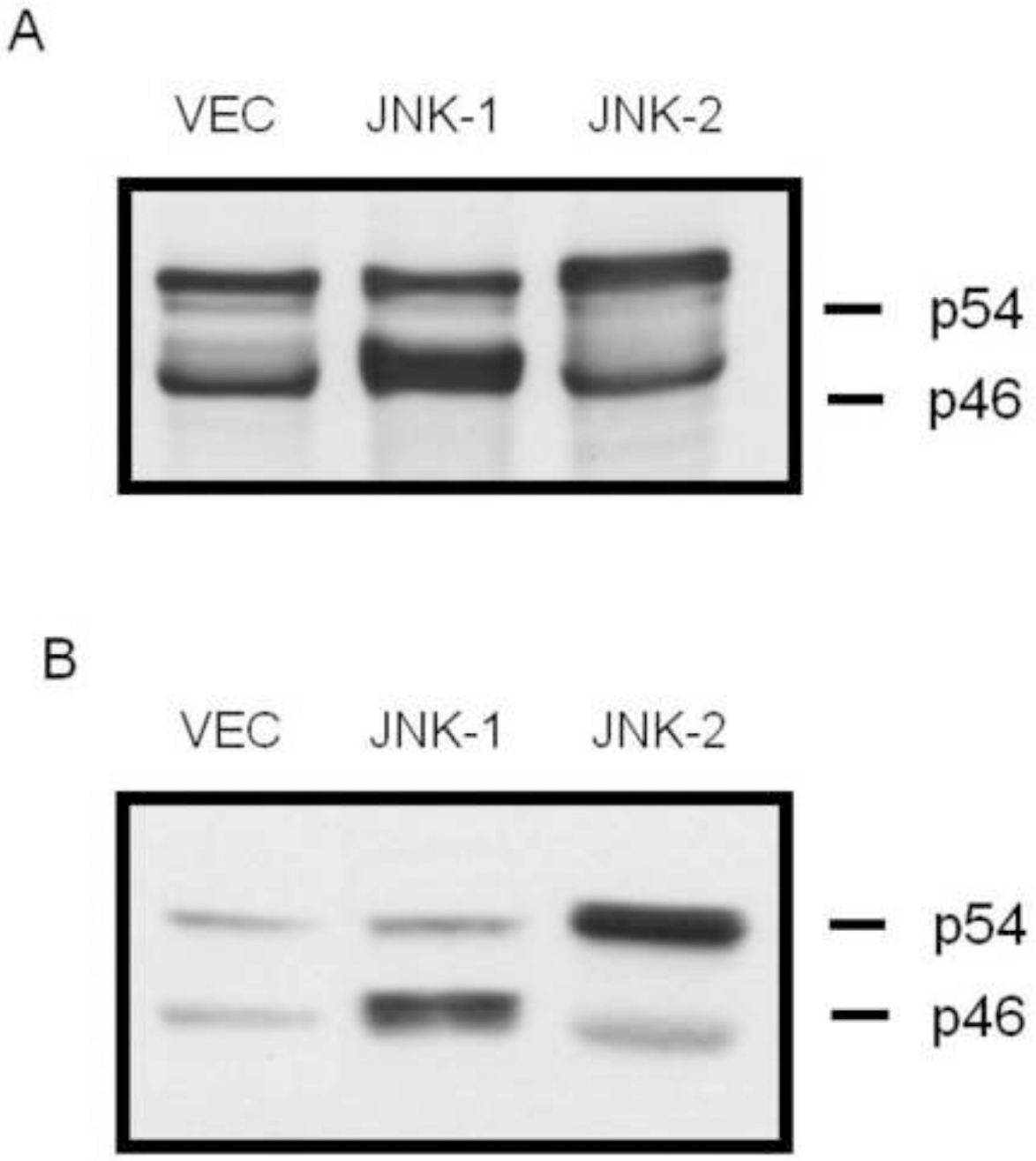


**Figure 4.**

Stable expression of dominant-negative JNK-1 or JNK-2 isoforms. (**A**) MCF-7 cells stably (**A**) or transiently (**B**) expressing either empty vector (VEC), dominant-negative JNK-1 (DN-JNK-1), or dominant-negative JNK-2 (DN-JNK-2) were generated as described in the Materials & Methods section. Pooled populations were harvested and immunoblot analysis was used to determine expression of either JNK-1 or JNK-1 expression. Molecular weight markers indicate migration of JNK-1 (p46) or JNK-2 (p55) bands.

