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Original Paper

Regulation of c-*fos* Transcription by Chemopreventive Isoflavonoids and Lignans in MDA-MB-468 Breast Cancer Cells

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Isoflavonoids and lignans are diet constituents with chemopreventive properties. We compared the ability of the isoflavonoids genistein and equol, the lignans enterodiol, enterolactone and nordihydroguaiaretic acid (NDGA) and the lignan metabolite methyl p-hydroxyphenyllactate to interfere with mitogenic and tumour promotional signal transduction pathways. Their effects on c-*fos* mRNA levels after induction by either epidermal growth factor (EGF) or the tumour promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA) was measured in human breast cancer-derived MDA-MB-468 cells. Of the six agents, only genistein decreased EGF-induced, c-*fos* transcription (by 63% compared to control at 100 μmol/l). In contrast, both genistein and equol at 100 μmol/l decreased TPA-induced c-*fos* levels, by 75 and 67%, respectively. NDGA and methyl p-hydroxyphenyllactate did not inhibit TPA mediated c-*fos* transcription and enterolactone and enterodiol had only a weak inhibitory effect. NDGA at 0.1–10 μmol/l increased c-*fos* mRNA levels. None of the agents inhibited protein kinase C and only genistein inhibited EGF receptor-linked protein tyrosine kinase obtained from MDA-MB-468 cells, with an IC_{50} of 60 μmol/l. NDGA and genistein arrested cell colony formation potently, genistein was 15-fold more growth-inhibitory than equol. The results suggest that both genistein and equol interfere similarly with TPA-induced signal transduction pathways. Inhibition by genistein of EGF-induced c-*fos* mRNA transcription is probably related to its interruption of EGF receptor-linked protein tyrosine kinase, whereas genistein-induced growth arrest is not. If ability to antagonise phorbol ester effects is important for chemopreventive efficacy, equol and genistein might be equi-efficacious chemopreventors, whereas enterolactone, enterodiol and NDGA should be much less potent. If phorbol ester antagonism together with antimitogenic activity determine optimal chemopreventive activity of this type of agent, genistein would be more potent than equol. © **1998 Elsevier Science Ltd. All rights reserved.**

Key words: chemoprevention, c-*fos*, transcription, genistein, equol, lignans, nordihydroguaiaretic acid

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INTRODUCTION

PLANT-DERIVED PHENOLS such as the isoflavonoid genistein and the lignan enterolactone are thought to possess cancer chemopreventive properties [1–3]. They occur in certain vegetables as their glycosides from which they are released by the bacterial microflora in the gut. The mechanisms by which these agents interfere with the process of carcinogenesis are only poorly understood. Among the mechanisms which have been discussed as potentially causing, or contributing towards, chemoprevention by these compounds are oestrogenic activity [4, 5], anti-oxidation [6] and inhibition of DNA

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topoisomerase II [7]. Moreover, chemopreventive polyphenols are thought to inhibit carcinogenesis in the initiated cell at the level of promotion via modulation of signal transduction. This suggestion has been based mainly on the finding that genistein is a potent inhibitor of protein tyrosine kinases, pivotal constituents of cellular signal transduction cascades which regulate proliferation and differentiation [8]. Genistein and related phenols are also inhibitors of cell growth [2, 9, 10]. Antipromotional and antiproliferative activities may be mechanistically linked, but the nature of this link is obscure. We compared six diet-derived phenols in terms of their potencies as disintegrators of signal transduction pathways important for mitogenesis on the one hand and tumour promotion on the other. The aim of this comparison was to contribute to the understanding of chemopreventive mechanisms and to help develop screening systems for the detection of novel chemopreventive substances. The compounds chosen for this comparison were the isoflavonoid genistein and the isoflavan equol (for structures see Figure 1) and the lignans enterodiol and enterolactone, because they are major metabolites of constituents of chemopreventive diets such as soybean and linseed [11, 12], the lignan nordihydroguaiaretic acid (NDGA), because it is an abundant ingredient of the leaves of the shrub *Larrea divaricata* with suspected anticarcinogenic and antitumorigenic [13], albeit also hepatotoxic [14] properties and methyl p-hydroxyphenyllactate, because it is a human lignan metabolite [15]. The study was conducted in human-derived MDA-MB-468 breast cancer cells which do not express the oestrogen receptor [16] and are thus unresponsive to the oestrogenicity of these phenols [17]. To assess interference with mitogenic and tumour promotional pathways, we measured mRNA levels of the early response gene c-*fos* after induction by either epidermal growth factor (EGF) or tumour promoting phorbol ester 12-*O*-tetradecanoylphorbol-13-acetate (TPA). Inhibition of EGF-induced signals reflects processes related to antimitogenic activity of a compound and interference with TPA-mediated events mirrors anti-tumour promotional properties. The study was complemented by an investigation of the ability of these dietary constituents to inhibit protein tyrosine kinase and protein kinase C (PKC), two enzymes crucial in the signal transduction cascades induced by EGF or TPA. We also studied the effects of the phenols on cell colony formation and compared them with those on growth factor- and tumour promotor-elicited gene transcription.

