

Trigonelline Is a Novel Phytoestrogen in Coffee Beans^{1,2}

Kimberly F. Allred,³ Katarina M. Yackley,³ Jairam Vanamala,^{4,5} and Clinton D. Allred^{3,4*}

³Department of Nutrition and Food Science and ⁴Institute for Obesity Research and Program Evaluation, Texas A&M University, College Station, TX 77843; and ⁵Department of Food Science and Human Nutrition, Colorado State University, Fort Collins, CO 80523

Abstract

Drinking coffee has been associated with the development of several endocrine-related cancers. The interpretation of these data has often been limited to the role that caffeine plays. Trigonelline (Trig), a niacin-related compound, is a natural constituent of coffee accounting for ~1% dry matter in roasted beans. Studies exploring the effects of this bioactive compound on mammalian cells are limited. The initial purpose of our studies was to determine whether Trig alters the actions of estradiol (E₂), using proliferation of estrogen-dependent human breast cancer (MCF-7) cells as a model system. When cells were cotreated with suboptimal doses of E₂ (10 pmol/L) and Trig (100 pmol/L), an additive enhancement of MCF-7 growth was observed. In the absence of E₂, Trig stimulated MCF-7 cell proliferation in a dose-responsive manner and significantly enhanced cell growth at concentrations as low as 100 pmol/L. Cotreatment of MCF-7 cells with Trig and ICI 182,780, an estrogen receptor (ER) antagonist, inhibited Trig-induced cell proliferation. Trig treatment also induced activation of estrogen response element reporter assays in MCF-7 cells and increased expression of ER target genes (pS2, progesterone receptor, and cyclin D1) similar to E₂. While our data demonstrate that Trig activates the ER, competitive binding assays showed that Trig does not compete E₂ off of the ER at any concentration. This suggests that Trig is activating the ER through a separate mechanism. Collectively, these data demonstrate that Trig even at low concentrations stimulates MCF-7 cell growth and that this effect is mediated through ER, clearly identifying Trig as a novel phytoestrogen. *J. Nutr.* 139: 1833–1838, 2009.

Introduction

Coffee is one of the most commonly consumed beverages in the world. Because of its popularity, it has become important to determine whether consumption of bioactive compounds from coffee may be associated with either health benefits or risks. Studies have examined the effects of drinking coffee on the incidence of several diseases. Coffee consumption has been correlated to reduced risk of colon cancer (1), type 2 diabetes (2), and Alzheimer's disease (3). Coffee may also be associated with the development of hormone-related cancers in women. For instance, women who consumed ≥ 4 cups of coffee daily had a reduced risk of developing invasive epithelial ovarian cancer compared with non-coffee drinkers (4). Other studies have not demonstrated this association (5,6). A recent study demonstrated that drinking coffee reduced women's risk of developing endometrial cancer (7). Similar to that seen in the ovary, the data examining the association between coffee consumption and breast cancer development has been variable. Several studies found a reduced risk of breast cancer in women who were coffee

drinkers (8–10). Others have shown no correlation between the beverage and onset of breast cancer (11) and 2 studies actually reported an increase in the number of breast cancer cases in women who drank coffee, although these differences did not reach the point of significance (5,12). Inconsistencies in data related to the development of these hormone-related cancers are likely due to several factors, including variations of coffee beans and preparation procedures. In addition, brewed coffee contains complex mixtures of bioactive compounds that may have different effects based on the stage of carcinogenesis.

Most of the research studying the impact of coffee consumption on endocrine-related cancers has focused on caffeine as the most likely bioactive component. It is hypothesized that caffeine consumption is associated with the modulation of endogenous estrogen and several studies have shown that the compound can alter expression of sex hormone-binding globulin in women (13–15). Although this correlation remains poorly defined, it seems logical that altering circulating concentrations of estradiol (E₂)⁶ would have an impact on the development of these estrogen-responsive cancers. However, several studies have clearly dem-

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* To whom correspondence should be addressed. E-mail: callred@tamu.edu.

