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Comparative study of oestrogenic properties of eight phytoestrogens in MCF7 human breast cancer cells

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Abstract

Previous studies have compared the oestrogenic properties of phytoestrogens in a wide variety of disparate assays. Since not all phytoestrogens have been tested in each assay, this makes inter-study comparisons and ranking oestrogenic potency difficult. In this report, we have compared the oestrogen agonist and antagonist activity of eight phytoestrogens (genistein, daidzein, equol, miroestrol, deoxymiroestrol, 8-prenylnaringenin, coumestrol and resveratrol) in a range of assays all based within the same receptor and cellular context of the MCF7 human breast cancer cell line. The relative binding of each phytoestrogen to oestrogen receptor (ER) of MCF7 cytosol was calculated from the molar excess needed for 50% inhibition of [^{3}H]oestradiol binding (IC_{50}), and was in the order coumestrol (35×)/8-prenylnaringenin (45×)/deoxymiroestrol (50×) > miroestrol (260×) > genistein (1000×) > equol (4000×) > daidzein (not achieved: 40% inhibition at 10^4-fold molar excess) > resveratrol (not achieved: 10% inhibition at 10^5-fold molar excess). For cell-based assays, the rank order of potency (estimated in terms of the concentration needed to achieve a response equivalent to 50% of that found with 17β-oestradiol (IC_{50})) remained very similar for all the assays whether measuring ligand ability to induce a stably transfected oestrogen-responsive ERE-CAT reporter gene, cell growth in terms of proliferation rate after 7 days or cell growth in terms of saturation density after 14 days. The IC_{50} values for these three assays in order were for 17β-oestradiol (1×10^{-11} M, 1×10^{-11} M, 2×10^{-11} M), and in rank order of potency for the phytoestrogens, deoxymiroestrol (1×10^{-10} M, 3×10^{-11} M, 2×10^{-11} M) > miroestrol (3×10^{-10} M, 2×10^{-10} M, 8×10^{-11} M) > 8-prenylnaringenin (1×10^{-9} M, 3×10^{-10} M, 3×10^{-10} M) > coumestrol (3×10^{-8} M, 2×10^{-8} M, 3×10^{-8} M) > genistein (4×10^{-8} M, 2×10^{-8} M, 1×10^{-8} M)/equol (1×10^{-7} M, 3×10^{-8} M, 2×10^{-8} M) > daidzein (3×10^{-7} M, 2×10^{-7} M, 4×10^{-8} M) > resveratrol (4×10^{-6} M, not achieved, not achieved). Despite using the same receptor context of the MCF7 cells, this rank order differed from that determined from receptor binding. The most marked difference was for coumestrol and 8-prenylnaringenin which both displayed a relatively potent ability to displace [^{3}H]oestradiol from cytosolic ER compared with their much lower activity in the cell-based assays. Albeit at varying concentrations, seven of the eight phytoestrogens (all except resveratrol) gave similar maximal responses to that given by 17β-oestradiol in cell-based assays which makes them full oestrogen agonists. We found no evidence for any oestrogen antagonist action of any of these phytoestrogens at concentrations of up to 10^{-6} M on either reporter gene induction or on stimulation of cell growth.

Keywords: Phytoestrogens; Resveratrol; Daidzein; Genistein; Equol; Coumestrol; 8-Prenylnaringenin; Miroestrol; Deoxymiroestrol; Oestrogen agonist; Oestrogen antagonist; Antioestrogen; Breast cancer cells; MCF7 cells

1. Introduction

Phytoestrogens are compounds produced naturally in plants and which have the ability to interfere with oestrogen action either by interacting directly with oestrogen receptors or indirectly by modulation of endogenous oestrogen concentrations [1,2]. Early studies noted adverse effects on fertility in animals that had been grazing on plants rich in phytoestrogens [3]. Today, there is a wide interest in phytoestrogens for their potential health benefits in countering menopausal symptoms and in lowering incidence of hormone-dependent diseases including breast cancer [4].

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Such diverse actions of phytoestrogens may involve non-oestrogen mediated mechanisms, such as inhibition of protein tyrosine kinases [2,5,6], inhibition of cell cycle progression [2,6–8], inhibition of DNA topoisomerase [2,6,9,10], inhibition of angiogenesis [2,6,11,12] or as antioxidants [2,6]. Other actions include alteration of levels of steroid hormone binding globulin (SHBG) [2,13], disruption of oestrogen metabolism [2,14] or alteration to cellular levels of oestrogen receptors [15,16]. However, a major mechanism of their action is thought to result from their ability to interact directly with oestrogen receptors [1,2]. Phytoestrogens could therefore interfere with endogenous oestrogen action either by acting as agonists in their own right at times of low endogenous oestrogen or by acting as antagonists at times of higher endogenous oestrogen levels. The molecular basis of oestrogen action involves the binding to intracellular receptors (ERα, ERβ), which function as ligand-activated transcription factors [17]. Therefore, phytoestrogen effects could result either from their competition for binding to ERα and/or ERβ, or from inducing patterns of gene expression different from those induced by 17β-oestradiol. In this respect, it is interesting that some phytoestrogens have been reported to bind more strongly to ERβ than to ERα [18] and that the two receptors have different patterns of tissue distribution [19]. More recent studies have begun to identify specific phytoestrogen-regulated genes [20].

In order to investigate these mechanisms further, studies have been carried out in a wide variety of in vitro assays. However, not all phytoestrogens have been tested in each assay, which makes both inter-study comparisons and ranking oestrogenic potency difficult [2]. Assays have varied from binding to rodent uterine receptors or to recombinant receptors (either full length or ligand binding domain), to gene expression assays in yeast, to reporter gene assays in human cell lines, to assay of endogenous oestrogen-regulated genes in human cell lines and then finally comparing to effects on growth of the oestrogen-sensitive MCF7 human breast cancer cell line [2,21–24]. Variations in the reported potency of phytoestrogens may relate as much to differing cellular receptor content and differing cellular context as to differences in the phytoestrogen actions. Insight into phytoestrogen actions in different cell types is not gained usefully by comparing action of one phytoestrogen in one cell type with action of another phytoestrogen in a different assay in another cell type. Many of such comparisons may turn out to be correct, but validation can only be achieved through performing for each phytoestrogen, all assays (ER binding, gene expression and cell proliferation) within each cell line. In this report, we have made a direct comparison between the oestrogenic actions of phytoestrogens in a range of assays all based in a single cell line, the MCF7 human breast cancer cell line.

Genistein and daidzein are isoflavones found in human diets in leguminous plants, especially soybeans [1,2]. Equol is a related phytoestrogen derived from metabolism of daidzein [1,2]. Equol was actually the major form of phytoestrogen present in sheep suffering fertility problems following the grazing on subterranean clover [25], but ability for metabolic conversion of daidzein can vary in the human population [2,26]. In MCF7 cells, genistein has been shown to have a biphasic effect on cell growth [27]. Low concentrations stimulate cell growth and enhance pS2 gene expression [28], whilst high concentrations (above 10 μM) inhibit cell growth by blocking the cell cycle at the G2-M phase [7,29]. The proliferative action of genistein at low concentrations can be inhibited by antioestrogen [16] indicating that it is an oestrogen receptor-mediated mechanism. However, the inhibition of cell growth at high concentrations is not prevented by antioestrogen or oestrogen [16], indicating it is not ER-mediated, but may be due to other mechanisms including inhibition of tyrosine phosphorylation [7,30].

