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Research Article

# Respective contribution exerted by AF-1 and AF-2 transactivation functions in estrogen receptor α induced transcriptional activity by isoflavones and equol: Consequence on breast cancer cell proliferation

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Estrogens used in hormone replacement therapy regimens may increase the risk of developing breast cancer. Paradoxically, high consumption of plant-derived phytoestrogens, particularly soybean isoflavones, is associated with a low incidence of breast cancer. To explore the molecular basis for these potentially different experimental/clinical outcomes, we investigated whether soybean isoflavones elicit distinct transcriptional actions from estrogens by performing transient transfections in different cell lines. Our results demonstrate that 17β estradiol (E2), isoflavones, and equol (EQ) effectively trigger the transcriptional activation with both estrogen receptors (ER), ERα and ERβ. ERα transcriptional activity is mediated through two transactivation domains AF-1 and AF-2, whose activity is tightly regulated in a cell-type and promoter-specific manner. Isoflavones, genistein, and daidzein (DAI), and EQ, the main estrogenic metabolite of DAI, are ERα agonists for transcriptional activation. The molecular mechanisms for ERα-induced transcriptional activity by isoflavones and EQ involve their capacity to act mainly through AF-1 regardless of the cell type. Therefore, our data indicate that estrogenic ligands, such as isoflavones and EQ, exert their effects on ERα transactivation similarly to the endogenous ligand E2, and suggest that the risk of estrogen-related diseases might not be reduced by soy-rich food and/or isoflavone- or EQ-based supplements.

**Keywords:** Breast cancer / Cellular background / Estrogen receptor / Phytoestrogens / Transactivation functions

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## 1 Introduction

Estrogens were used in hormone replacement therapy (HRT) not only to prevent menopausal symptoms such as hot flushes, urogenital atrophy but also osteoporosis in postmenopausal women. Unfortunately, HRT has not lived up to its potential to improve health in women. Estrogens have been associated with an increased incidence of breast and endometrial cancers, which has led to the use of antiestrogens and selective estrogen receptor modulators (SERM) such as tamoxifen and raloxifen that have a safer profile. However, since undesirable effects persist, numerous investigators continue to search for better SERM for HRT.

There is a growing interest in using natural dietary phytoestrogens, particularly those found in soy products, as a potential alternative to the estrogens in HRT. Interest in phytoestrogens has been fuelled by observational studies showing a marked difference in the incidence rates of breast cancer in women from western countries compared to those in non-western countries [1]. Investigators have hypothesized that an Asian diet, which is typically high in soy content, may be one factor that explains the lower incidence of

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**Abbreviations: AF,** transactivation function; **DAI,** daidzein; **EQ,** equol; **ER,** estrogen receptor; **FCS,** fetal calf serum; **GEN,** genistein; **HRT,** hormone replacement therapy; **SERM,** selective estrogen receptor modulator

breast cancer in these countries compared with other countries on a traditional diet that lacks soy as a common component [2].

The isoflavones genistein (GEN) and daidzein (DAI), which are abundant in soybeans, and available widely as herbal tablets, are especially popular among postmenopausal women who are taking phytoestrogens in an effort to alleviate menopausal symptoms without the risk of developing breast cancer. Despite their popularity and putative health benefits, it is clear that we need to know much more about the molecular mechanisms, safety, and efficacy of isoflavones, as natural SERM, before they can be recommended to postmenopausal women as an alternative to estrogens for HRT.

Dietary isoflavones, such as GEN and DAI, and its main metabolite equol (EQ), are strikingly similar in chemical structure to steroidal and nonsteroidal estrogens and are capable of binding weakly to estrogen receptors (ER) [3]. There are experimental evidences for and against the action of phytoestrogens as promoters or protectants of breast cancer (for review [4]). Since many *in vitro* studies have used a similar cellular background (MCF-7) and experimental conditions, the discrepancies in the findings are puzzling.