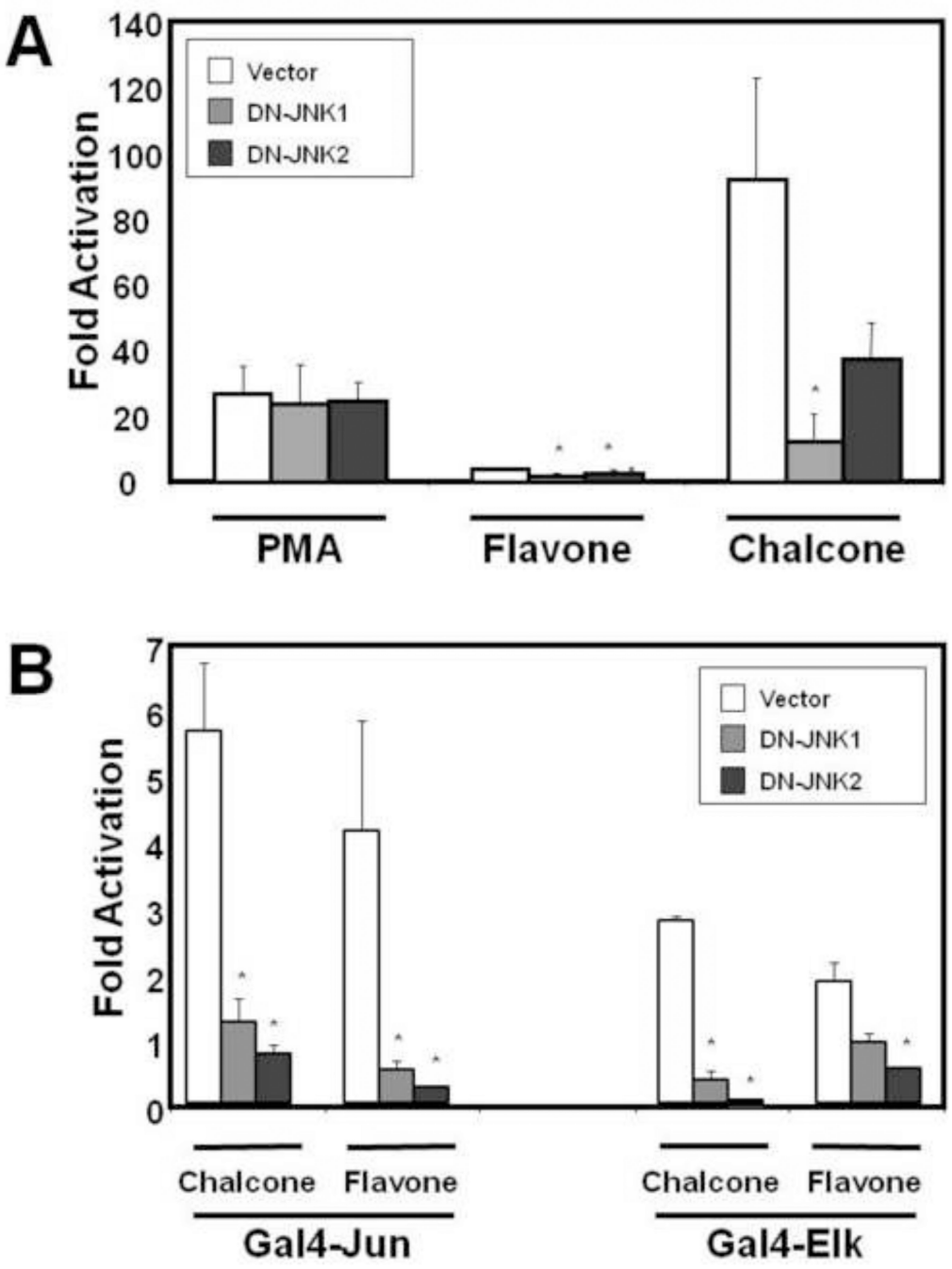


**Figure 5.**

Regulation of flavonoid-stimulated AP-1, c-Jun, and Elk transcriptional activation by DN-JNK isoform expression. (**A**), Stable expressing MCF-7/Vec, MCF-7/DN-JNK1, and MCF-7/DN-JNK2 cells were transfected with an AP-1-luciferase reporter construct 6 hours followed by treatment with either vehicle (DMSO), PMA (20 ng/ml), flavone (25 μM) or chalcone (25 μM). (**B**), Stable expressing MCF-7/Vec, MCF-7/DN-JNK1, and MCF-7/DN-JNK2 cells were transfected with a GAL4-UAS-Luciferase construct in conjunction with either a GAL4-DBD-Jun or GAL4-DBD-Elk plasmid followed by treatment with either

vehicle (DMSO), chalcone (25 μM) or flavones (25 μM). Cells were harvested the following day and analyzed for luciferase activity as indicated in the Materials and Methods. Data are represented as fold activation from vehicle control samples for each cell line ±SEM of four experiments in duplicate (AP-1) or as a representative experiment in duplicate (c-Jun, Elk) (*, $p < 0.05$).