Deoxymiroestrol and its derivative miroestrol have been reported as phytoestrogens with high oestrogenic potency [31], but their action in MCF7 cells has only ever been reported once and then only on their ability to antagonize antioestrogen action [32]. 8-Prenylnaringenin is a phytoestrogen found in hops and found to be a potent stimulator of Ishikawa cell growth [33] and of E-cadherin-dependent aggregation in MCF7 cells [34].

Coumestrol is a phytoestrogen found in alfalfa and animal foodstuffs and is thought to have potent oestrogenic activity. However, reported relative binding affinities have varied from <0.01% in sheep uterus to 94% for some human ER [1], oestrogen-responsive reporter gene expression has been reported in yeast, HeLa, LeC9 and prostate cells [1], and no studies have been reported on MCF7 cell growth [22].

Resveratrol, a polyphenolic compound found in grapes and wine, has a variety of biological effects, among which its oestrogenic activity is thought to contribute to the cardiopreventive effects associated with red wine consumption [35]. Like genistein, resveratrol has been reported to give a biphasic response on cell growth, but the differences in concentrations between stimulatory (3–22 μM) and inhibitory (above 25–44 μM) responses are rather smaller [36–38]. One report attests to an ability of resveratrol to function as a superagonist to reporter gene expression in MCF7 cells, producing a greater maximal response than oestradiol [36], but this may be promoter and receptor dependent [39] and relate to the presence of steroid response elements in the luciferase reporter gene [40]. Despite these reported superagonist effects in vitro [36], resveratrol is too weak in its oestrogenic activity to allow any measurable oestrogenic response in an in vivo uterotrophic assay [41].

In this study, we have made a comparison between the oestrogenic activities of eight phytoestrogens (genistein, daidzein, equol, miroestrol, deoxymiroestrol, 8-prenylnaringenin, coumestrol and resveratrol) in a variety of assays all based in the same MCF7 cell system, so that direct comparisons can be made within the same receptor and cellular context. We have compared their relative ability to bind to oestrogen receptors of MCF7 cell cytosol, their relative ability to induce a stably transfected oestrogen-responsive

reporter gene (ERE-CAT) in MCF7 cells, and their relative ability to stimulate growth of MCF7 cells. Using the same cells, we have then compared their abilities to antagonise the action of oestradiol in both reporter gene expression and cell growth assays.

2. Materials and methods

2.1. Chemicals

Genistein (98% purity) was purchased from Sigma (Poole, UK). Daidzein (98% purity), DL-equol (97–98% purity), (−)8-prenylnaringenin (98% purity), coumestrol (98–99% purity) and *trans*-resveratrol (98% purity) were all purchased from Plantech (Reading, UK). The purity of the samples of coumestrol and other phytoestrogens of commercial origin was calculated from the relative magnitude of the phytoestrogen peak and any minor peaks due to impurities as revealed by high pressure liquid chromatography (HPLC). Miroestrol and deoxymiroestrol were prepared and supplied by Ishikawa [32]. 17β-Oestradiol was purchased from Steraloids (Croydon, UK).

All compounds were made as stock solutions in ethanol and diluted into culture medium. Stock solutions of genistein, equol, 8-prenylnaringenin and resveratrol were prepared at 10^{-2} M in ethanol. Stock solutions of daidzein, coumestrol, miroestrol and deoxymiroestrol were prepared at 10^{-3} M in ethanol (solubility in ethanol prevented solutions of 10^{-2} M being made).

For genistein, equol, 8-prenylnaringenin and resveratrol, cell culture experiments using 10^{-5} M concentrations were performed by diluting the stock 10^{-2} M solutions at 1 in 1000 (v/v) in culture medium and controls were performed with equivalent concentrations of ethanol. For concentrations of 10^{-6} M and below, stock solutions were serially diluted such that the dilution into culture medium was always 1 in 10,000 (v/v) and controls were performed with equivalent concentrations of ethanol.

For daidzein, coumestrol, miroestrol and deoxymiroestrol, cell culture experiments using 10^{-6} M concentrations were performed by diluting the stock 10^{-3} M solutions at 1 in 1000 (v/v) in culture medium and controls were performed with equivalent concentrations of ethanol. For concentrations of 10^{-7} M and below, stock solutions were serially diluted such that the dilution into culture medium was always 1 in 10,000 (v/v) and controls were performed with equivalent concentrations of ethanol.

2.2. Culture of stock cells

MCF7 McGrath human breast cancer cells were kindly provided by C.K. Osborne at passage number 390 [42]. This cell line is dependent on oestrogen for growth as described previously [43]. Stock MCF7 cells were grown as monolayer cultures in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen) supplemented with 5% (v/v) foetal calf serum (FCS) (Invitrogen), 10 μg/ml insulin (Sigma) and 10^{-8} M 17β-oestradiol in a humidified atmosphere of 10% carbon dioxide in air at 37 °C. Cell stocks were sub-cultured at weekly intervals by suspension with 0.06% trypsin/0.02% EDTA (pH 7.3).

2.3. Competitive binding assay to ER of MCF7 cytosol

MCF7 human breast cancer cells were grown as monolayer cultures in phenol red-free RPMI 1640 medium (Invitrogen) with 5% dextran–charcoal-stripped FCS (DCFCS) [44] for a minimum of 3 days to deplete steroid hormone levels in the cells. Cells were then harvested, pelleted and homogenised in eight volumes of buffer (10 mM Tris–HCl, 1 mM EDTA, 2 mM dithiothreitol, 10% (v/v) glycerol, 0.5 M NaCl, pH 7.4) at 4 °C. Homogenates were centrifuged at 105,000 × *g* for 1 h at 4 °C and the resulting supernatant was stored in aliquots at −70 °C. Competitive binding assays were performed on the cytosol using the dextran-coated charcoal method as described previously [45]. Competition was assayed between the binding of [2,4,6,7-^{3}H]oestradiol (Amersham International, Bucks, UK) at 16×10^{-10} M and 1–100,000-fold molar excess of unlabelled compounds.

2.4. Assay of stably transfected ERE-CAT reporter gene in MCF7 cells

The ERE-CAT vector consisted of the oestrogen response element (ERE) of the vitellogenin A2 gene from −331 to −295 bp cloned into the pBLCAT2 vector [46] upstream of the thymidine kinase (tk) promoter (kindly provided by M. Parker). A clonal line of MCF7 cells stably transfected with this vector was previously characterized and the assay was carried out exactly as published previously [47]. *P*-values were calculated using a two-tailed student *t*-test, two-sample assuming unequal variance, within the MS Excel 2000 software package.

2.5. Cell proliferation experiments

Cells were added to the required volume of phenol red-free RPMI 1640 medium containing 5% DCFCS at a concentration of about 0.2×10^5 cells/ml and plated in monolayer in 0.5 ml aliquots into 24-well plastic tissue culture dishes (Nunc). After 24 h, the medium was changed to phenol red-free RPMI 1640 medium supplemented with 5% DCFCS with or without supplements of 17β-oestradiol and/or phytoestrogen. Culture medium was changed routinely every 3–4 days in all experiments. Cell counts were performed by counting released nuclei on a model ZBI Coulter Counter, as described previously [48]. Doubling time of the cells was calculated as a function of the slope of the linear plot of $\log_{10}$ (cell number) against time. Doubling time was calculated as $\log_{10}2$/slope. *P*-values were calculated using

a two-tailed student's *t*-test, two-sample assuming unequal variance, within the MS Excel 2000 software package.