The effects of 17β-estradiol (E2) and related compounds, such as phytoestrogens, are mediated by two members of the nuclear receptor superfamily, ERα and ERβ, which are coded on separate genes. ER uses two transactivation functions (AFs), located in their N-terminal (AF-1) and C-terminal (AF-2) domains. Once activated by ligand binding, these AF recruit coregulators of gene transcription. Constitutively active when isolated, AF-1 has been subdivided into three regions, box 1 (amino acids 38–64), box 2 (amino acids 87–108), and box 3 (amino acids 108–129), which correspond to sequences surrounding the main phosphorylated serine residue at position 118 [5]. The transcriptional activity of the AF-2 region is dependent on ligand binding. The transcriptional activity of ERα can be promoted through functional cooperation between both AF-1 and AF-2 or through each AF acting independently [6]. Importantly, the respective activities of AF-1 and AF-2 are controlled in a cell-type and promoter-specific manner [7, 8]. Furthermore, it has been shown that the relative contribution that AF-1 and AF-2 exert on the transcriptional activity of ERα varies in a cell differentiation stage-dependent manner [9]. Consequently, during the epithelial–mesenchymal transition, the cell permissiveness shift from AF-1 to AF-2 can be predicted to cause ERα to regulate different gene patterns. More recently, the sensitivity of ERα transcriptional activity toward the cell differentiation stage has been shown to be controlled by the AF-1 box 1 subregion [10].

Therefore, isoflavones and EQ may produce clinical and/or experimental effects distinct from estrogens by differentially triggering ERα transcriptional activity. To test this hypothesis, we compared the effects of GEN, DAI, and EQ on the transcriptional activity of ERα in different cellular backgrounds. Furthermore, the present study investigated the roles of the AF domains, and more particularly of the AF1 box 1 subregion, in the ability of isoflavones (GEN, DAI) and EQ in ERα transactivation. For these purposes, transient transfections of ERα constructs were performed in an ER positive mammary epithelial cell line (MCF-7) as well as in the ER-negative HeLa and HepG2 cell lines, which offer opposite AF permissiveness. The ability of GEN, DAI, and EQ, alone or in combination with E2, to modulate ERα protein levels and to exert growth promoting effects in MCF-7 cells were also examined.

## 2 Materials and methods

### 2.1 Reagents and antibodies

E2 and the monoclonal anti-β-actin AC-15 were purchased from Sigma (France). Polyclonal anti-ERα (HC-20) was obtained from Tebu-bio (France) and ICI 182 780 (ICI) from Tocris (UK). The isoflavones GEN and DAI, and its metabolite EQ, were obtained by chemical synthesis by Dr. S. Shinkaruk at the Laboratoire de Chimie Organique et Organométallique (Institut des Sciences Moléculaires ISM, UMR 5255, CNRS, France). Purity of these compounds was assessed by means of NMR spectra analyses and determined as ≥97%. Stock solution of EQ was a mixture (1:1) of racemic compounds (*R*-EQ/*S*-EQ).

### 2.2 Plasmids

Expression vectors pSG5, pSG human (h)ERα66, pSGERα46 pSGERαΔ79, pSGERαΔ79 S118A, and pCMV-β galactosidase as well as ERE-TK-LUC and hC3-LUC reporter genes were previously described [10]. Expression vector for human (h)ERβ (pSG5ERβ) was provided by Professor J. A. Gustafsson's department (Department of Biosciences and Medical Nutrition, Karolinska Institute, Sweden).

### 2.3 Cell culture and proliferation

All cell lines (HeLa, HepG2, and MCF-7) were routinely maintained in DMEM (Sigma), supplemented with 5% fetal calf serum (FCS, Sigma) and antibiotics (Invitrogen, France). For experimental conditions, phenol red-free medium DMEM/F12 (Invitrogen) supplemented with 2.5% charcoal-stripped FCS was used (experimental medium). For cell proliferation, MCF-7 cells (25 000/well) were seeded in a 24-well plate in DMEM supplemented with 5% FCS. After 24 h, the medium was changed to an experimental medium either supplemented or not with FCS and with the indicated treatment for up to 5 days. Cell number was determined after 3 and 5 days with a Coulter cell counter (Beckman Coulter, France).

