Pergamon

European Journal of Cancer, Vol. 33, No. 14, pp. 2384–2389, 1997

Printed in Great Britain
0959-8049/97 $17.00+0.00

PII: S0959-8049(97)00303-1

Original Paper

Differential Effects of Dietary Phyto-oestrogens Daidzein and Equol on Human Breast Cancer MCF-7 Cells

N. Sathyamoorthy and T.T.Y. Wang

Laboratory of Nutritional and Molecular Regulation, NCI-Frederick Cancer Research and Development Center, P.O. Box B, Frederick, Maryland 21702–1201, U.S.A.

The *in vitro* effects of two closely related phyto-oestrogens daidzein and equol on the oestrogen receptor positive human breast cancer cells MCF-7 were examined. There is differential metabolism of daidzein in humans, and the conversion of daidzein to equol by intestinal microbes occurs only in 30% of the population. The differential potency of these two compounds is thus of considerable importance since it may be likely that the relative risk of hormone-dependent cancers may be higher in 'non-responders'. In the present study, we compared the ability of both these compounds to induce mRNA expression of the oestrogen-responsive pS2 gene, to compete with oestradiol for binding to the oestrogen receptor (ER) and to affect cellular proliferation. Our studies demonstrate that equol is a 100-fold more potent than daidzein in stimulating an oestrogenic response. Equol was also more effective than daidzein in competing with ^{3}H-oestradiol for binding to the ER. These results suggest that equol has a higher affinity for the ER. Both compounds stimulated the growth of MCF-7 cells in a concentration-dependent manner (10^{-8}–10^{-5} M). Although equol exhibits oestrogenic activity, exposure of MCF-7 cells to equol simultaneously with oestradiol was effective in reducing pS2 mRNA expression. This was not observed with daidzein. However, long-term exposure of MCF-7 cells to both daidzein and equol resulted in the downregulation of ER mRNA expression. © **1997 Elsevier Science Ltd.**

Key words: MCF-7 cells, daidzein, equol, pS2 expression, oestrogen receptor binding, cellular growth

Eur J Cancer, Vol. 33, No. 14, pp. 2384–2389, 1997

INTRODUCTION

POLYPHENOLIC COMPOUNDS present in fruits, vegetables and grains are bioactive molecules which elicit a wide range of responses both *in vivo* and *in vitro* [1–8]. Most significantly, polyphenolic compounds present in soy products have been associated with chemopreventive properties [9–11]. Genistein and daidzein are naturally occurring components of soy food products, whereas equol is derived from daidzein by the action of gut microflora [12, 13]. There has been considerable attention focused recently on the anti-oestrogenic and anticarcinogenic properties of genistein [7, 9–11]. However, much less is known about the effect of daidzein which is the other major isoflavone present in soy food and which has been shown to be the more bioavailable isoflavone [14]. In addition, the precursor/product relationship of daidzein and equol (Figure 1) is of special interest because others have reported a differential metabolism of daidzein in humans. In the tested population, 30% of individuals were 'responders' [15, 16]; the levels of equol in the plasma or urine were 150–900-fold higher in the 'responders' than 'non-responders' [15, 17]. However, the physiological significance of this observation is not clear. Hence, we felt that a careful examination of the biological activities of both daidzein and equol would be highly relevant. Using the oestrogen receptor (ER)-positive human breast cancer cell MCF-7, we compared the effects of daidzein and equol on (a) pS2 expression, (b) binding of oestradiol to the oestrogen receptor (ER), (c) ER mRNA expression and (d) cellular proliferation.

Correspondence to N. Sathyamoorthy.
Received 20 Aug. 1996; revised 11 May 1997; accepted 17 May 1997.

MATERIALS AND METHODS

Chemicals

β-Oestradiol and tamoxifen were obtained from Sigma St. Louis, Missouri, U.S.A.; daidzein was purchased from Indo-

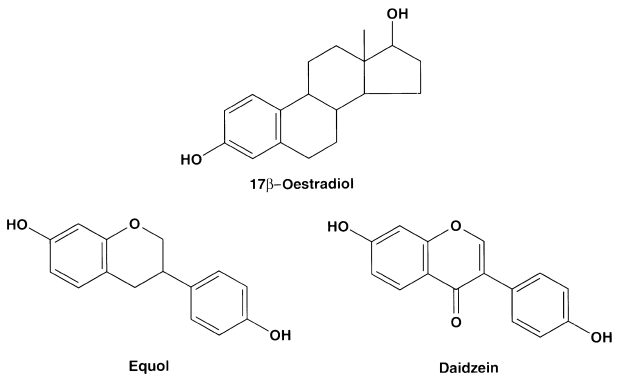

Figure 1. Structure of oestradiol, daidzein and equol.

fine Chemical Company, Somerville, New Jersey, U.S.A.; equol was synthesised by the method of Pelissero and associates [18]. Equol used in the initial experiments was a generous gift from Dr K.D.R. Setchell, Children's Hospital Medical Center, Cincinnati, Ohio, U.S.A. The cDNA coding for pS2 [19] and the human breast cancer cell line MCF-7 were obtained from American Type Culture Collection.