⁶ Abbreviations used: β -GAL, β -galactosidase; DMSO, dimethyl sulfoxide; ER, estrogen receptor; ERE, estrogen response element; E₂, 17 β -estradiol; FBS, fetal bovine serum; ICI, ICI 182,780; MCF-7, human breast cancer cell; PGR, progesterone receptor; RBA, relative binding affinity; Trig, trigonelline.

onstrated that changes in cancer incidence associated with drinking coffee are not the result of caffeine intake (7).

Other biologically active compounds in coffee have been identified. One of these is trigonelline (Trig), a niacin-related compound. After caffeine, Trig is the second most abundant alkaloid compound in raw coffee beans (16) and makes up ~1% of the dry matter of roasted coffee beans (17). Trig has been demonstrated to have several biological activities, including an antimicrobial agent inhibiting the growth of *Streptococcus mutans*, a bacterium closely associated with the development of dental caries in humans (18). In vitro Trig (30–100 $\mu\text{mol/L}$) was associated with regeneration of dendrites and axons in cortical neurons and this effect was correlated to improved spatial memory in rats (19). In addition, treatment with Trig (2.5–40 $\mu\text{mol/L}$) has been shown to inhibit the invasion of liver cancer cells in vitro (20). However, to date, no studies to our knowledge have focused on how this compound may influence estrogen-related cancers.

Epidemiological and clinical studies suggest that coffee consumption can influence the development of estrogen-sensitive cancers in women. The presented studies sought to determine whether Trig, a major constituent in coffee, would suppress estrogen action in estrogen-dependent breast cancer (MCF-7) cells. This is a well-defined model for testing the estrogenic potential of compounds. Initial studies demonstrated that Trig did not inhibit E_2 -stimulated growth of these cells. In fact, the compound actually increased total cell number following treatment at low concentrations. Subsequent experiments were designed to clearly define the molecular actions of Trig in these cells and to determine whether the enhanced growth of the MCF-7 cells was the result of Trig acting as a phytoestrogen.

Materials and Methods

Reagents

DMEM, ICI 182,780 (ICI), Trig, and 17β - E_2 were purchased from Sigma-Aldrich. MS analysis was performed on the Trig stock solutions used in the presented experiments to verify the molecular weight and purity of the compound. ICI, Trig, and E_2 were dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich). Fetal bovine serum (FBS) was purchased from Hyclone. ESCORT transfection reagent was purchased from Sigma-Aldrich. Luciferase assay and β -galactosidase (β -GAL) enzyme assay kits were purchased from Promega. TRIzol reagent was purchased from Invitrogen. Cyclin D1, pS2, progesterone receptor (PGR), and 18s gene expression assays and TaqMan one-step RT-PCR master mix were purchased from Applied Biosystems.

Cells and cell culture

MCF-7 cells were purchased from ATCC. Cells were maintained in phenol red free DMEM containing 10% FBS at 37°C in a 5% CO_2 atmosphere. Seventy-two hours prior to treatment, cells were placed in DMEM containing charcoal dextran-treated FBS. Briefly, cells were washed with PBS, $1\times$ trypsin EDTA was added, cells were incubated for 5 min at 37°C , transferred to a 15-mL tube, and centrifuged at $800\times g$ for 5 min to form a cell pellet. Media was removed and cell pellet was resuspended in 5 mL fresh DMEM containing charcoal dextran-treated FBS. Then 10 μL cell suspension was added to a hemacytometer and cells were counted and 150,000 cells were added per well in 6 well plates. Cells were treated 24 h later (see below). After 3 d media was changed and cells were treated again. Cells were removed from plates 72 h later and resuspended in 1.5 mL of media. Cells were counted on a Coulter Counter (Z1, Beckman Coulter).

Dose response. Cells were treated with vehicle (DMSO), 10 pmol/L E_2 , and 1, 10, and 100 pmol/L Trig. Experiments were repeated 3 times with 3 wells per treatment for each experiment ($n = 9$).

Combination E_2 and Trig. Treatments consisted of vehicle (DMSO), 10 pmol/L E_2 , and 10 pmol/L $E_2 + 100$ pmol/L Trig. Experiments were repeated 3 times with 3 wells per treatment for each experiment ($n = 9$).