 

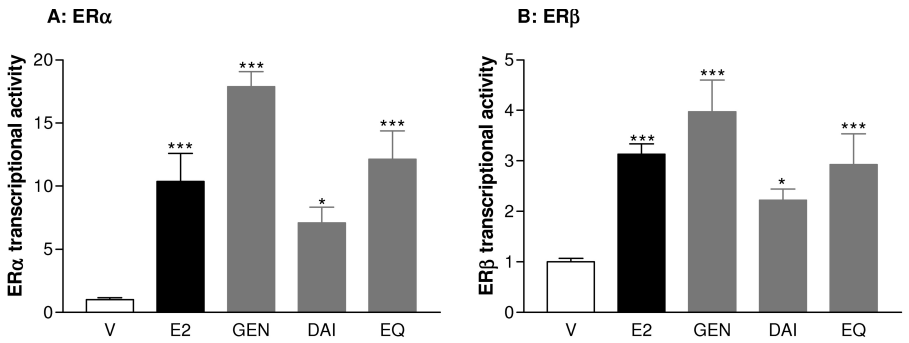


**Figure 1.** Transcriptional activation of ER by isoflavones and EQ in MCF-7. The effects of V (EtOH, 0.1%), E2 (10 nM), GEN, DAI, and EQ (10 μM) on ER transcriptional activity. Cells were cotransfected with ERα (A) or ERβ (B) and the hC3-LUC. Data are expressed as reporter fold induction compared to V-treated cells. Shown are means ± SEM of six to ten independent transfection experiments, each performed in triplicate wells. Comparisons between experimental conditions were performed by a one way ANOVA followed by Dunnett's test and/or a Student's *t*-test when applicable. Statistical significance is indicated by * and *** for comparison to control (V-treated cells) for $p < 0.05$ and $p < 0.001$, respectively.

### 2.4 Transient transfection experiments

Transfections were carried out with FuGENE™ 6 as recommended by the manufacturer (Roche Diagnostics, France) and as described previously [10] with 50 ng of total DNA consisting of the expression vector, the reporter gene and the pCMV-β galactosidase internal control (10, 20, and 20 ng, respectively). Following an overnight incubation with the transfection mixture, cells were treated either with different concentrations of E2, isoflavones (10 nM, 0.1–10 μM, respectively), ICI (1 μM), or vehicle (0.1% ethanol), or a combination of these compounds as indicated. Following 36 h of transient expression, cells were harvested and luciferase and β-galactosidase assays were performed as previously described [10]. The reporter gene activity was obtained after normalization of the luciferase activity with the β-galactosidase activity.

### 2.5 ERα mRNA and protein expression

Total RNA was extracted using Tri-Reagent® (Euromedex, France) after 24 h of treatment in an experimental medium. RT was performed on 2 μg of total RNA. PCR amplifications of human ERα and β-actin as the housekeeping gene were performed as published [11]. Expressions of ERα and β-actin were examined by Western blot as previously published [11]. Densitometry analysis was performed using ImageJ 1.17 (National Institute of Health, USA), to determine relative amounts of amplicons and protein. Data obtained were normalized to β-actin signals.

### 2.6 Statistical analysis

Shown are the means ± SEM of three to ten independent experiments, each performed in triplicate (transfections) or duplicate. One-way ANOVA and Dunnett's multiple comparison *post-hoc* test or Student's *t*-test were performed for the statistical analysis between experimental conditions (GraphPad Prism®, USA). Dose-dependant effects were assessed by linear regression (GraphPad Prism). Statistical significance is indicated by 1, 2, and 3 symbols for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

## 3 Results and discussion

In MCF-7 cells, GEN, DAI, and EQ (0.1–10 μM) induce ERα and ERβ transactivation on the hC3-LUC reporter gene in a dose-dependant fashion as calculated by linear regression ($R^2 = 0.99$; 0.97; 0.78 for ERα and 0.97; 0.99; 0.87 for ERβ, respectively). For ERα a significant increase in transcriptional activity is obtained with 1 and 10 μM of GEN, DAI, and EQ (12.8 ± 3.6; 17.9 ± 1.2, 4.8 ± 1.0; 7.1 ± 1.2, and 11.7 ± 3.4; 12.1 ± 2.2-fold increase compared to control, respectively). For ERβ, 1 and 10 μM of GEN and DAI induce a significant increase (2.9 ± 0.4; 3.9 ± 0.6, and 2.1 ± 0.4; 2.3 ± 0.2-fold increase compared to control) while only the highest concentration of EQ induces a significant effect (2.9 ± 0.6-fold increase compared to control). GEN, DAI, and EQ (10 μM) induce a significant ERα and ERβ transactivation similar to the one induced by 10 nM E2 (Fig. 1). E2, GEN, DAI, and EQ effects on both ER are completely abolished by treatment with 1 μM ICI (data not shown). The ERβ-induced transactivation by E2 and isoflavones in MCF-7 cells is lower overall than that of ERα. For both ER, transactivation is slightly lower overall when using the estrogen sensitive reporter gene ERE-TK-LUC (data not shown). We observe that similar effects to E2 are typically achieved at concentrations that are two or three orders of magnitude higher, which can be reached physiologically with a phytoestrogen-rich diet. Serum concentrations of GEN, DAI, or EQ are quite different between women from various geographic areas and/or specific diets