Cells and cell culture

MCF-7 cells were cultured in Falcon flasks (150 cm^2) and grown in the presence of 5% CO_2 in air at 37°C. The cells were maintained in medium A [RPMI 1640 with 2 mM L-glutamine, hydrocortisone (3.5 ng/ml), insulin (1.5 ng/ml), penicillin (100 units/ml) and streptomycin (100 μg/ml)] with 5% fetal bovine serum. One week before the onset of the experiment, the cells were switched to medium A supplemented with 5% charcoal dextran treated calf serum (CDCS). Cells were grown to confluence and passaged using trypsin–EDTA. Viable cells were determined by Trypan-Blue exclusion. A day prior to treatment, the cells were switched to medium B [phenol red-free RPMI 1640 containing 2 mM glutamine, penicillin (100 units/ml), streptomycin (100 μg/ml)] and 5% CDCS.

Total RNA isolation

Cells were grown in six-well Costar plates (1×10^6 cells/well in 3 ml of media B). Twenty-four hours after plating, the test compounds (dissolved in ethanol; final concentration of ethanol in the medium did not exceed 0.2%) were added. The medium was changed every 24 h after plating and the various compounds were added fresh with each change. Total RNA was isolated 72 h after plating as described earlier [8, 20].

Probe for pS2 and ER

Digoxigenin [DIG]-labelled pS2 cRNA probe and DIG-ER cRNA probe were synthesised using the nucleic acid labelling kit following the manufacturer's instructions (Boehringer Mannheim Biochemicals, Indianapolis, Indiana,

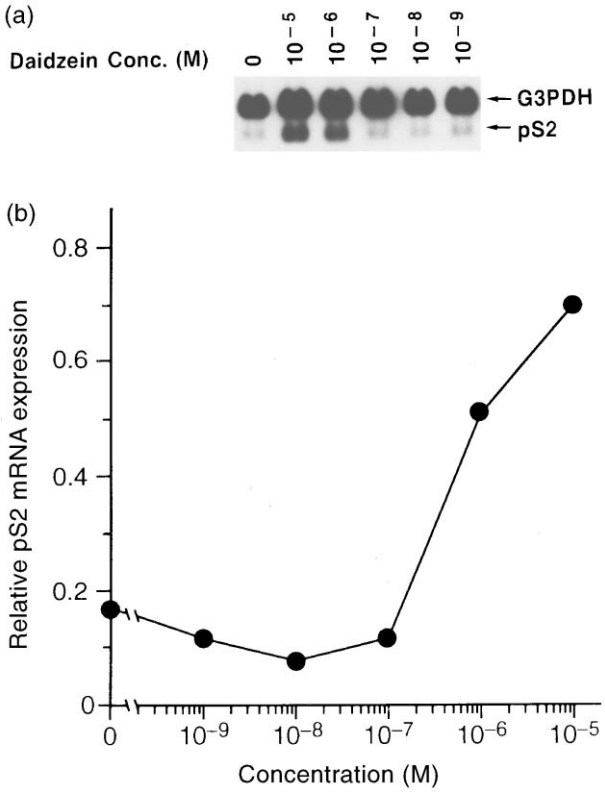

Figure 2. Concentration curve for the stimulation of pS2 expression by daidzein. MCF-7 cells were cultured in media B in the presence of various concentrations of daidzein (10^{-9}–10^{-5} M). Total RNA was isolated after 48 h. The levels of pS2 were normalised to that of G3PDH. (a) Representative RNA blots probed for G3PDH and pS2. (b) Quantitative representation of pS2 expression based on densitometric readings.