Trig and ICI. Treatments of vehicle (DMSO), 100 pmol/L E_2 , 100 pmol/L Trig, 100 pmol/L $E_2 + 1$ $\mu\text{mol/L}$ ICI, 100 pmol/L Trig + 1 $\mu\text{mol/L}$ ICI were used. Experiments were repeated 3 times with 3 wells per treatment for each experiment ($n = 9$).

Transfection assays

MCF-7 cells were transfected with 4 μg estrogen response element (ERE)-Luc and 0.5 μg β -GAL plasmid per 24-well plate. Plasmids were introduced into cells using ESCORT transfection reagent over a 4-h period. Cells were subsequently treated with vehicle (DMSO), 100 pmol/L E_2 , 100 pmol/L Trig, 1 $\mu\text{mol/L}$ ICI, 100 pmol/L Trig + 1 $\mu\text{mol/L}$ ICI, and 100 pmol/L $E_2 + 1$ $\mu\text{mol/L}$ ICI for 18 h and then lysed in 100 μL reporter lysis buffer. The quantification of induced firefly (*Phontius pyralis*) luciferase protein was performed using a Luciferase Assay kit (Promega) on an Infinite 200 Tecan plate reader. β -GAL activity was utilized as a constitutively active reporter and was measured using a β -GAL Enzyme Assay system. Experiments were repeated 3 times with 4 wells per treatment for each experiment ($n = 12$).

RNA preparation and qRT-PCR

RNA was collected from MCF-7 cells using the TRIzol method. Briefly, 1 mL TRIzol reagent was added to cells and incubated for 5 min at room temperature. Then 200 μL chloroform was added and tubes were shaken vigorously and centrifuged at $7800\times g$ for 15 min. The aqueous phase was transferred to a new tube and 500 μL isopropanol was added, incubated for 10 min, and centrifuged at $7800\times g$ for 10 min. RNA pellet was washed with 1 mL 75% ethanol, briefly vortexed, and centrifuged at $6100\times g$ for 5 min. Ethanol was removed and pellet was allowed to air dry. A total of 50 μL RNase-free water was added to pellet and RNA was stored at -70°C . Using qRT-PCR, RNA was analyzed for cyclin D1, pS2, and PGR gene expression. Premixed gene expression assays for cyclin D1, pS2, PGR, and 18s (control) were purchased from Applied Biosystems and TaqMan One-Step RT-PCR Master mix was used. Briefly, 10 μL $2\times$ Master mix, 0.5 μL $40\times$ MultiScribe, 1 μL gene expression primer mix, 7.5 μL water, and 100 ng RNA were added per well. Run time parameters were 30 min at 48°C , 10 min at 95°C , 40 cycles of 15 s at 95°C , and 1 min at 60°C . A Bio-Rad icycler was used. Amplicons were analyzed as triplicates in separate wells to allow quantification of target genes normalized to a control, 18s. Data were analyzed using the Ct method and fold-change in gene expression was determined.

ER binding assays

Relative binding affinities (RBA) were determined by competitive radio-metric binding assays with 2 nmol/L [^3H] E_2 as tracer ([2,4,6,7- ^3H]estra-1,3,5 (10), -triene-3,17 β -diol, 70–120 Ci/mmol), (GE Healthcare) as a modification of methods previously described (21,22). The source of ER was purified full-length human ER α and ER β purchased from Pan Vera/Invitrogen. For all binding experiments, the ER was diluted into binding buffer (50 mmol/L Tris, pH 8.0, 10% glycerol, 10 mmol/L mercapto-ethanol, 0.3 g/L ovalbumin). Incubations were done at 0°C for 18–24 h and hydroxyapatite (Bio-Rad) was used to absorb the purified receptor-ligand complexes and unbound ligand was washed away (22). The binding affinities are expressed as RBA values, where the RBA of E_2 is 100%; under these conditions, E_2 binds to ER α with a K_d of 0.2 nmol/L and to ER β with a K_d of 0.5 nmol/L. The determination of these RBA values is reproducible in separate experiments with a CV of 0.3 and the values shown represent the mean \pm range of 2 or more separate determinations.