 

[12]. For instance, isoflavones concentrations in plasma from 0.2 to 10 μM have been reported [2, 13]. This is also in accordance with the fact that the relative binding affinity of phytoestrogens, measured on recombinant ER, is generally in the order of 1000–10 000 times less than that of E2 (except for GEN which has a much higher relative binding affinity in the order of 10–100 times less than E2) [3]. We observed a stronger transactivation (approximately five times) of ERα compared to ERβ (Fig. 1). This is in agreement with previously published data on HEK 293 cells and in Ishikawa cells using different (3ERE-TATA LUC) or similar reporter constructs [3, 14] but in contrast with those of Harris *et al*. [15], which showed no difference between ERα and ERβ in transfected MCF-7 cells. However, this stronger activation seems to be corroborated by the study of Manas *et al*. [16], in 2004 showing essentially no difference in the structural determinations between the ERα-ligand binding domain (LBD) and the ERβ-LBD bound to GEN.

Previously published data have often given contradictory results for phytoestrogens, suggesting that the estrogenic and/or antiestrogenic activity of these structurally diverse natural compounds is complex. Therefore, it was of particular interest to determine the precise mechanisms of the action of GEN, DAI, and EQ on ERα transcriptional activation. The use of ERα constructs expressed in ER-negative backgrounds has been a powerful technique for studying the function of various domains in the ER [7–10]. To examine the role of the different AF of the ERα-induced transactivation by GEN, DAI, and EQ in different cellular backgrounds, we used expression vectors of full length ERα (ERα66) or truncated ERα in the A/B domain (ERαΔ79, ERα46). ERαΔ79 construct is deleted from the first 79 amino acids and therefore from the AF-1, box 1 subregion. ERα46 is deleted from the A/B domains and therefore from the entire AF-1 (box 1 and box 2/3 subregions). Thus, a similar activity of the three receptors is inherent in a strict AF-2 activity, whereas higher transcriptional activity of ERα66 or ERα66 and ERαΔ79 defines a box 1 or box 2/3 activity, respectively. This was done by comparing the transcriptional efficiency of different ERα constructs on estrogen sensitive reporter genes in MCF-7 as well as in HeLa and HepG2 epithelial cell lines, which both have opposite AF permissiveness.

Similar expression of the different ERα variants in all cell lines was controlled by Western blots (data not shown). In MCF-7 cells, GEN, DAI, and EQ (10 μM) present the same transactivation profile of ERα constructs as E2 (10 nM), *i.e*., a higher transactivation of both ERα66 and ERαΔ79 than ERα46 (Fig. 2A). Furthermore, while the E2 and EQ effects are quantitatively similar on the three constructs, GEN-induced transactivation of ERα66 and ERαΔ79 is higher and DAI-induced transactivation of ERαΔ79 is lower compared to the E2 effects. ICI (1 μM) completely blocked the effects of E2 and of isoflavones and EQ (data not shown). These results indicate that, in MCF-7 cells, transactivation of ERα by isoflavones and EQ is mainly mediated though AF-1 and that deletion of AF-1 box 1 subregion has no effect on ERα-induced transactivation.

It is well known that ERα serine 118 (Ser 118) phosphorylation modulates box 2/3 activity of AF-1 function [5]. To further investigate the mechanisms of the action of isoflavones and EQ to induce ERα transcriptional activity, cells were transfected with another construct, ERαΔ79S118A, deleted from the first 79 amino acids and with the Ser 118 replaced by an Ala. The replacement of Ser 118 inhibits the ERαΔ79-induced transcriptional activity by E2, GEN, DAI, and EQ in MCF-7 cells (Fig. 2B) as well as in Hela and HepG2 cell lines (data not shown). Therefore, in MCF-7 cells, GEN, DAI, EQ, and E2, induce ERα transcriptional activity mainly through the box 2/3 of the AF-1 domain, and involve the phosphorylation of the Ser 118.