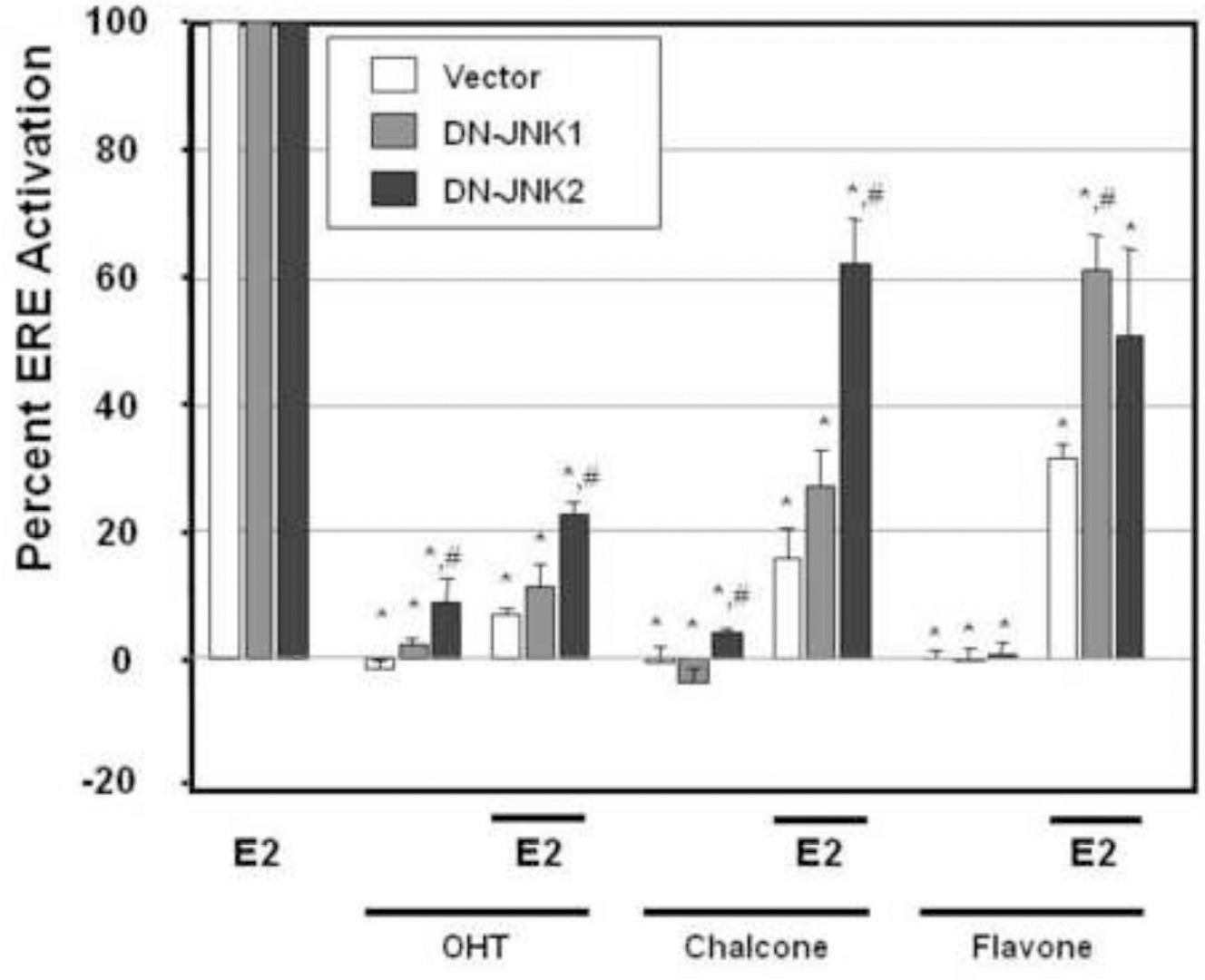


**Figure 6.**

Antiestrogenic effects of chalcone and flavone require intact JNK signaling. Stable expressing MCF-7/Vec, MCF-7/DN-JNK1, and MCF-7/DN-JNK2 cells were transfected with an ERE-luciferase reporter construct using Lipofectamine for 6 hours followed by treatment with either vehicle (DMSO), 4-OH-tamoxifen (100 nM), chalcone (25 μM), or flavones (25 μM) alone or in combination with 17β-estradiol for 24 hours and harvested for luciferase assay. Data are represented as normalized percent activation as compared to 1 nM 17β-estradiol (100%) ± SEM from four independent experiments each in duplicate (*, $p < 0.05$ compared to E2 control, #, $p < 0.05$ compared to vector control).