3. Results

3.1. Experimental strategy

The chemical structures of the eight phytoestrogens included in this study are given in Fig. 1. Oestrogen agonist activity was assessed for a range of concentrations for each phytoestrogen using a variety of assays all based in MCF7 human breast cancer cells: (1) ligand ability to bind to ER from MCF7 cell lysates in a competitive binding assay; (2) ligand ability to regulate expression of a stably transfected oestrogen-responsive reporter gene (ERE-CAT) in MCF7 cells; (3) ligand ability to regulate the proliferation of oestrogen-dependent MCF7 cells.

The action of each phytoestrogen in the presence of 17β-oestradiol was also assayed on regulation of the ERE-CAT reporter gene in MCF7 cells and on growth of MCF7 cells. 17β-Oestradiol was used at the lowest concentration for maximal response (10^{-10} M). Any genuine oestrogen antagonist property of these phytoestrogens should be visible as a reduction in response to 17β-oestradiol when using a concentration of phytoestrogen below that needed to produce full agonist response when administered alone. Inhibitory responses seen as concentrations of phytoestrogen increased from 10^{-6} to 10^{-5} M in both the presence and absence of 17β-oestradiol were associated with toxicity in the cells and not due to oestrogen antagonism via ER.

The receptor content of MCF7 human breast cancer cells has been accepted as mainly ERα, although ERβ can be detected in MCF7 cells [49]. In our MCF7 McGrath human breast cancer cells, ERβ is weakly detectable after 35 cycles of PCR using 1 μg whole cell RNA in the one-step Qiagen RT-PCR kit (data unpublished).

3.2. Ligand binding to ER of MCF7 cell lysates

Since the first step in the action of any oestrogenic compound involves the binding of ligand to an intracellular receptor [17], experiments began by determining the relative effectiveness of these eight phytoestrogens in binding to ER of MCF7 cell cytosol. In a single point competitive binding assay, ERs from MCF7 human breast cancer cells were incubated at 4 °C for 18 h with 16×10^{-10} M [2,4,6,7-^{3}H]oestradiol and the extent of inhibition of binding was determined with increasing concentrations of each of the phytoestrogens in turn. Fig. 2 shows that [^{3}H]oestradiol binding could be inhibited almost completely by coumestrol (95%), 8-prenylnaringenin (96%) and deoxymiroestrol (95%) at 1000-fold molar excess. Miroestrol was less effective than deoxymiroestrol in this assay, inhibiting [^{3}H]oestradiol binding by 78% at 1000-fold molar excess. Genistein was the next most effective phytoestrogen at displacing [^{3}H]oestradiol, showing a 50% inhibition at 1000-fold molar excess and needing 100,000-fold molar excess for near complete inhibition (94%). Equol was the next most effective giving a 50% inhibition at 4000-fold molar excess, but complete inhibition could not be achieved within the solubility range of equol in this assay. For daidzein, a 40% inhibition was achieved at 10,000-fold molar excess, but again higher concentrations could not be assayed due to the solubility of daidzein. Resveratrol was very weak in this assay and even at 100,000-fold molar excess gave only a 10% inhibition of [^{3}H]oestradiol binding. Control experiments showed that [^{3}H]oestradiol binding was inhibited by 96% by 10-fold molar excess and by 100% by 100-fold molar excess of diethylstilboestrol. Previous work using the same assay in our laboratory has shown that the glucocorticoid, dexamethasone has no effect on [^{3}H]oestradiol binding at the concentrations tested of up to 100,000-fold molar excess [47]. A summary of these results is given in Table 1 by calculating for each phytoestrogen the molar excess needed for 50% inhibition of [^{3}H]oestradiol binding.

3.3. Ligand ability to increase expression of ERE-CAT reporter gene in MCF7 cells

The ability of each phytoestrogen to regulate gene expression was tested using a stably transfected oestrogen-sensitive reporter gene (ERE-CAT) in MCF7 human breast cancer cells [47]. Using a clonal line of MCF7 cells containing a stably integrated ERE-CAT reporter gene, cells were deprived of steroid for 7 days and then CAT gene expression was assayed after 24 h of treatment with varying concentrations of either 17β-oestradiol or phytoestrogen. The results are shown in Fig. 3, which is divided into two parts (A and B) for ease of graphical representation. The values for 17β-oestradiol in A and B are the same but are duplicated for comparative purposes. All data in Fig. 3 represent one single experiment carried out for all phytoestrogens at the same time.

CAT activity in this experiment was increased from a basal level with no oestrogenic compound added (5947 ± 340 dpm ^{14}C-acetyl transferred/h/10^4cells) to a maximal level at 10^{-10} M 17β-oestradiol ($23{,}982 \pm 271$ dpm ^{14}C-acetyl transferred/h/10^4cells) and this represented a four-fold induction ($P < 0.001$). Comparison of the relative concentration of each phytoestrogen needed to increase CAT reporter gene activity above basal levels showed the order of potency of the phytoestrogens to be deoxymiroestrol > miroestrol > 8-prenylnaringenin > coumestrol > genistein/equol > daidzein > resveratrol. Miroestrol and deoxymiroestrol increased CAT gene expression at the lowest concentrations. Although both miroestrol and deoxymiroestrol at 10^{-9} M concentrations increased CAT gene expression to the same level as 17β-oestradiol, at 10^{-10} M concentrations, CAT activity was increased to a greater extent by deoxymiroestrol ($13{,}930 \pm 353$ dpm ^{14}C-acetyl transferred/h/10^4cells) than miroestrol ($10{,}024 \pm 246$ dpm ^{14}C-acetyl transferred/