In HepG2, a strict AF-1 permissive cell line, all compounds have the same transactivation profile for the three ERα constructs (Fig. 2C). For the phytoestrogens tested, HepG2 cells are highly permissive to the transcriptional activity of ERα, which relies mainly on the box 1 of the AF-1 domain (≈60%). Furthermore, GEN, DAI, and EQ effects were not quantitatively different from those induced by E2.

In HeLa, the AF-2 permissive cell line, ERα is induced slightly compared to HepG2 (Fig. 2D). This transactivation is mainly ensured by AF-2 (ERα66≈ERα46). However, deletion of the box 1 and the A domain (ERαΔ79), previously identified as an internal structural repressor [6], enhances ERα activity therefore allowing a stronger transactivation through AF-1 box 2/3 (ERαΔ79 *vs*. ERα66≈ERα46). GEN and EQ present the same transactivation profile as E2, with a similar transactivation of ERα66 and ERα46 and a higher ERαΔ79 transactivation. The GEN, EQ, and E2 effects are quantitatively similar. Interestingly, DAI transactivates equally both ERα66 and ERαΔ79 in a significantly higher manner than E2 while ERα46-induced transactivation is similar. This suggests that DAI is more potent than E2 in an AF-2 cellular background in inducing transactivation through box 2/3 and/or in preventing structural repression by box 1.

It appears that the ability of phytoestrogens to act as ER agonists varies between cell types. Our results indicate that regardless of the cellular background, *i.e*., AF sensitivity, GEN, DAI, EQ, and E2 induce a similar transcriptional activation of ERα. More particularly, ERα transcriptional activation by isoflavones and EQ involve their capacity to act mainly through AF-1 regardless of the cellular background. Previous studies show that differences among SERM, including antiestrogens, xenoestrogens, and phytoestrogens, can be observed in cells transfected with variant forms of ERα [7, 8, 17–19]. They conclude that the estrogenic potency of compounds is a complicated phenomenon which results from a number of factors, such as the differ-

 