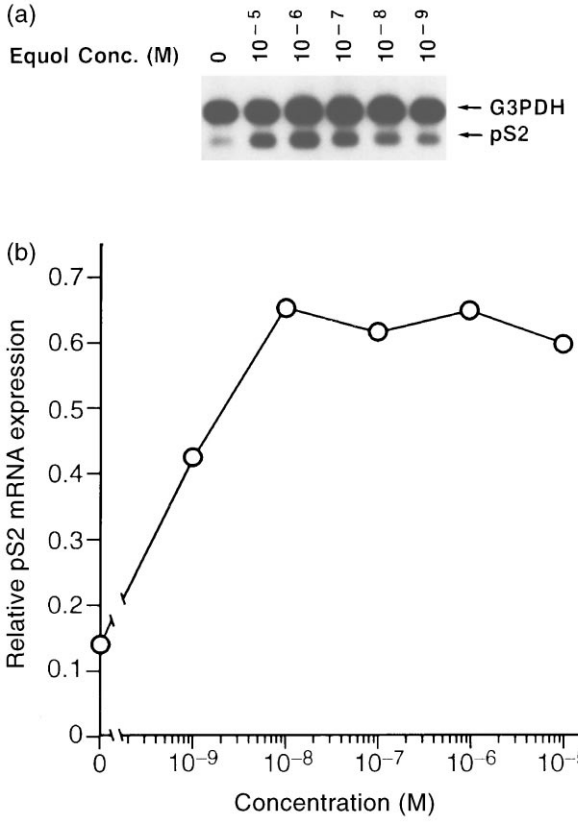

Figure 3. Concentration curve for the stimulation of pS2 expression by equol. MCF-7 cells were cultured in media B in the presence of various concentrations of equol (10^{-9}–10^{-5} M). Total RNA was isolated after 48 h. The levels of pS2 were normalised to that of G3PDH. (a) Representative RNA blots probed for G3PDH and pS2. (b) Quantitative representation of pS2 expression based on densitometric readings.

U.S.A.). Details for synthesis of the pS2 probe [8] and ER probe have been described previously [7].

Northern blot hybridisation and detection

For detection of pS2 expression, 10 µl of total RNA was separated on 1% formaldehyde agarose gel and transferred on to a nylon membrane (Boehringer Mannheim Biochemicals) using the PossiBlot Pressure Blotter (Stratagene, La Jolla, California, U.S.A.). The RNA was fixed on to the membrane by UV cross-linking in Stratalinker (Stratagene) for 3 min at the highest power. The membrane was then hybridised with the DIG-labelled RNA probe and detected by chemiluminescence nucleic acid detection method according to the manufacturer's protocol (Boehringer Mannheim Biochemicals). All Northern blots were also probed for G3PDH (glyceraldehyde-3-phosphate dehydrogenase). The blots were quantitated using a densitometer (Analtech Uniscan, Newark, Delaware, U.S.A.) and the data normalised for G3PDH RNA content.

Growth assay

Each well of a 24-well plate (Costar, Cambridge, Massachusetts, U.S.A.) was seeded with 1×10^5 cells in 1 ml of media B. Twenty-four hours after plating, oestradiol, daidzein or equol at the indicated concentration were added. All compounds were dissolved in ethanol (final concentration of ethanol in the medium was 1%). The medium was changed every 24 h and the compounds were replenished with each change. Cell growth was determined on the sixth day by the sulphorhodamine colorimetric assay as described by Skehan and associates [21]. After colour development, aliquots were pipetted into a 96-well microtitre plate and the absorbance was determined at 570 nm using an Elisa microplate reader (Molecular Devices, Menlo Park, California, U.S.A.).

Oestrogen receptor (ER) binding study

MCF-7 cells were grown to confluency in 150 cm^2 Falcon flasks and cultured in medium A supplemented with 5% CDCS at 37°C in the presence of 5% CO_2 in air. The cells were rinsed twice with cold phosphate-buffered saline (PBS), scraped off in 5 ml of PBS and pelleted at 1500 rpm for 10 min. After aspiration of the supernatant, the cell pellet was suspended in 10 mM Tris–HCl, pH 7.4, containing 1.5 mM EDTA and 0.5 mM dithiothreitol. The cell suspension was homogenised using a Tekmar Tissumizer (Tekmar Co., Cincinnati, Ohio, U.S.A.) for 45 s and the homogenate was then centrifuged at 40 000 rpm for 50 min and the supernatant saved. After determination of the protein concentration, the supernatant was used for ER binding assays. The ER binding studies were performed essentially as described by McGuire [22]. Briefly, 200 µl of the cytosol fraction (2.0 mg/ml protein) was incubated with ^{3}H-oestradiol at the indicated concentration for 18 h at 4°C. After incubation, the unbound oestradiol was removed by addition of 0.5 ml of dextran-coated charcoal to the assay mixture and incubated again at 4°C for 30 min with vortexing at 5 min intervals. After the incubation period, the tubes were centrifuged and 0.5 ml of the supernatant was removed and radioactivity measured using a scintillation counter. For competition experiments, the binding assays were performed as described above in the presence of increasing amounts of either daidzein or equol (0–10^{-6} M), while the concentration of oestradiol was held constant at 10^{-9} M.

RESULTS

We reported in an earlier paper that both daidzein and equol at 10^{-6} M were able to stimulate the expression of pS2 [8]. In the present study, we compared the ability of daidzein and equol to stimulate the expression of pS2 mRNA, compete with oestradiol for binding to the ER, alter the cellular response to oestradiol, regulate ER mRNA expression and affect the proliferation of MCF-7 cells.