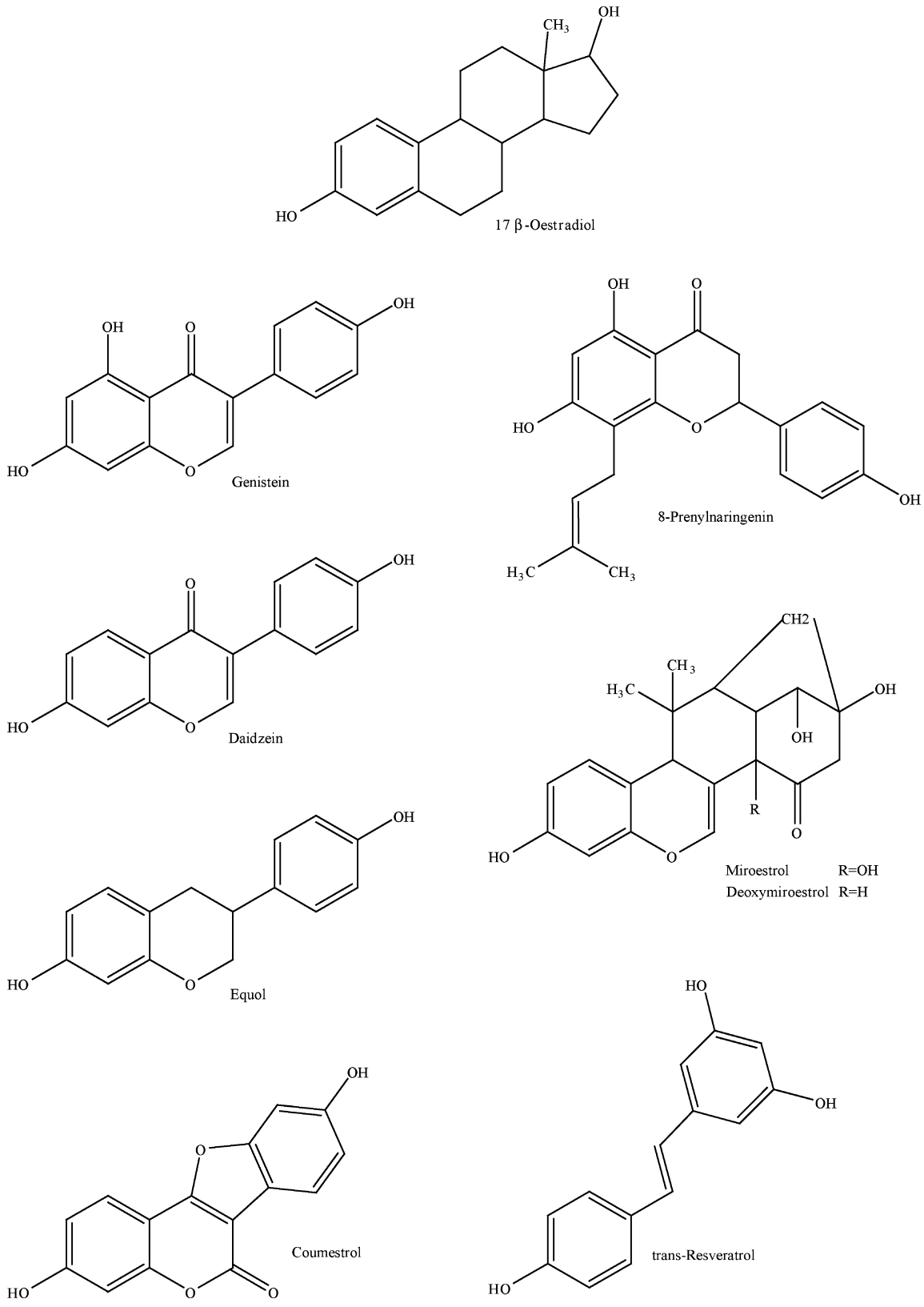

Fig. 1. Comparison of the chemical structures of 17β-oestradiol, genistein, daidzein, equol, coumestrol, 8-prenylnaringenin, miroestrol, deoxymiroestrol and resveratrol.

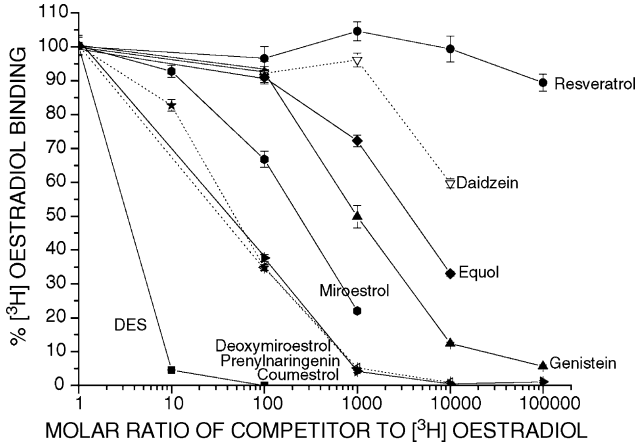

Fig. 2. Competitive binding of eight phytoestrogens to ERα from MCF7 human breast cancer cells. In single point competitive binding assays, 16×10^{-10} M [2,4,6,7-^{3}H]oestradiol was incubated with cytosol plus the stated molar excess of unlabelled diethylstilboestrol (DES) (solid square, solid line), deoxymiroestrol (solid star, dotted line), 8-prenylnaringenin (solid arrow pointing right, solid line), coumestrol (open arrow pointing left, dotted line), miroestrol (solid hexagon, solid line), genistein (solid arrow pointing up, solid line), equol (solid diamond, solid line), daidzein (open arrow pointing down, dotted line) or resveratrol (solid circle, solid line). Error bars represent the mean ± S.E. of triplicate assays.

h/10^{4}cells) ($P < 0.001$). 8-Prenylnaringenin did not increase CAT activity to the levels seen with 17β-oestradiol until at a concentration of 10^{-7} M. Coumestrol, genistein, equol and daidzein all reached maximal induction at 10^{-6} M concentrations. Resveratrol was the least active phytoestrogen in this assay and at 10^{-5} M still only gave an increase in CAT activity equivalent to 69% of that seen with 17β-oestradiol.

Inevitably, increase in CAT gene expression at higher concentrations of phytoestrogens was limited by issues of solubility. However, in as far as concentrations above 10^{-6} M could be studied without increasing the level of ethanol vehicle above a dilution of 1/1000 (v/v), it is interesting to note that 8-prenylnaringenin gave lower CAT gene expression at 10^{-5} M than at 10^{-6} M concentrations ($P = 0.004$). A small reduction was also found for genistein ($P = 0.038$) but that with equol ($P = 0.109$) was not significant. No reduction was observed for resveratrol, which continued to show an increase in CAT gene expression from 10^{-6} to 10^{-5} M. These results are summarized in Table 1 by calculating for each phytoestrogen the concentration needed to achieve an increase in CAT gene expression equivalent to 50% of that seen with 17β-oestradiol.

3.4. Ligand ability to stimulate proliferation of MCF7 human breast cancer cells

MCF7 human breast cancer cells are dependent on oestrogen for their proliferation in monolayer culture [43]. Cell growth assays showed that each of these eight phytoestro-

Table 1
Comparison of the relative oestrogen agonist activities of each of 8 phytoestrogens in different assay systems in MCF7 human breast cancer cells

Compound	Binding to ER; inhibition of 3H-E binding		Reporter gene; ERE-CAT induction		Cell proliferation			
	Molar excess to achieve 50% inhibition		Molar concentration to achieve 50% of response with 10^{-8} M oestradiol		Growth after 7 days		Growth after 14 days	
					Molar concentration to achieve 50% of response with 10^{-8} M oestradiol		Molar concentration to achieve 50% of response with 10^{-8} M oestradiol	
DES/oestradiol	**1 (DES)**	**3x**	**1 (E)**	**1×10^{-11} M**	**1 (E)**	**1×10^{-11} M**	**1 (E)**	**2×10^{-11} M**
Coumestrol	2	35x	5	3×10^{-8} M	5/6	2×10^{-8} M	6	3×10^{-8} M
8-Prenylnaringenin	3	45x	4	1×10^{-9} M	4	3×10^{-10}M	4	3×10^{-10} M
Deoxymiroestrol	4	50x	2	1×10^{-10} M	2	3×10^{-11} M	2	2×10^{-11} M
Miroestrol	5	260x	3	3×10^{-10} M	3	2×10^{-10} M	3	8×10^{-11} M
Genistein	6	1000x	6	4×10^{-8} M	5/6	2×10^{-8} M	5	1×10^{-8} M
Equol	**7**	**4000x**	**7**	**1×10^{-7} M**	**7**	3×10^{-8} M	**7**	**2×10^{-8} M**
Daidzein	**8**	**Not achieved**	**8**	**3×10^{-7} M**	**8**	2×10^{-7} M	**8**	**4×10^{-8} M**
Resveratrol	**9**	**Not achieved**	**9**	**4×10^{-6} M**	**9**	Not achieved	**9**	**Not achieved**

The rank order of potency is indicated on the left-hand side of each column. The relative potency in cell-based assays can be predicted for some phytoestrogens from their relative binding affinity to oestrogen receptor (bold script) but not for others (normal script).