Statistics

Cell growth assay data were tested by 1-way ANOVA using the StatTools add-on for Excel. After determining an overall treatment effect based on the ANOVA F-test, a post-hoc Tukey's pairwise comparison test was performed to identify significance between treatments. ERE data were analyzed using a 2-way ANOVA, performed using MiniTab 15. For RT-

PCR data, 1-way ANOVA was performed followed by Tukey's pairwise comparison test.

Results

Cotreatment of E₂ and Trig stimulates the growth of MCF-7 cells more than E₂ alone. We first wanted to determine how Trig would alter the growth of MCF-7 cells in the presence of E₂. A suboptimal dose of E₂ was chosen so that either additive or inhibitory actions of Trig treatment could be measured (Fig. 1). The 10 pmol/L E₂ treatment alone increased cell numbers in MCF-7 cells compared with vehicle control ($P < 0.001$). Cotreatment with 10 pmol/L E₂ plus 100 pmol/L Trig resulted in an increase in cell number compared with either vehicle or E₂ treatment alone ($P < 0.001$).

Trig increases cell number in a dose-responsive manner. We next wanted to determine whether Trig altered the growth of MCF-7 cells in the absence of E₂. Treatment of MCF-7 cells with 10 pmol/L E₂ again resulted in an increased number of cells compared with vehicle (Fig. 2). Trig also significantly induced MCF-7 cell growth in a dose-dependent manner. A dose of 1 pmol/L Trig did not differ from vehicle control-treated cells. However, total cell number was increased at concentrations as low as 10 pmol/L ($P < 0.001$). Trig treatment at 100 pmol/L increased cell number to the point that it was higher than both the vehicle control and those cells treated with E₂ ($P < 0.001$).

ICI inhibits the effects of Trig. Next, we wanted to determine whether the physiological effects of Trig in MCF-7 cells were the result of estrogen receptor (ER) activation. First, we utilized an approach in which cells were cotreated with ICI, an ER α antagonist (Fig. 3). Treatment with 100 pmol/L E₂ alone or 100 pmol/L Trig alone resulted in increased cell numbers compared with vehicle control-treated cells ($P < 0.001$). However, cotreatment with 1 μ mol/L ICI and either E₂ or Trig resulted in significantly reduced cell growth compared with treatment with E₂ or Trig alone. In fact, final cell counts in the cotreatment groups did not significantly differ from the vehicle control group, suggesting that the growth-promoting effect of Trig is completely inhibited by blocking the ER.

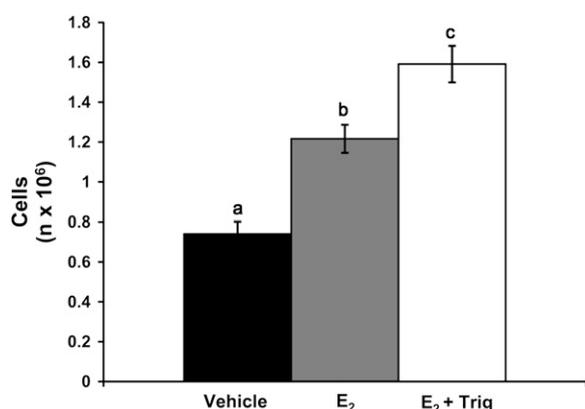


FIGURE 1 Cotreatment with E₂ and Trig increases cellular proliferation of MCF-7 cells. Cells were treated for 6 d with DMSO (vehicle), 10 pmol/L E₂, or 10 pmol/L E₂ + 100 pmol/L Trig. These data are from 3 separate experiments. Values are means \pm SEM, $n = 9$ wells. Bars without a common letter differ, $P < 0.001$.