Stimulation of pS2 mRNA expression

Daidzein was able to induce pS2 mRNA expression at concentrations of 10^{-6}–10^{-5} M (Figure 2). Equol was able to stimulate pS2 mRNA expression at concentrations as low as 10^{-9} M and maintained maximal effect from 10^{-8} to 10^{-5} M (Figure 3). Thus, equol was approximately 100-fold more potent than daidzein in stimulating pS2 expression.

Inhibition by tamoxifen

The effect of adding increasing concentrations of tamoxifen (10^{-9}–10^{-5} M) on daidzein- or equol-induced pS2 expression was evaluated. Tamoxifen at 10^{-5} M was able to inhibit completely pS2 expression by 10^{-6} M daidzein (Figure 4). However, the same concentration of tamoxifen only partially inhibited pS2 stimulation by 10^{-6} M equol. We therefore added 10^{-5} M tamoxifen to decreasing amounts of equol to determine the concentration at which pS2 expression would be totally inhibited. We observed complete inhibition at equol concentrations of 10^{-8} M (Figure 5). The fact that tamoxifen was able to inhibit the pS2 stimulating activity of both daidzein and equol indicated that their response was mediated via the ER.

Affinity for ER

The differential potency and sensitivity of daidzen and equol to tamoxifen may be due to their relative affinities for binding to the ER. Thus, we directly assessed the ability of daidzein and equol to compete with oestradiol for binding to the oestrogen receptor. Equol was very effective in inhibiting the binding of ^{3}H-oestradiol to the ER (Figure 6). Equol at a concentration of 5×10^{-7} M produced a 50% inhibition of

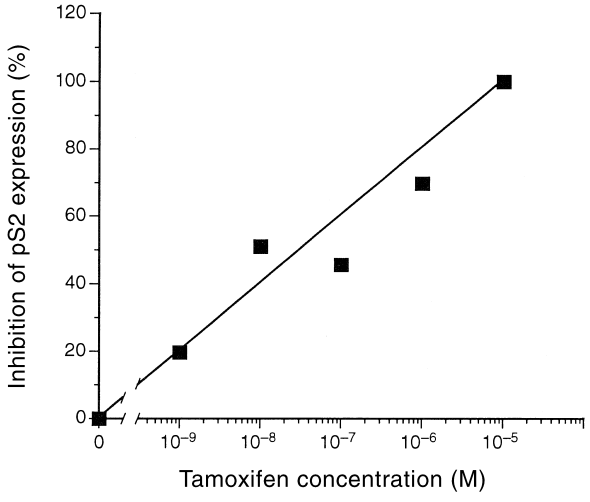

Figure 4. Inhibition of daidzein induced pS2 expression by the addition of tamoxifen. Cells were plated at a density of 1×10^6 per well in six-well Costar plates and exposed to either 10^{-6} M daidzein alone or daidzein plus different concentrations of tamoxifen for 48 h.

binding. In contrast, daidzein only slightly inhibited (~ 20%) oestradiol binding to the ER, even at a concentration of 10^{-6} M.

Interaction with oestradiol

We have previously demonstrated that genistein can act both as an agonist and antagonist of the ER-dependent pathway [7]. In the present study we examined the effect of the addition of either daidzein or equol on oestradiol-stimulated pS2 mRNA expression. The addition of either equol or daidzein to cell culture along with oestradiol did not result in an additive response (data not included). On the contrary, treating MCF-7 cells simultaneously with 10^{-6} or 10^{-7} M equol along with 10^{-10} M oestradiol caused a significant decrease in pS2 mRNA expression (Figure 7). This is similar to what we had observed with genistein [7]. We also evaluated the effect of long-term exposure of cells to either daidzein or equol on ER mRNA levels (Figure 8). We found that the steady-state ER mRNA levels were reduced to 25% of that seen in the untreated cells, similar to what we and others have observed with oestradiol [7, 23].

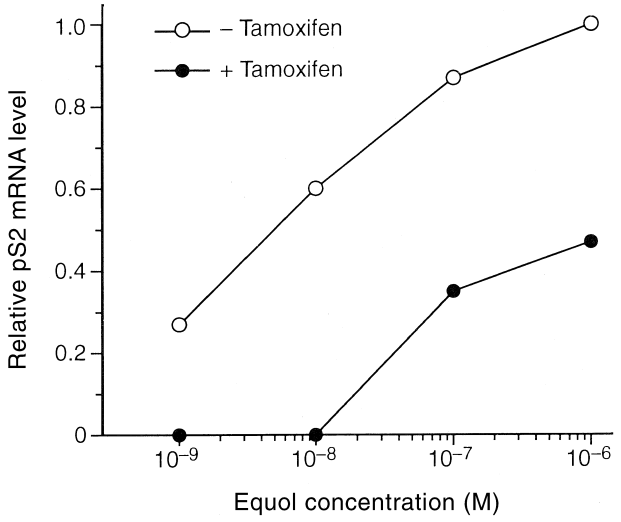

Figure 5. Inhibition of equol induced pS2 expression by the addition of tamoxifen. Cells were plated at a density of 1×10^6 per well in six-well Costar plates and exposed to equol at different concentrations with or without 10^{-5} M tamoxifen for 48 h.