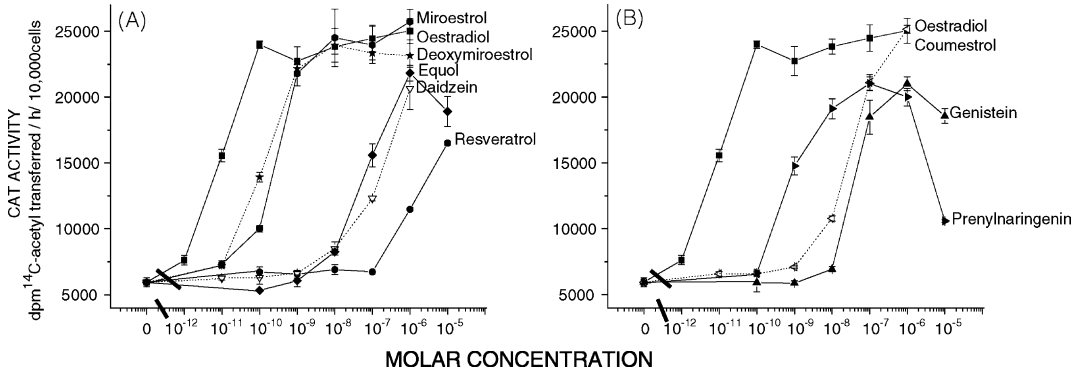

Fig. 3. Regulation by eight phytoestrogens of CAT gene expression from a stably transfected ERE-CAT gene in MCF7 human breast cancer cells. Cells were grown in RPMI 1640 medium/5% DCFCS for 7 days, and then in the same medium for a further 24 h with no addition (given at 0 molar concentration) or with either 17β-oestradiol or each phytoestrogen alone at the molar concentrations indicated. The results are presented graphically with CAT activity expressed as disintegrations per minute (dpm) of ^{14}C-acetyl transferred to chloramphenicol in 1 h per 10,000 cells. As this was a clonal stably transfected cell line, it was not necessary to normalise for transfection efficiency. Symbols on graph A are as follows: 17β-oestradiol (solid square, solid line), deoxymiroestrol (solid star, dotted line), miroestrol (solid hexagon, solid line), equol (solid diamond, solid line), daidzein (open arrow pointing down, dotted line), resveratrol (solid circle, solid line). Symbols on graph B are as follows: 17β-oestradiol (solid square, solid line) (as on graph A for comparison), 8-prenylnaringenin (solid arrow pointing right, solid line), coumestrol (open arrow pointing left, dotted line), genistein (solid arrow pointing up, solid line). Error bars are the standard error of triplicate wells.

gens could increase proliferation of MCF7 cells after 7 days (Fig. 4) or 14 days (Fig. 5), although requiring varying concentrations. Since these growth assays were not set up as one single experiment and plating densities varied, comparison was achieved by expressing the results as the percentage number of doublings with the phytoestrogen compared with the number of doublings with 10^{-8} M 17β-oestradiol in that same assay. The results are presented in Figs. 4 and 5, each of which is divided into two parts (A and B) for ease of graphical representation. The values for 17β-oestradiol in A and B of each figure are the same but are duplicated for comparative purposes.

The growth of MCF7 cells was increased after 7 days from 0.56–1.21 doublings without added oestrogen to 2.76–4.70 doublings with 10^{-8} M 17β-oestradiol, and after 14 days from 0.80–2.83 doublings without added oestrogen to 4.68–6.54 doublings with 10^{-8} M 17β-oestradiol. Comparison of the relative concentration of each phytoestrogen needed to increase cell proliferation above basal levels showed the order of potency of the phytoestrogens to be deoxymiroestrol > miroestrol > 8-prenylnaringenin > coumestrol / genistein/equol > daidzein > resveratrol, and this was similar whether considering proliferation after 7 or 14 days in culture. Although both

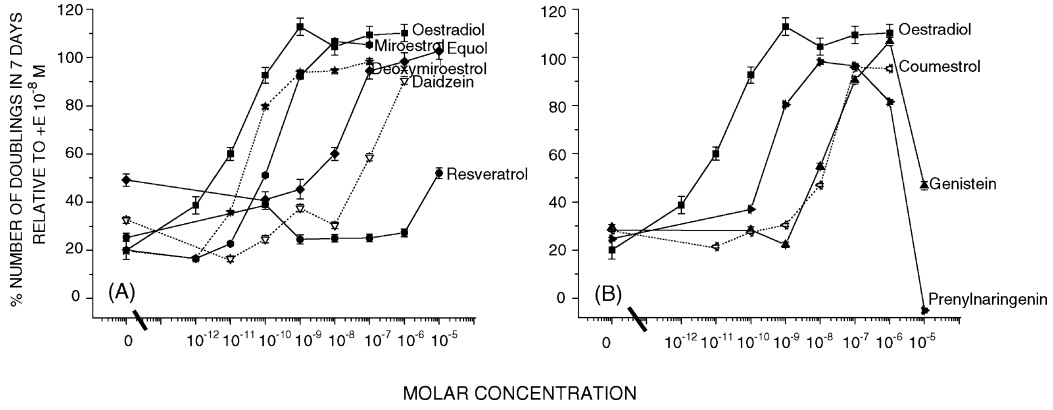

Fig. 4. Effects of different concentrations of each of eight phytoestrogens on the proliferation of MCF7 human breast cancer cells in monolayer culture. Cells were grown for 7 days in phenol red-free RPMI 1640 medium/5% DCFCS with no further addition (given at 0 molar concentration) or with either 17β-oestradiol or each phytoestrogen alone at the molar concentrations indicated. The results are presented graphically as the percentage of number of doublings with the phytoestrogen compared with the number of doublings with 10^{-8} M 17β-oestradiol in that same assay. Symbols on graph A are as follows: 17β-oestradiol (solid square, solid line), deoxymiroestrol (solid star, dotted line), miroestrol (solid hexagon, solid line), equol (solid diamond, solid line), daidzein (open arrow pointing down, dotted line), resveratrol (solid circle, solid line). Symbols on graph B are as follows: 17β-oestradiol (solid square, solid line) (as on graph A for comparison), 8-prenylnaringenin (solid arrow pointing right, solid line), coumestrol (open arrow pointing left, dotted line), genistein (solid arrow pointing up, solid line). Error bars are the standard error of all nine values from triplicate dishes with 10^{-8} M 17β-oestradiol and triplicate dishes with phytoestrogen.