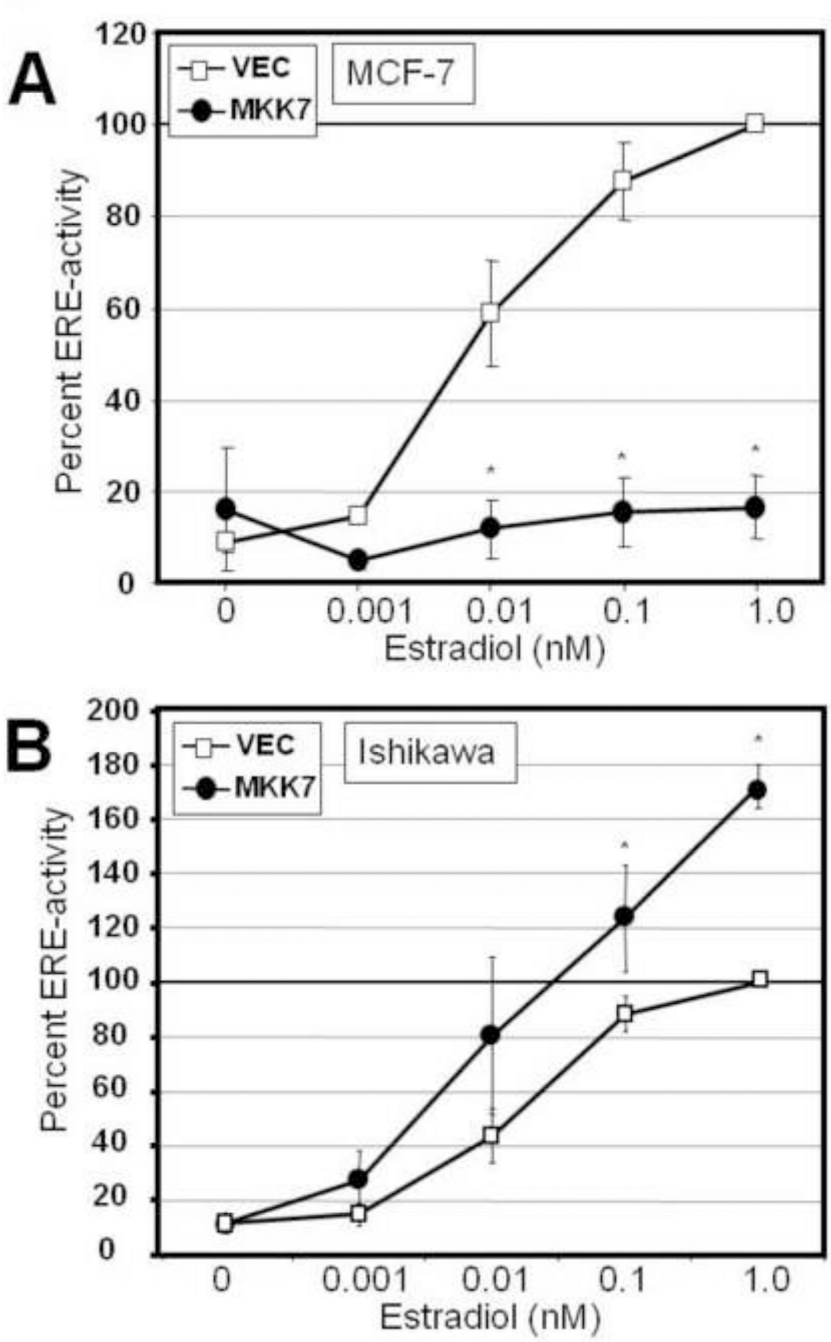


**Figure 7.**

Effects of constitutive active MKK7 on estrogen receptor transcriptional activation. MCF-7 cells (**A**) or Ishikawa(+) cells (**B**) were transfected with ERE-Luc along with either empty vector (VEC) or constitutive active MKK7 (CA-MKK7) for 6 hours and treated with DMSO or increasing concentration of estradiol ($10^{-12}$ to $10^{-9}$ M) and harvested 18 hours later for luciferase assay. Data are represented as percent ERE-activation normalized to vector

transfected and 1 nM E2 treated (100%) ± SEM from four independent experiments each in duplicate (*, $p < 0.05$).