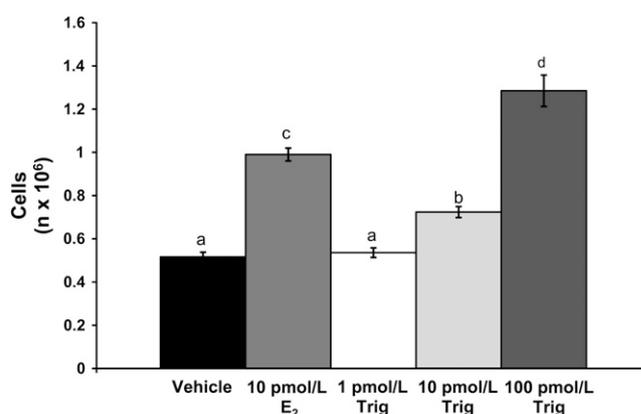


FIGURE 2 MCF-7 cell growth increased by Trig alone. Cells were treated for 6 d. These data are from 3 separate experiments. Values are means \pm SEM, $n = 9$ wells. Bars without a common letter differ, $P < 0.001$.

Trig enhances ERE reporter activation in MCF-7 cells. We then wanted to further demonstrate that Trig activates the ER resulting in enhanced transcriptional regulation. Treatment with ICI alone did not affect ERE reporter activity (Fig. 4). However, 100 pmol/L E₂ enhanced reporter activity in these cells ($P < 0.001$). Likewise, 100 pmol/L Trig significantly increased reporter activity compared with control, but the effect was not as elevated as that of E₂. Cotreatment with 1 μ mol/L ICI completely blocked the effects of both E₂ and Trig.

Trig increases expression of ER target genes. Finally, the previous data suggested that Trig is capable of modulating ER-mediated transcription. However, we wanted to demonstrate this effect in genomic DNA. Three genes were chosen because they are known downstream targets of ER (cyclin D1, pS2, and PGR). Trig enhanced gene expression of cyclin D1 ($P < 0.001$), pS2 ($P < 0.0001$), and PGR ($P < 0.0001$) similar to the enhancement with E₂ treatment (Fig. 5). In conjunction with previous data demonstrating that Trig can stimulate an ERE-reporter construct, these data suggest that Trig binds the ER,

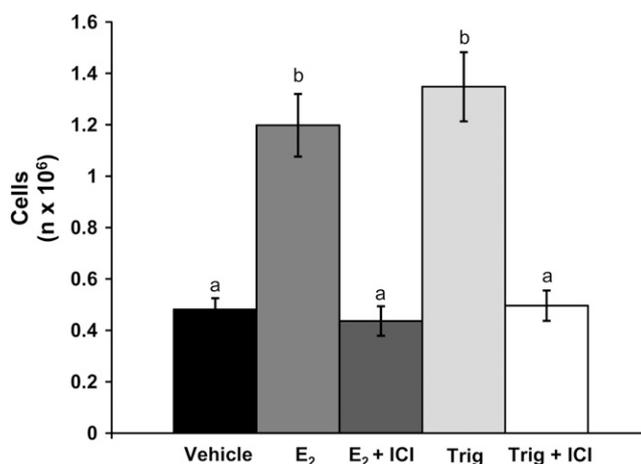


FIGURE 3 ICI suppressed E₂ and Trig induced cellular proliferation in MCF-7 cells. Cells were treated for 6 d with 100 pmol/L E₂, 100 pmol/L Trig, 100 pmol/L E₂ + 1 μ mol/L ICI, or 100 pmol/L Trig + 1 μ mol/L ICI. These data are from 3 separate experiments. Values are means \pm SEM, $n = 9$ wells. Bars without a common letter differ, $P < 0.001$.