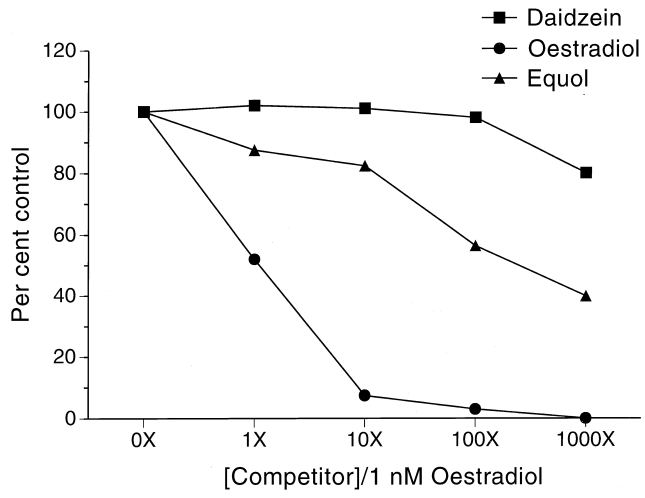

Figure 6. Effects of daidzein and equol on the binding of ^{3}H-oestradiol to the ER. Competitors were added at the indicated concentrations with 10^{-9} M ^{3}H-oestradiol. The results are expressed as percentage of control.

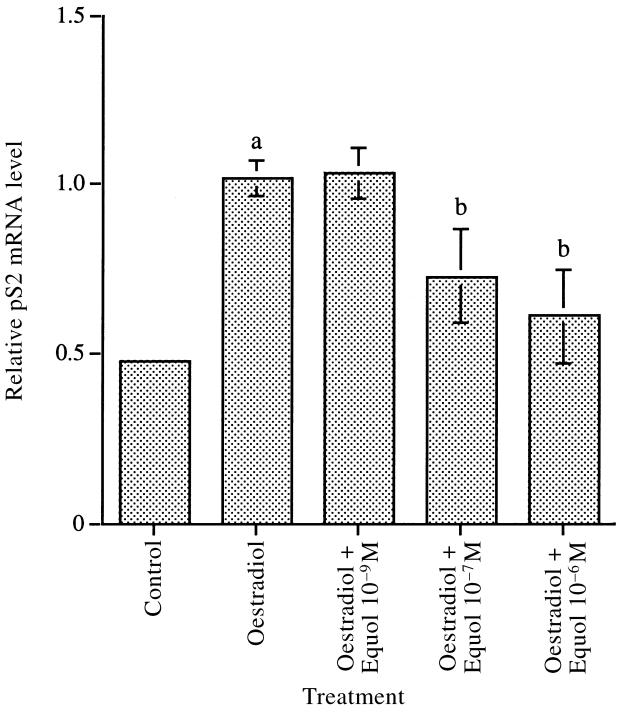

Figure 7. Effect of equol on oestradiol-stimulated pS2 mRNA expression. MCF-7 cells were incubated with either oestradiol (10^{-10} M) alone or oestradiol (10^{-10} M) with different concentrations of equol (10^{-9}–10^{-6} M). Total RNA was isolated after 48 h. The relative level of pS2 mRNA expressed is presented (mean ± SE; $n = 4$). Data were analysed using ANOVA and *post hoc* comparison using Fisher's PLSD test. Bars with different letters were significantly different from each other ($P < 0.05$).

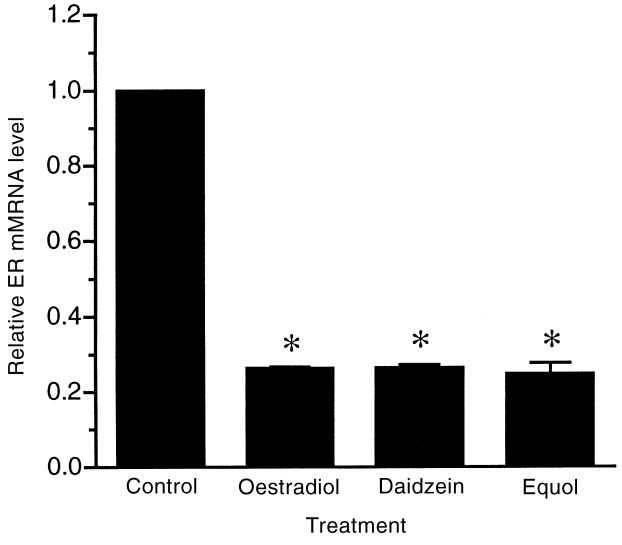

Figure 8. Effect of long-term treatment with daidzein or equol on ER mRNA level. MCF-7 cells were treated with either oestradiol (10^{-10} M), daidzein (10^{-6} M) or equol (10^{-6} M) for 6 days. The media and the test compounds were changed daily. RNA was isolated on the seventh day (mean ± SE; $n = 4$). Data were analysed using ANOVA and *post hoc* comparison using Fisher's PLSD test. *Significant difference ($P < 0.01$).