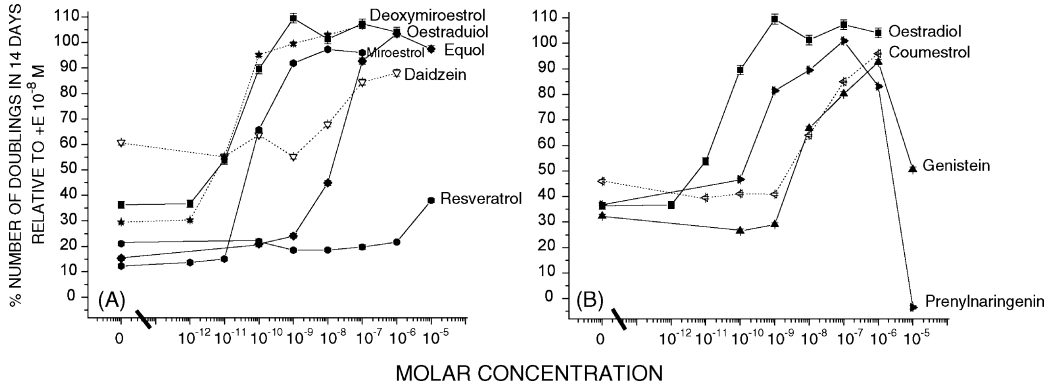

Fig. 5. Effects of different concentrations of each of eight phytoestrogens on the proliferation of MCF7 human breast cancer cells in monolayer culture. Cells were grown for 14 days in phenol red-free RPMI 1640 medium/5% DCFCS with no further addition (given at 0 molar concentration) or with either 17β-oestradiol or each phytoestrogen alone at the molar concentrations indicated. The results are presented graphically as the percentage of number of doublings with the phytoestrogen compared with the number of doublings with 10^{-8} M 17β-oestradiol in that same assay. Symbols on graph A are as follows: 17β-oestradiol (solid square, solid line), deoxymiroestrol (solid star, dotted line), miroestrol (solid hexagon, solid line), equol (solid diamond, solid line), daidzein (open arrow pointing down, dotted line), resveratrol (solid circle, solid line). Symbols on graph B are as follows: 17β-oestradiol (solid square, solid line) (as on graph A for comparison), 8-prenylnaringenin (solid arrow pointing right, solid line), coumestrol (open arrow pointing left, dotted line), genistein (solid arrow pointing up, solid line). Error bars are the standard error of all nine values from triplicate dishes with 10^{-8} M 17β-oestradiol and triplicate dishes with phytoestrogen.

miroestrol and deoxymiroestrol at 10^{-9} M concentrations increased cell growth to a similar level as 17β-oestradiol, at 10^{-10} M concentrations, cell proliferation was increased to a greater extent by deoxymiroestrol than miroestrol after both 7 and 14 days. 8-Prenylnaringenin did not increase cell proliferation to the levels seen with 17β-oestradiol until at a concentration of 10^{-8} M at either 7 or 14 days. Coumestrol, genistein and equol, all reached maximal induction at 10^{-7} M concentrations after 7 or 14 days. Daidzein reached maximal induction at 10^{-6} M concentrations after 7 days, although 10^{-7} M allowed maximal growth after 14 days. Resveratrol was the least active phytoestrogen in this assay and at 10^{-5} M still only gave a partial growth response after 7 and 14 days compared with 17β-oestradiol.

As for CAT reporter gene assays (see above), studies on cell growth at higher concentrations of phytoestrogens were limited by issues of solubility. However, in as far as concentrations above 10^{-6} M could be studied without increasing the level of ethanol vehicle above a dilution of 1/1000 (v/v), it is interesting to note that several of the phytoestrogens gave reduced cell proliferation at 10^{-5} M than at 10^{-6} M concentrations. This was true for genistein after 7 days ($P < 0.001$) and 14 days ($P = 0.009$), and to a lesser extent for equol after 14 days ($P = 0.014$). 8-Prenylnaringenin at 10^{-5} M resulted

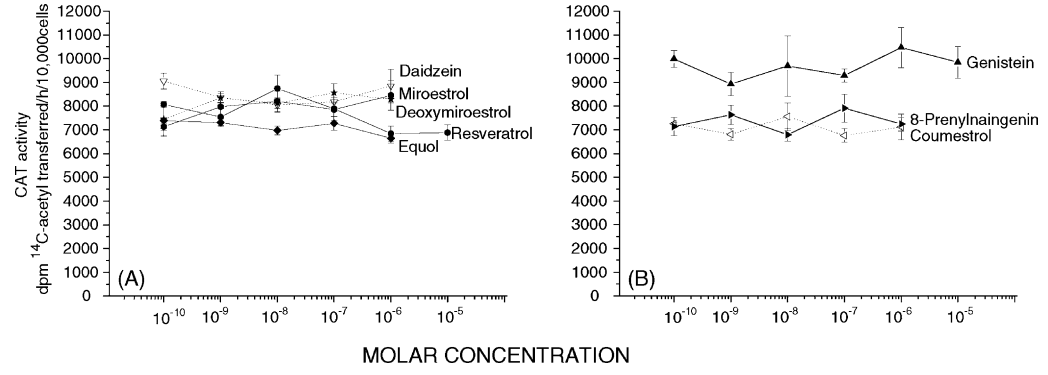

Fig. 6. Effect of each of eight phytoestrogens on oestrogen stimulation of CAT gene expression from a stably transfected ERE-CAT gene in MCF7 human breast cancer cells. Cells were grown in RPMI 1640 medium/5% DCFCS for 7 days, and then in the same medium for a further 24 h in the presence of 10^{-10} M 17β-oestradiol together with each stated phytoestrogen at the molar concentrations indicated. The results are presented graphically with CAT activity expressed as disintegrations per minute (dpm) of ^{14}C-acetyl transferred to chloramphenicol in 1 h per 10,000 cells. As this was a clonal stably transfected cell line, it was not necessary to normalise for transfection efficiency. Symbols on graph A are as follows: 10^{-10} M 17β-oestradiol with deoxymiroestrol at the indicated concentrations (solid star, dotted line), 10^{-10} M 17β-oestradiol with miroestrol at the indicated concentrations (solid hexagon, solid line), 10^{-10} M 17β-oestradiol with equol at the indicated concentrations (solid diamond, solid line), 10^{-10} M 17β-oestradiol with daidzein at the indicated concentrations (open arrow pointing down, dotted line), 10^{-10} M 17β-oestradiol with resveratrol at the indicated concentrations (solid circle, solid line). Symbols on graph B are as follows: 10^{-10} M 17β-oestradiol with 8-prenylnaringenin at the indicated concentrations (solid arrow pointing right, solid line), 10^{-10} M 17β-oestradiol with coumestrol at the indicated concentrations (open arrow pointing left, dotted line), 10^{-10} M 17β-oestradiol with genistein at the indicated concentrations (solid arrow pointing up, solid line). Error bars are the standard error of triplicate wells.

in complete loss of cell proliferation since cell numbers were not significantly different from the plating density after 7 days ($P = 0.480$) or 14 days ($P = 0.578$). However, the opposite effect was observed for resveratrol, which continued to give an increase in cell proliferation from 10^{-6} to 10^{-5} M. These results are summarized in Table 1 by calculating for each phytoestrogen the concentration needed to achieve an increase in cell proliferation after either 7 days or 14 days equivalent to 50% of that seen with 17β-oestradiol.

3.5. Ligand ability to reduce oestrogen stimulation of ERE-CAT gene expression

Since oestrogenic compounds are generally expected to display a spectrum of antagonist as well as agonist properties, each phytoestrogen was tested for its ability to reduce the increase in CAT reporter gene expression induced by 10^{-10} M 17β-oestradiol. No significant reduction in oestradiol-induced CAT activity was found with any of the concentrations of any of the phytoestrogens tested (Fig. 6).

3.6. Ligand ability to reduce oestrogen stimulation of MCF7 cell proliferation

Similarly, in anticipating antagonist as well as agonist activity, each phytoestrogen was assayed for its ability to antagonize the growth-promoting action of 10^{-10} M 17β-oestradiol. No significant antagonism of oestradiol stimulation of cell proliferation was found up to concentrations of 10^{-6} M of any of the phytoestrogens after either 7 days (Fig. 7) or 14 days (Fig. 8).