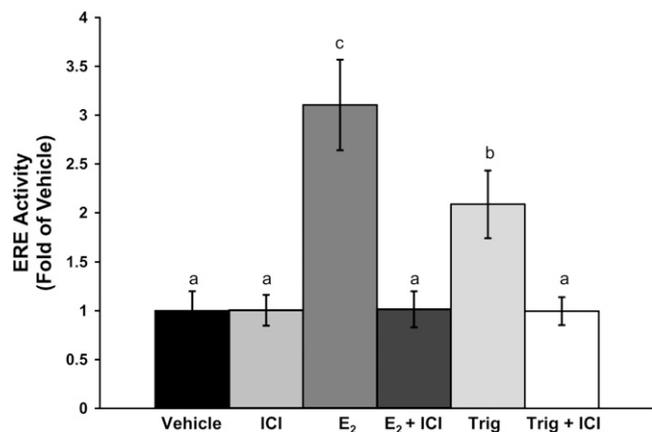


FIGURE 4 Estrogenic compounds induce ERE in MCF-7 cells. Cells were transiently transfected with an ERE luciferase reporter vector and treated with 1 $\mu\text{mol/L}$ ICI, 100 pmol/L E₂, 100 pmol/L Trig, 100 pmol/L Trig + 1 $\mu\text{mol/L}$ ICI, or 100 pmol/L E₂ + 1 $\mu\text{mol/L}$ ICI for 18 h. Luciferase activity was normalized to a constitutively active $\beta\text{-GAL}$ reporter. Luciferase: $\beta\text{-GAL}$ ratios are expressed as the fold of vehicle control cells. Values are means \pm SEM of 3 experiments, $n = 12$. Bars without a common letter differ, $P < 0.001$.

resulting in transcriptional activation by a mechanism similar to E₂. In competitive binding assays, unlabeled E₂ competed with the radiolabeled E₂ in a dose-responsive manner in both ER α and ER β . However, at no tested concentration (100 pmol/L to 100 $\mu\text{mol/L}$) could Trig compete with the radiolabeled E₂ in either ER subtype (Fig. 6). Therefore, although it is clear that Trig activates the ER, resulting in enhanced ERE activity, it is unlikely that it does so by binding the ligand binding domain of the receptor.

Discussion

At the onset of these studies, we wanted to determine whether Trig could alter the actions of E₂ at the cellular level, because coffee is thought to modulate endogenous estrogen levels. However, data demonstrated that cotreatment with Trig and E₂ resulted in enhanced cellular number compared with E₂

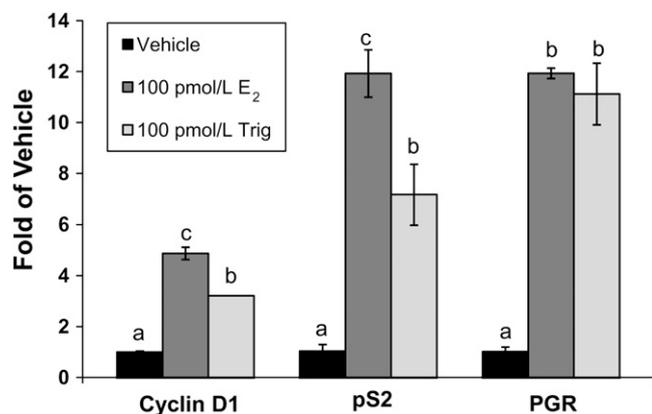


FIGURE 5 Trig increases expression of ER target genes. Cells were treated with DMSO (vehicle), 100 pmol/L E₂, or 100 pmol/L Trig and RNA was subsequently collected. Using qRT-PCR, RNA was analyzed for cyclin D1, pS2, and PGR gene expression. Values are means \pm SEM, $n = 3$. Bars without a common letter differ. Cyclin D1, $P < 0.001$; pS2, $P < 0.0001$; PGR, $P < 0.0001$.