Effect on cellular proliferation

The biological effects of flavonoids and related polyphenolic agents are not mediated through the ER alone. Antiproliferative effects have been described which may be mediated through inhibition of protein kinases and topoisomerase [24, 25]. In addition to a specific end point mediated through interaction with the ER, the effects of daidzein and equol on cellular proliferation were also examined (Figure 9). Both daidzein and equol stimulated cellular proliferation in a concentration-dependent manner from 10^{-9} to 10^{-5} M.

DISCUSSION

Daidzein, an isoflavone found in high quantity in soybeans, is recovered in the urine of humans ingesting soy food products. Genistein is the other major isoflavone in soy foods, but it has been reported that daidzein is the more bioavailable isoflavone [14]. Daidzein is further metabolised to equol by the action of intestinal microflora [12, 13]. The two compounds are structurally similar to oestradiol and there have been several reports identifying daidzein and equol as having oestrogen-like properties [2, 26–29]. Published reports indicate that only one-third of the human population is able to metabolise daidzein to equol [11, 12]. Hence, we felt it was worthwhile to evaluate the individual effects of these isoflavones in a well-characterised *in vitro* system in order to fully comprehend the physiological significance of being a 'responder' versus a 'non-responder'. Data obtained from the present study indicate that the flavonoids with very similar structures may not produce an identical biological response. It emphasises the point that flavonoids need to be evaluated on an individual basis.

We compared the two isoflavones daidzein and equol and our studies demonstrate that equol is approximately 100-fold more oestrogenic than daidzein in the pS2 mRNA expression assay; while daidzein at a concentration of 10^{-6} M is able to stimulate increased expression of pS2 mRNA, equol is moderately active even at 10^{-9} M. A similar pattern is evident in the inhibition experiments carried out in the presence of tamoxifen. While 10^{-5} M tamoxifen was able to abolish the pS2 response of 10^{-6} M daidzein, the same concentration of tamoxifen was able to achieve a 55% inhibition of pS2 mRNA expression in the case of equol. Again, in the competition experiments, equol was more effective than daidzein in displacing ^{3}H-oestradiol from binding to the oestrogen receptor. Thus, there appears to be a correlation between the ability of the compound to compete for binding to the ER and its ability to stimulate pS2 expression. Recently, Martin and associates have also reported that equol was more potent than daidzein in displacing oestradiol or testosterone binding to the human sex steroid binding protein [30].

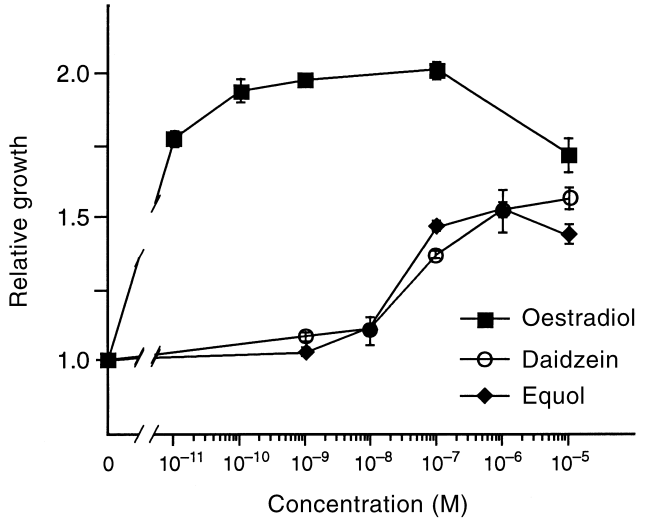

Figure 9. Effect of varying concentrations of oestradiol, daidzein and equol on the growth of MCF-7 cells. Cells were cultured in the presence of various concentrations of the compounds for 5 days. The results (mean ± SE; $n = 4$) are expressed as the growth observed relative to cells grown in the presence of vehicle alone.