Inhibition of oestradiol-stimulated cell growth was found when using phytoestrogen concentrations of 10^{-5} M after 7 days for genistein, equol and resveratrol (Fig. 7) and after 14 days for genistein and resveratrol (Fig. 8). At this concentration of phytoestrogen, cell numbers increased between 7 and 14 days for both equol ($P < 0.001$) and resveratrol ($P = 0.020$) indicating a reduced cell proliferation rate. For 10^{-5} M genistein, however, although cell numbers increased from a plating density of $0.142 \pm 0.016 \times 10^5$ cells per well to $0.429 \pm 0.039 \times 10^5$ cells per well after 7 days ($P = 0.006$), cell numbers then fell between 7 and 14 days to $0.305 \pm 0.025 \times 10^5$ cells per well ($P = 0.076$), indicating cell death.

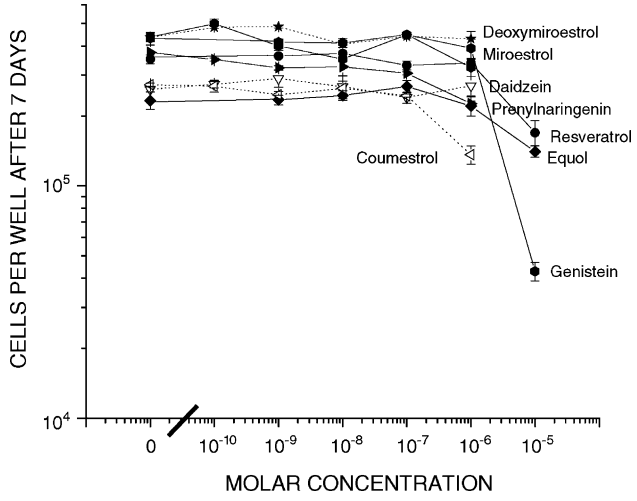

Fig. 7. Effect of each of eight phytoestrogens on oestrogen regulation of proliferation of MCF7 human breast cancer cells in monolayer culture. Cells were grown for 7 days in RPMI 1640 medium/5% DCFCS in the presence of 10^{-10} M 17β-oestradiol either alone or together with each stated phytoestrogen at the molar concentrations indicated. Symbols are as follows: 10^{-10} M 17β-oestradiol with deoxymiroestrol at the indicated concentrations (solid star, dotted line), 10^{-10} M 17β-oestradiol with miroestrol at the indicated concentrations (solid hexagon, solid line), 10^{-10} M 17β-oestradiol with equol at the indicated concentrations (solid diamond, solid line), 10^{-10} M 17β-oestradiol with daidzein at the indicated concentrations (open arrow pointing down, dotted line), 10^{-10} M 17β-oestradiol with resveratrol at the indicated concentrations (solid circle, solid line),10^{-10} M 17β-oestradiol with 8-prenylnaringenin at the indicated concentrations (solid arrow pointing right, solid line), 10^{-10} M 17β-oestradiol with coumestrol at the indicated concentrations (open arrow pointing left, dotted line), 10^{-10} M 17β-oestradiol with genistein at the indicated concentrations (solid arrow pointing up, solid line). The results are presented as number of cells in each well after 7 days and error bars are the standard error of triplicate wells.

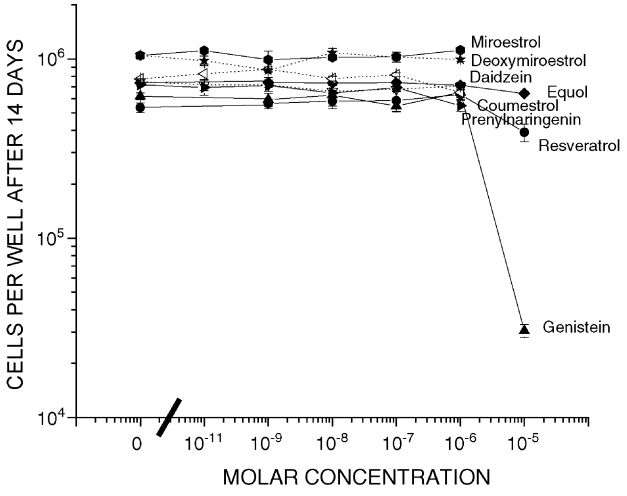

Fig. 8. Effect of each of eight phytoestrogens on oestrogen regulation of proliferation of MCF7 human breast cancer cells in monolayer culture. Cells were grown for 14 days in RPMI 1640 medium/5% DCFCS in the presence of 10^{-10} M 17β-oestradiol either alone or together with each stated phytoestrogen at the molar concentrations indicated. Symbols are as follows: 10^{-10} M 17β-oestradiol with deoxymiroestrol at the indicated concentrations (solid star, dotted line), 10^{-10} M 17β-oestradiol with miroestrol at the indicated concentrations (solid hexagon, solid line), 10^{-10} M 17β-oestradiol with equol at the indicated concentrations (solid diamond, solid line), 10^{-10} M 17β-oestradiol with daidzein at the indicated concentrations (open arrow pointing down, dotted line), 10^{-10} M 17β-oestradiol with resveratrol at the indicated concentrations (solid circle, solid line), 10^{-10} M 17β-oestradiol with 8-prenylnaringenin at the indicated concentrations (solid arrow pointing right, solid line), 10^{-10} M 17β-oestradiol with coumestrol at the indicated concentrations (open arrow pointing left, dotted line), 10^{-10} M 17β-oestradiol with genistein at the indicated concentrations (solid arrow pointing up, solid line). The results are presented as number of cells in each well after 7 days and error bars are the standard error of triplicate wells.

4. Discussion

The oestrogen agonist and antagonist properties of eight phytoestrogens (genistein, daidzein, equol, miroestrol, deoxymiroestrol, 8-prenylnaringenin, coumestrol and resveratrol) have been assayed in terms of their relative ability to compete with [^{3}H]oestradiol for binding to receptors of MCF7 cell cytosol, to induce a stably transfected oestrogen-responsive reporter gene (ERE-CAT) in the same MCF7 cell line and to stimulate growth of the MCF7 cells (summary in Table 1). This now enables a direct comparison between all these phytoestrogens in terms of their relative potency in different assays within the same receptor and cellular context. The rank order of potency remained very similar for all cell-based assays, whether measuring reporter gene expression, cell growth in terms of proliferation rate after 7 days or cell growth in terms of saturation density after 14 days. However, this did not follow the same rank order as determined from receptor binding, despite using the same receptor context. Correlation of the relative binding affinity to receptor and cellular response has also been shown to be limited for polychlorinated biphenyls in MCF7 cells [50] and several xenoestrogens in rat adenocarcinoma cells [51]. Explanation for such discrepancy may lie either in the relative ability of the ligand–receptor complex to transactivate gene expression or in the fate of the ligand within the cell, which could be influenced by cellular uptake and/or metabolic processes.

The ability of coumestrol to act in the cell-based assays was markedly reduced compared with its very potent ability to displace [^{3}H]oestradiol from cytosolic ER of the same cell. The rank potency of coumestrol in terms of its relative binding activity (RBA) determined here is in line with that described in previous publications [1], and this study demonstrates that the weaker activity of coumestrol in the cell-based assays is not therefore related to the receptor context. Since coumestrol was weaker in inducing reporter gene expression as well as cell growth, it might be that coumestrol-receptor dimers have a relatively impaired ability to regulate gene expression [52]. However, since the receptor binding studies are carried out in a cell-free system devoid of metabolizing enzymes, it is equally possible that metabolic conversion of coumestrol reduced its concentration within the MCF7 cell context. Whatever the mechanism, coumestrol cannot be considered to have potent oestrogenic activity within the MCF7 cell and this may also explain anomalies in its atypical activity in in vivo rat uterine studies [53].