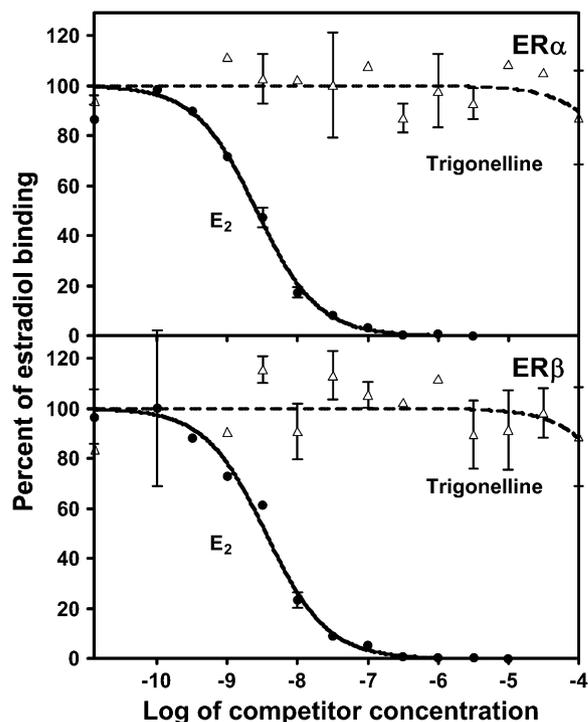


FIGURE 6 Trig does not displace E₂ in ER binding assays. Relative binding affinities were determined by competitive radiometric binding assays with 2 nmol/L [³H]E₂ as tracer ([2,4,6,7-³H]estra-1,3,5 (10), -triene-3,17 β -diol, 70–120 Ci/mmol). Binding affinities are expressed as RBA values, where the RBA of E₂ is 100%. The determination of these RBA values is reproducible in separate experiments with a CV of 0.3 and the values shown represent the mean \pm range of 2 or more separate determinations.

treatment alone. Subsequent experiments showed that Trig induced MCF-7 cell growth in a dose-dependent manner. We next wanted to determine whether this effect was ER mediated. Cotreatment of MCF-7 cells with Trig and ICI, an ER antagonist, resulted in a suppression of Trig activity. These data suggested that Trig was stimulating the growth of these estrogen-dependent breast cancer cells by activating the ER. Then we transfected the MCF-7 cells with a ERE-reporter plasmid. Trig induced the reporter, suggesting that not only can it activate the receptor, but it also induces the molecular action of ER α , the predominant form of the receptor in MCF-7 cells. ICI again fully inhibited this response. Finally, we analyzed gene expression of several well-known ER α target genes following the treatment of the cells with Trig. In each case, Trig treatment induced gene expression in a similar manner as E₂ treatment. Collectively, these data clearly demonstrate that Trig can function as an ER agonist in estrogen-responsive breast cancer cells and that the compound results in transcriptional activation of the ER, which alters gene expression of downstream target genes.

As mentioned previously, the epidemiological and clinical data exploring the effects of frequent coffee consumption and its role in breast tumor formation has been inconsistent (5,8–12). Some studies have shown a reduced risk of breast cancer associated with coffee consumption, whereas others have actually observed an increased risk in coffee drinkers. Our data suggest that exposure to Trig could in part contribute to some of the differences between findings. Most of those studies focused on coffee itself and/or caffeine as the target compound. However, it would seem that accounting for Trig exposure in these studies would be very useful in interpreting their outcomes.

For example, soy phytoestrogens are known to play dual roles in the development and progression of breast cancer. Both human and rodent tumor models have demonstrated that exposure to these compounds early in life, prior to tumor formation, seems to reduce risk of mammary cancer later in life (23,24). Conversely, dietary consumption of these compounds after a tumor has formed induces growth of estrogen-dependent mammary tumors in rodents (25–27). Although our data falls short of demonstrating what effect consumption of Trig may have on breast tumor formation and/or growth in humans, any future study designed to evaluate the effects of coffee on breast tumor formation should consider the estrogenicity of this compound. Additional studies will be needed to identify correlations between Trig and estrogen-responsive diseases. When evaluating clinical data, it will be important to realize that human exposure to Trig in studies and in general will vary depending on several factors involved in coffee preparation. Trig concentrations differ between species of coffee bean. *Coffea arabica* contains the highest concentrations of Trig compared with other varieties (28) and is the species most commonly used when preparing the beverage. In addition to bean type, degree of roasting also decreases Trig content (29).