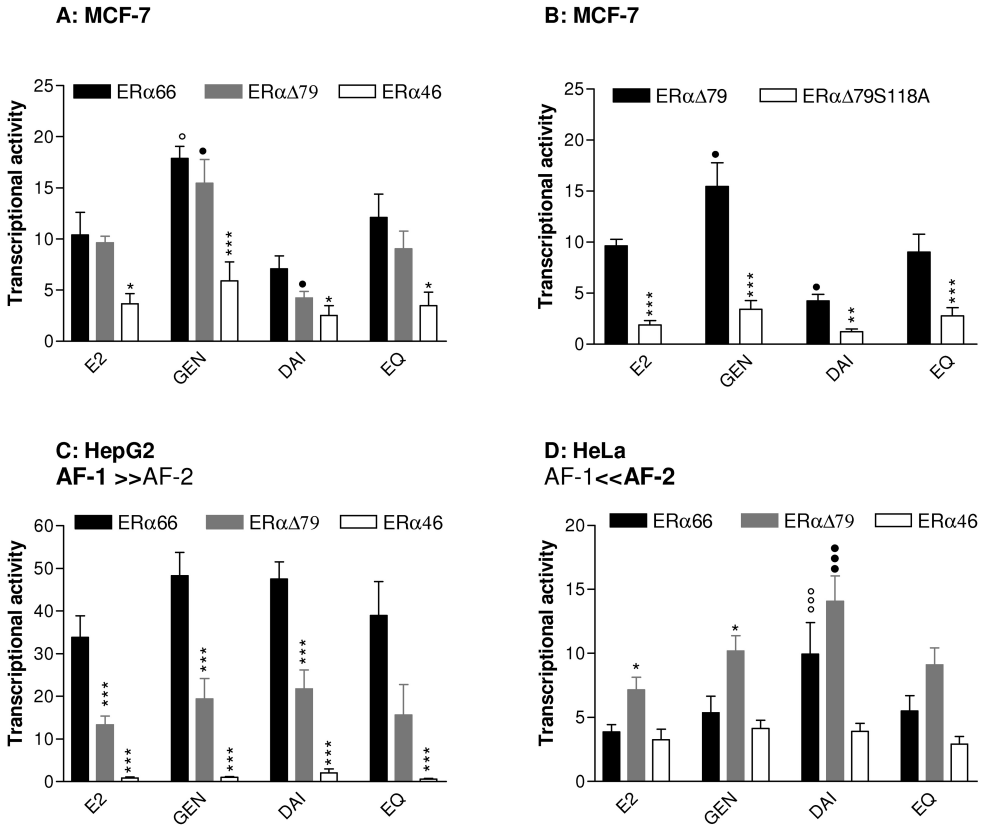


**Figure 2.** Transcriptional activation of ERα by isoflavones and EQ in different cell lines. Transcriptional activity of different ERα constructs was determined using hC3-LUC in MCF-7 cells (A and B) or ERE-TK-LUC in HepG2 (C) and HeLa cells (D). Data are expressed as reporter fold induction. Shown are means ± SEM of six to ten independent transfection experiments, each performed in triplicate wells. Comparisons between experimental conditions were performed by a one way ANOVA followed by Dunnett's test and/or a Student's *t*- test when applicable. Statistical significance is indicated by * symbol for comparison to ERα-66-transfected cells (black bars) within groups for a same compound, symbols ○ or ● were used for comparison to E2-treated cells for a same ERα construct. 1, 2, and 3 symbols were used to express significance for $p < 0.05$, $p < 0.01$, and $p < 0.001$, respectively.

ential effects on the transactivation functionalities of the receptor, the particular coactivators recruited, the cell- and target promoter-contexts and the cell differentiation stage [7–10].

These particular phytoestrogens have the same mechanisms of action than E2 on ERα in MCF-7. Having established that isoflavones and EQ are ER activators, we wanted to confirm these effects on more complex physiological responses, such as ERα expression and cell proliferation. E2 is known to downregulate the levels of ERα in breast cancer cell lines through an increased turnover of the E2-activated ERα protein and a reduced transcription rate of its own gene [20]. This downregulation represents an additional hallmark of the activation of ERα by an agonist. This prompted us to check whether ERα mRNA and protein levels are sensitive to isoflavones and EQ in MCF-7 cells. GEN, DAI, and EQ (10 μM, 24 h) downregulate ERα mRNA levels in a similar extent to 10 nM of E2 (data not shown). Using the same treatment, we also observe a downregulation of ERα66 protein content with GEN, DAI, and EQ similar to the one induced by E2 (Fig. 3A). These results confirm previously published studies in MCF-7 cells [21, 22]. In contrast, only GEN significantly decreases ERα46 protein content, while there was no change with E2, DAI, and EQ. This effect could be explained by GEN having an ER-independent effect such as the inhibition of tyrosine kinase or the induction of c-fos [23]. The effects of GEN, DAI, and EQ on ERα protein expression were unchanged in presence of E2 (data not shown).

Estrogens are decisive actors responsible for the proliferation and differentiation of normal mammary epithelial cells as well as the development and progression of breast cancer. We then analyzed the effects of these compounds on the proliferation of estrogen-dependant MCF-7 cells, in the presence or absence of charcoal-stripped FCS. Figures 3B and C show that isoflavones, EQ, and E2 stimulate the proliferation of MCF-7 cells to a similar extent in the presence or absence of FCS. The effects of GEN, DAI, and EQ on MCF-7 cell proliferation were similar at 1 μM and unchanged in the presence of E2 (data not shown). ICI

 