By contrast to coumestrol, deoxymiroestrol and miroestrol maintained an activity in cell-based assays in line with their relative binding to receptor. As reported previously, deoxymiroestrol acted at lower concentrations than miroestrol in every assay [32], with deoxymiroestrol giving oestrogenic responses at no more than 10-fold higher concentrations than 17β-oestradiol and miroestrol around five-fold higher than deoxymiroestrol. Thus, these phytoestrogens can be considered as having potent oestrogenic activity in every assay in vitro and this has also been the case in reported in vivo assays over several decades [31].

In line with coumestrol but to a lesser extent, 8-prenylnaringenin also displayed reduced oestrogenic activity in cell-based assays compared with its very potent ability to displace [^{3}H]oestradiol from total oestrogen receptor of the same cell. The ability of 8-prenylnaringenin to bind to MCF7 cytosolic ER was not dissimilar to that found for deoxymiroestrol and coumestrol, but it required higher concentrations for oestrogenic responses in every cell-based assay compared with deoxymiroestrol. Oestrogenic activity of 8-prenylnaringenin has not been previously reported in MCF7 cells nor have uterotrophic studies been published. However, the concentrations required for half-maximal response in the MCF7 cell-based assays are in line with those reported for alkaline phosphatase stimulation in Ishikawa cells [33].

The phytoestrogens with weaker oestrogenic activity in MCF7 cells (lower down on Table 1) showed similar rank orders of potency across the receptor binding and cell-based assays. Studies of receptor binding by [^{3}H]oestradiol displacement were limited for equol, daidzein and resveratrol by issues of their solubility. Despite the relative inability of resveratrol to displace [^{3}H]oestradiol from receptor at 10^5-fold molar excess (Fig. 2), increase to 10^6-fold molar excess did allow both induction of reporter gene expression and stimulation of cell growth at levels of 10^{-5} M resveratrol compared with the required 10^{-11} M for 17β-oestradiol in the same assays (Figs. 3–5). However, responses with resveratrol were all to a lesser extent than with 17β-oestradiol, and therefore in as far as compound solubility is a limitation, resveratrol should be considered to be only a partial oestrogen agonist in MCF7 cells. Since in cell-based assays, daidzein relative to equol required higher concentrations in line with receptor binding activity, it would seem that metabolic conversion of daidzein to equol was not occurring in any major measure in the MCF7 cells, and both these compounds just about attained the status of full oestrogen agonist in MCF7 cells.

It is noteworthy that seven of the eight phytoestrogens (all except resveratrol) gave similar maximal responses to that given by 17β-oestradiol, albeit at varying concentrations. When expressed as percentages relative to 17β-oestradiol at 10^{-8} M as 100%, the responses ranged from 85 to 105% in the CAT assay to 88–103% and 88–105% in the cell growth assays at 7 and 14 days, respectively. This similarity among the seven phytoestrogens suggests that when bound to ER they all have similar effectiveness and similar effectiveness to 17β-oestradiol itself in promoting gene expression and cell proliferation via the expression of oestrogen-sensitive genes. Thus, all these seven phytoestrogens could be described as full oestrogen agonists. This contrasts with compounds such as tamoxifen which bind quite strongly to MCF7 cytosolic ER [47,54,55] but which only ever give reduced responses in cell-based assays compared with 17β-oestradiol [56,57] and can be described as partial oestrogen agonists.

Discussion of the extent to which phytoestrogens can act as oestrogen antagonists has been confused, and often mixed with the described inhibitory responses to these compounds at high concentrations of above 10^{-6} M. There seems now to be a general consensus view that in MCF7 cells, genistein has biphasic effects on cell growth such that at low concentrations it acts as an oestrogen agonist through the ER [16,27,28] whilst at higher concentrations (above 10 μM) it inhibits cell growth via non-ER pathways [7,16,29,30]. The results here are in agreement with this biphasic action of genistein on cell growth since alone it stimulated MCF7 cell growth at concentrations of 10^{-8} to 10^{-6} M, but cell growth was much reduced at 10^{-5} M (Figs. 4 and 5), and this reduction at 10^{-5} M genistein could not be reversed by the presence of 10^{-10} M 17β-oestradiol (Figs. 7 and 8). Interestingly, 8-prenylnaringenin also showed a similar biphasic response (Figs. 4 and 5) and this has not been reported previously. Assay at 10^{-5} M was not possible for deoxymiroestrol and miroestrol due to limited availability of material and not possible for coumestrol and daidzein by limitations in solubility, and so it remains unknown as to whether these phytoestrogens would behave in a similar inhibitory manner if sufficient concentrations could be tested. However, the reason for an inhibitory growth response seen with equol and resveratrol at 10^{-5} M only in the presence of 10^{-10} M 17β-oestradiol (Figs. 7 and 8) and not in the absence of oestradiol (Figs. 4 and 5) deserves further study. In summary, it would seem that many phytoestrogens can show biphasic effects on cell growth, stimulatory at low concentrations and inhibitory at higher concentrations. Since the inhibitory responses at concentrations above 10^{-6} M are seen also in the presence of oestradiol, they must be distinguished from any oestrogen antagonist responses, but it remains to be resolved as to whether they result from specific non-ER mediated mechanisms or from general toxicity.

However, what remains far less clear is whether any of these phytoestrogens can exhibit genuine oestrogen antagonist properties. Uterotrophic studies, measuring [^{3}H]oestradiol uptake, demonstrated oestrogen antagonist properties for genistein in in vivo assays in mice [58,59], but administration of dietary genistein did not antagonize the action of oestrogen in either oestrogen-supplemented ovariectomised rats or in intact rats [60]. Reports on the use of phytoestrogens to prevent breast cancer have also been mixed [2,61–63]. Animal models have given conflicting results showing genistein capable of reducing development of chemically induced mammary tumours in rats [64–66] especially in younger rats [67], whilst stimulating growth of subcutaneous MCF7-derived tumours in mice [68,69]. Assumption that soy diets of the East were the reason for reduced breast cancer in those parts of the world have similar conflicting epidemiological reports and dietary trial results [2]. Any true oestrogen antagonist action by any one of these compounds would be expected to give rise to a reduction in oestradiol-stimulated response at a concentration when the compounds given alone gave less than full agonist response and were not inhibitory. Dose–response curves for all eight of these phytoestrogens were carried out on oestradiol-stimulated reporter gene expression and cell growth response and no evidence was found for any oestrogen antagonist action by any of these phytoestrogens at concentrations up to 10^{-6}M (Figs. 6–8). This agrees with previous work on the phytoestrogens coumestrol, genistein and zearalenone given in the diet [70] and also our own previous work in vitro with the xenoestrogens polychlorinated biphenyls [50,71] and parabens [47,54,55]. This suggests either that the 17β-oestradiol was not displaced from the receptor by any of the concentrations of phytoestrogen or that if displacement did occur that the phytoestrogen-ER complex was as effective as the 17β-oestradiol-ER complex in these assays. This contrasts with compounds such as tamoxifen and its metabolite 4-hydroxytamoxifen [72,73] and faslodex [74], which can compete with 17β-oestradiol for binding to receptor and in doing so can reduce the overall oestrogenic response [74]. It also contrasts with the reported oestrogen antagonist activity of genistein in an immature mouse uterine weight assay, which was also associated with displacement of 17β-oestradiol by genistein [58,59]. These anomalies remain to be resolved, but it would appear that the beneficial effects of phytoestrogen on breast cancer are probably via pathways other than antagonism of oestrogen action.

Acknowledgments

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