Few studies have been conducted to examine how Trig influences function of mammalian cells much less the effects it may have on fully transformed tumor cells. One investigation examined the cellular actions of Trig on hepatoma cells. In these studies, Trig suppressed cellular invasion in a dose-responsive manner from 2.5 to 40 $\mu\text{mol/L}$ while the same concentrations did not affect cellular proliferation (20). These findings are significant, because our studies demonstrated that Trig induced cellular growth of MCF-7 cells at concentrations as low as 10 pmol/L, and 100 pmol/L was considered an optimal dose in most of our assays. The opposing actions of Trig in these systems may be related to the fact that they are different cell types, but it may also be related to level of cellular exposure. It is difficult to estimate what tissue levels of Trig exposure might be, but several studies have demonstrated that the compound is absorbed into the small intestine (30) and that it retains its activity when consumed and absorbed from the diet. As before, invasion of hepatoma cells was reduced when the cells were treated with serum from rats fed Trig (20). Although concentrations of Trig were higher than those used in the present study, this result suggests that Trig can be absorbed by the gastrointestinal tract and that the compound itself or its metabolite(s) retain biological function. In addition, oral Trig treatment improved spatial memory in mice, which corresponded to prevention of dendritic and axonal atrophy *in vitro* (19).

Given the chemical structure of Trig, perhaps the most intriguing aspect of this study is the fact that it demonstrated such profound estrogenic activity in MCF-7 cells. Trig is an alkaloid compound that is formed by the addition of a methyl group to the nitrogen atom of nicotinic acid (Fig. 7). To date, compounds that have been identified as phytoestrogens share some similar structural characteristics. Specifically, the presence of a correctly positioned phenolic ring and proper distance between the 2 opposing phenolic oxygens give these compounds chemical structures similar to E_2 (Fig. 7). It is these properties that allow phytoestrogens such as genistein, a commonly studied isoflavone, to bind the active site of the ligand binding domain of the ER and at appropriate concentrations displace E_2 (31). Trig does not share these chemical characteristics. Furthermore, competitive binding assays demonstrated that Trig did not compete with E_2 binding of either $\text{ER}\alpha$ or $\text{ER}\beta$ at any concentration (Fig. 6). However, Trig enhanced ERE reporter

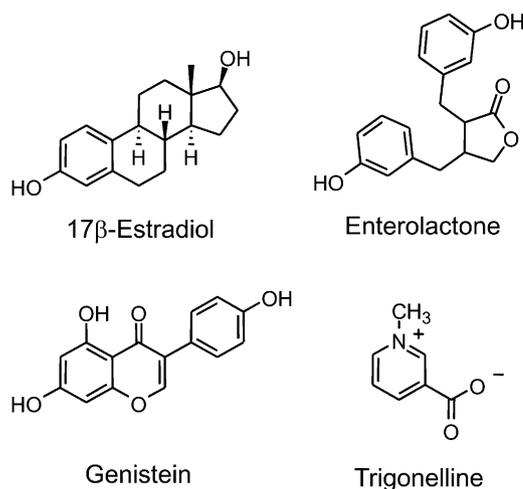


FIGURE 7 Chemical structures of E_2 , enterolactone, genistein, and Trig.

activity in MCF-7 cells in a manner similar to that of E_2 and resulted in increased expression of known ER target genes in these same cells. All of these effects were in the absence of E_2 . These data demonstrate that Trig is capable of activating the ER, resulting in increased transcriptional activity. However, this effect is most likely due to a mechanism that does not involve the compound binding the active site of the receptor. One possibility is that Trig treatment results in a conformational change of the receptor promoting the binding of a transcriptional coregulator. A number of coactivators and corepressors of the ER have been discovered and future experiments will focus on the ability of Trig to alter expression and function of these compounds resulting in unique activation of the ER.

Data presented in these studies demonstrate that Trig, a common component in coffee beans and associated beverages, stimulates the growth of estrogen-dependent breast cancer cells *in vitro*. This effect appears to be the result of ER activation, as inhibiting the receptor fully blocked the stimulatory actions of Trig and the compound regulated ERE transcriptional activation in both reporter assays and by increasing expression of known ER target genes. Each of these observations was the result of very low concentrations of Trig and previous studies suggest that dietary Trig is absorbed intact. To begin to assess the overall physiological relevance of this compound in the diet, future studies will test whether consumption of Trig stimulates the growth of estrogen-dependent breast tumors *in vivo*. Collectively, the presented data identify Trig as a novel phytoestrogen compound. However, additional studies are necessary before conclusions can be drawn about the potential health risks or benefits that may be associated with its consumption.

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