It is interesting that although both daidzein and equol by themselves exhibit oestrogenic activity, they do not cause an additive response when added along with oestradiol. Despite the fact that equol is more potent than daidzein in inducing pS2 mRNA expression, when added with oestradiol, equol but not daidzein was able to cause a significant decrease in pS2 mRNA expression (Figure 7). This is very similar to the antagonist effect demonstrated by genistein [7]. Thus, it would suggest that equol is, in fact, more anti-oestrogenic than daidzein. In a recent study, Cassidy and associates [16] monitored the effect of a diet rich in isoflavones on the menstrual cycle of premenopausal women. The results from their study demonstrated that a high intake of soy protein increased the length of the follicular phase and/or delayed menstruation. The midcycle surges of luteinising hormone and follicle-stimulating hormone were also significantly suppressed during the soy-diet period. The above observations suggest that the agonist–antagonist effect of the phyto-oestrogens present in soy may be responsible for these hormonal modifications to the cycle. An increase in the menstrual cycle length reduces the life-time exposure to oestrogens; this as well as the concomitant lengthening of the follicular phase would be beneficial in lowering breast cancer risk since the mitotic activity of the breast tissue is reported to be 4-fold higher in the luteal phase than during the follicular phase [31, 32]. What is of greater interest in the context of our findings is the fact that these workers found that equol was the most effective; two of the women who had the highest urinary equol excretion showed the largest increase in follicular phase length [12].

Results from our current study indicate that long-term exposure of MCF-7 cells to either daidzein or equol results in a significant downregulation of ER mRNA expression (Figure 8). This implies that in a situation where there is a chronic exposure to either one of these compounds, there is a decreased response to oestradiol. However, with short-term treatment, we found that equol was far more anti-oestrogenic than daidzein (Figure 7). It has been reported that daidzein and genistein have a substantially shorter half-life than equol [33]. Hence, based on our experimental data, one could hypothesise that in individuals who regularly consume a diet rich in soy foods, both 'responders' and 'non-responders' would benefit, since long-term exposure to either agent would result in the downregulation of ER mRNA. However, in the case of individuals who consume soy-food products infrequently, the 'responders' would have significantly higher circulating levels of the more potent anti-oestrogen equol than the 'non-responders'. It is not clear what the physiological impact of this might be and is worth examining.

Since the conversion of daidzein to equol occurs by the action of gut microflora, it has been proposed that the type of bacteria found in the colon of the 'responders' is different from those of 'non-responders'. Moreover, it has been reported that an individual is consistently either a 'responder' or 'non-responder' [15], suggesting that the reason for this individual variability may be intrinsic or perhaps even genetically determined. Hence, it would be worthwhile examining the relative risk of hormone-dependent cancers among 'responders' and 'non-responders' to see if one group is more susceptible than the other.