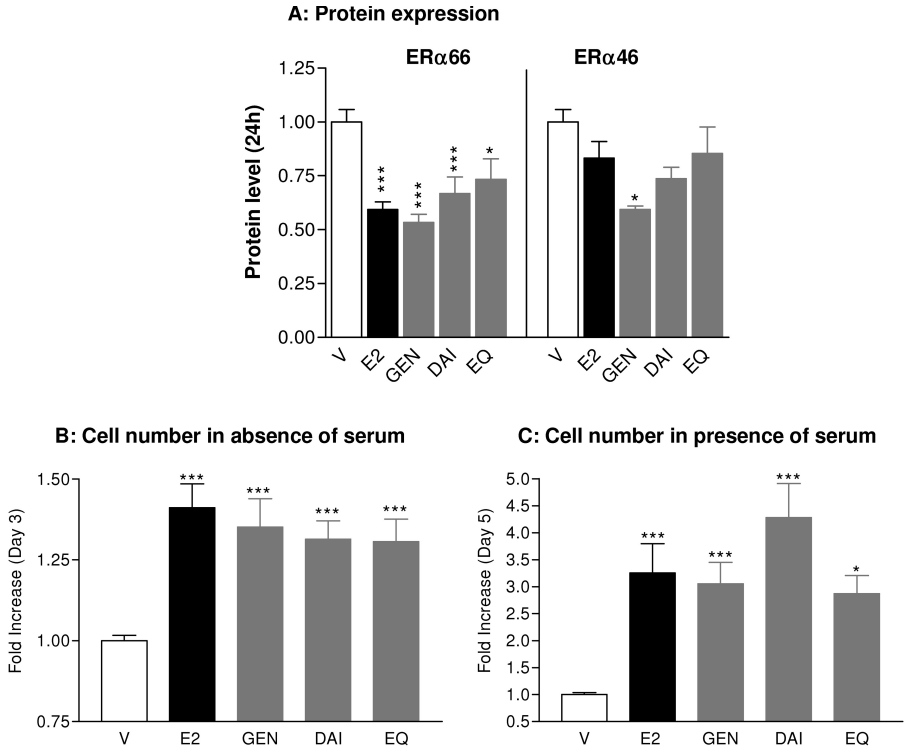


**Figure 3.** Effects of isoflavones and EQ on ERα protein levels and cell proliferation in MCF-7. The effects of V (EtOH 0.1%), E2 (10 nM), GEN, DAI, and EQ (10 μM) on ERα protein levels (A) and on the number of MCF-7 cells in the absence (B, day 3) or the presence (C, day 5) of 2.5% charcoal-stripped FCS. Data are expressed as fold increase compared to control (V-treated cells). Shown are means ± SEM of four to five independent experiments, each performed in duplicate wells. Comparisons between experimental conditions were performed by a one way ANOVA followed by Dunnett's test. Statistical significance *versus* control is indicated by * and *** for $p < 0.05$ and $p < 0.001$, respectively.

(1 μM) completely blocked the E2, the isoflavones, and E-induced MCF-7 cell proliferation (data not shown). There seems now to be a general consensus view that in MCF-7 cells, GEN has biphasic effects on cell growth such that, at low concentrations, it acts as an estrogen agonist through the ER, whilst at higher concentration it inhibits cell growth through non-ER pathways [21]. However, our results are in accordance with those classically described for lower concentrations of phytoestrogens in MCF-7 cells [22, 24, 25] and are consistent with the demonstration that full activity of the AF-1 domain is required for the E2-dependant proliferation of ERα positive breast cancer cell lines [17].

## 4 Concluding remarks

Understanding how natural estrogens elicit clinical selective effects is a key to the development of safer estrogens for HRT. We have shown that isoflavones (GEN and DAI), EQ, and estrogens (E2) have similar effects on ERα transcriptional activation, on ERα expression regulation and on MCF-7 cell proliferation. More precisely, the molecular mechanisms for ERα transcriptional activation by isoflavones and EQ involve their capacity to act mainly through AF-1 and GEN, DAI, and EQ have similar estrogenic properties to E2 on ERα expression and on MCF-7 cell proliferation. This study confirms that phytoestrogens are SERM with estrogenic activities.

The declining use of HRT may mean that more women are using phytoestrogens supplements as a natural alternative to HRT despite the fact that there is no good evidence that they reduce menopausal symptoms much beyond a placebo effect. These supplements are available as over the counter drugs and they vary widely in their phytoestrogen content [26]. In conclusion, advocating soy-rich food or even isoflavone-based pills for women without medical follow-up should be considered critically. Moreover, it also follows that the diet of breast cancer patients should be matched carefully with their particular tumor types.

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*ERα46, and pSGERαΔ79 and the luciferase reporter plasmids ERE-TK-LUC and hC3-LUC. Many thanks to Professor J. A. Gustafsson's department (Department Biosciences and Medical Nutrition, Karolinska Institute, Sweden) for providing the expression vector for ERβ (pSG5hERβ). We are indebted to Russell Wallace for the revision of the English text.*

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