1. Martin PM, Horwitz KB, Ryan DS, McGuire WL. Phytoestrogen interaction with estrogen receptors in human breast cancer cells. *Endocrinology* 1978, **103**, 1860–1867.
2. Welshons WV, Murphy CS, Koch R, Calaf G, Jordan VC. Stimulation of breast cancer cells *in vitro* by the environmental estrogen enterolactone and the phytoestrogen equol. *Breast Cancer Res Treat* 1987, **10**, 169–175.
3. Adlercreutz H, Fotsis T, Heikkinen R, *et al.* Excretion of the lignans enterolactone and enterodiol and of equol in omnivorous and vegetarian women and in women with breast cancer. *Lancet* 1982, **2**, 1295–1299.
4. Fotsis T, Pepper M, Adlercreutz H, *et al.* Genistein, a dietary derived inhibitor of *in vitro* angiogenesis. *Proc Natl Acad Sci USA* 1993, **90**, 2690–2694.
5. Peterson G, Barnes S. Genistein and Biochanin A inhibit the growth of human prostate cancer cells but not epidermal growth factor receptor tyrosine autophosphorylation. *The Prostate* 1993, **22**, 335–345.
6. Hodnick WF, Duval DL, Pardini RS. Inhibition of mitochondrial respiration and cyanide-stimulated generation of reactive oxygen species by selected flavonoids. *Biochem Pharmacol* 1994, **47**, 573–580.
7. Wang TTY, Sathyamoorthy N, Phang JM. Molecular effects of genistein on estrogen receptor mediated pathways. *Carcinogenesis* 1996, **17**, 271–275.
8. Sathyamoorthy N, Wang TTY, Phang JM. Stimulation of pS2 expression by diet-derived compounds. *Cancer Res* 1994, **54**, 957–961.
9. Lee HP, Gourley L, Duffy SW, Esteve J, Lee J, Day NE. Dietary effects on breast cancer risk in Singapore. *Lancet* 1991, **337**, 1197–1200.
10. Adlercreutz H, Honjo H, Higashi A, *et al.* Urinary excretion of ligands and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. *Am J Clin Nutr* 1991, **54**, 1093–1100.
11. Murrill WB, Brown NM, Zhang JX, Manzolillo PA, Barnes S, Lamartiniere CA. Prepubertal genistein exposure suppresses mammary cancer and enhances gland differentiation in rats. *Carcinogenesis* 1996, **17**, 1451–1457.
12. Borriello SP, Setchell KDR, Axelson M, Lawson AM. Production and metabolism of lignans by the human fecal flora. *J Appl Bacteriol* 1985, **58**, 37–43.
13. Setchell KDR, Adlercreutz H. Mammalian lignans and phytoestrogens: recent studies on their formation, metabolism and biological role in health and disease. In Rowland IR, ed. *Role of the Gut Flora in Toxicity and Cancer*. London, Academic Press, 1988, 315–345.
14. Xu X, Wang HJ, Murphy PA, Cook L, Hendrich S. Daidzein is a more bioavailable soymilk isoflavone than is genistein in adult women. *J Nutr* 1994, **124**, 825–832.
15. Kelley GE, Nelson C, Waring MA, Joannou GE, Reeder AY. Metabolites of dietary (soya) isoflavones in human urine. *Clin Chim Acta* 1993, **223**, 9–22.
16. Cassidy A, Bingham S, Setchell KD. Biological effects of a diet of soy protein rich in isoflavones on the menstrual cycle of premenopausal women. *Am J Clin Nutr* 1994, **60**, 333–340.
17. Morton MS, Wilcox G, Wahlqvist, Griffiths K. Determination of lignans and isoflavonoids in human female plasma following dietary supplementation. *J Endocrinol* 1996, **142**, 251–259.
18. Pelissero C, Bennetau B, Babin P, Le Menn F, Dunogues J. The estrogenic activity of certain phytoestrogens in the Siberian sturgeon *Acipenser baeri*. *J Steroid Biochem Mol Biol* 1991, **38**, 293–299.
19. Masiakowski P, Breathnach R, Bloch J, Gannon F, Krust A, Chambon P. Cloning of cDNA sequences of hormone-regulated genes from the MCF-7 human breast cancer cell line. *Nucleic Acids Res* 1982, **10**, 7895–7903.
20. Xie W, Rothblum LI. Rapid small scale RNA isolation from tissue culture cells. *Biotechniques* 1991, **11**, 325–327.
21. Skehan P, Storeng R, Scudiero D, *et al.* New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* 1990, **82**, 1107–1112.
22. McGuire W. Quantitation of estrogen receptor in mammary carcinoma. In O'Malley BW, Hardmann JG, eds. *Methods in Enzymology*. New York, Academic Press Inc., 1975, **36**, 248–254.
23. Read LD, Greene GL, Katzenellenbogen BS. Regulation of estrogen receptor messenger ribonucleic acid and protein levels in human breast cancer cell lines by sex steroid hormones, their antagonist, and growth factors. *Mol Endocrinol* 1989, **3**, 295–304.
24. Akiyama T, Ishida J, Nakagawa S, *et al.* Genistein, a specific inhibitor of tyrosine-specific protein kinase. *J Biol Chem* 1987, **262**, 5592–5595.
25. Okura A, Arakawa H, Oka H, Yoshinari T, Monden Y. Effect of genistein on topoisomerase activity on the growth of [Val12] Haras-transformed NIH 3T3 cells. *Biochem Biophys Res Commun* 1988, **157**, 183–189.
26. Mayr U, Butsch A, Schneider S. Validation of two *in vitro* test systems for estrogenic activities with zearalenone, phytoestrogens and cereal extracts. *Toxicology* 1992, **74**, 135–139.
27. Markiewicz L, Garey J, Adlercreutz H, Gurpide E. *In vitro* bioassays of non-steroidal phytoestrogens. *J Steroid Biochem Mol Biol* 1993, **45**, 399–405.
28. Miksicek RJ. Interaction of naturally occurring nonsteroidal estrogens with expressed recombinant human estrogen receptor. *J Steroid Biochem Mol Biol* 1994, **49**, 153–160.
29. Miksicek RJ. Estrogen flavonoids: Structural requirements for biological activity. *Proc Soc Exp Biol Med* 1995, **208**, 44–50.
30. Martin ME, Haourigui M, Pelissero C, Benassayag C, Nunez EA. Interactions between phytoestrogens and human sex steroid binding protein. *Life Sci* 1996, **58**, 429–436.
31. Treolar AE, Boynton RE, Behn BG, Brown BW. Variation of the human menstrual cycle throughout reproductive life. *Int J Fertil* 1970, **12**, 77–126.
32. Ferguson DJP, Anderson TJ. Morphological evaluation of cell turnover in relation to the menstrual cycle in the ''resting'' human breast. *Br J Cancer* 1981, **44**, 177–181.
33. Kelley GE, Joannou GE, Reeder AY, Nelson C, Waring MA. The variable metabolic response to dietary isoflavones in humans. *Proc Soc Exp Biol Med* 1995, **95**, 40–43.