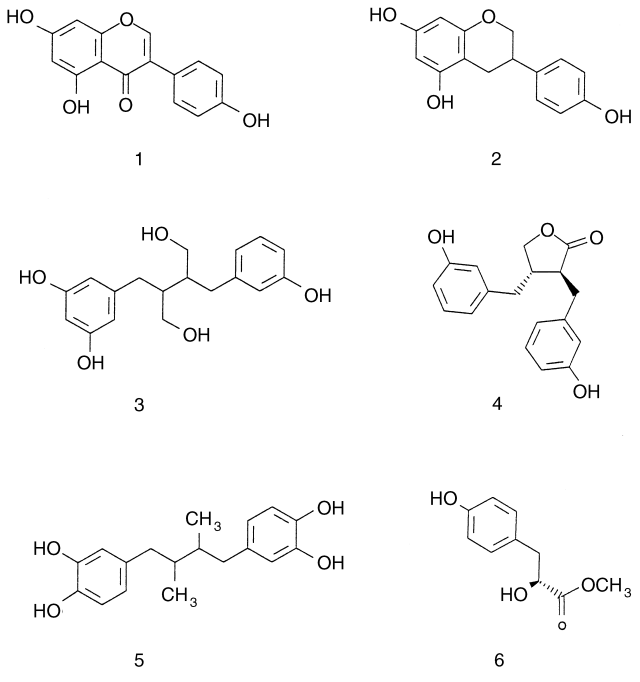

Figure 1. Structures of the isoflavonoids genistein (1) and equol (2), the lignans enterodiol (3), enterolactone (4) and NDGA (5) and the flavonoid metabolite methyl p-hydroxyphenyllactate (6).

MATERIALS AND METHODS

Chemicals

NDGA was obtained from Fluka Chemicals (Gillingham, U.K.), equol from Apin Chemical Company (Abingdon, U.K.), genistein and TPA from Sigma Chemical Comp. (Poole, U.K.), GF109203X, human recombinant epidermal growth factor and platelet-derived growth factor (BB-chain homodimer, human recombinant) from Calbiochem (Nottingham, U.K.). The following compounds were gifts: enterolactone, enterodiol and equol from H. Adlerkreutz (University of Helsinki, Finland), methyl p-hydroxyphenyllactate from M.D. Threadgill (University of Bath, U.K.), and CGP 52411 from E. Buchdunger (Ciba-Geigy AG, Basle, Switzerland).

Cell culture

Human-derived MDA-MB-468 breast cancer cells (obtained from J. Carmichael, University of Nottingham, U.K.) were routinely cultured in RPMI 1640 medium (Gibco/BRL, Paisley, U.K.) containing 10% heat-inactivated fetal calf serum (FCS), 2 Mm L-glutamine, 50 IU/ml penicillin, 50 µg/ml streptomycin at 37°C in air/CO_2 (5%). For the RT-PCR analysis, cells were seeded (1.5×10^5/dish) in 6-well plates (Falcon, Rotherham, U.K.) and grown for 72 h to 80% confluency. The cells were kept in medium without FCS for 48 h with one medium change after 24 h. Agents were added to cultures 90 min prior to stimulation with either EGF (10 ng/ml) or TPA (100 ng/ml) for 30 min. The direct effect of the agents on their own on c-*fos* mRNA levels was determined by exposure of quiescent cells for 30 min. RNA was isolated immediately afterwards.

Clonogenic assay

MDA-MB-468 cells (100–500 per dish) were seeded in 6-well plates and incubated with conditioned medium (normal medium plus medium from confluent cultures, 1:1) which had previously been filtered (pore size 0.23 µm). Cells were left for 4 h before the addition of agents. Medium including agent was replenished at 2-day intervals. After incubation for 7–9 days cells were fixed with methanol and stained (Giemsa). Colonies of 10 or more cells were counted. The colony forming efficiency of control cells was $49 \pm 10\%$ ($n = 7$) for 100 cells/dish, $65 \pm 19\%$ ($n = 6$) for 200 cells and $50 \pm 11\%$ ($n = 3$) for 500 cells. The correlation coefficient for the plot describing log of number of colonies versus log of concentration of isoflavonoid or lignan was 0.957 or higher.

RNA isolation and RT-PCR analysis

Total cellular RNA was isolated using RNAzol B (Biogene, Cambridge, U.K.) and diluted to a final concentration of 100 ng/µl. RT-PCR was carried out using an internal RNA standard. The internal standard was a modified sequence of

cellular c-*fos* mRNA which was amplified. Internal standard and cellular c-*fos* mRNA contained the same primer sites. The modification made the internal standard sequence slightly longer by duplication of a piece of sequence between the primer sites. Details of primer construction are given below. In the analysis 100 ng of cellular RNA was mixed with between 0.05 and 40 pg of the internal standard RNA in a final reaction volume of 10 µl containing Tris/HCl (20 mmol/l) pH 8.4, KCl (50 mmol/l), $MgCl_2$ (2.5 mmol/l), RNAsin (Promega, Southampton, U.K.) (20 U/µl), MMLV-reverse transcriptase (Gibco/BRL) (10 U/µl), dNTP (1 mmol/l), hexamers (Pharmacia, St. Albans, U.K.) (approximately 15 pmoles/µl) and dithiothreitol (1 mmol/l). Hexamers were annealed at 23°C for 10 min, products extended at 42°C for 45 min and the reaction terminated by heating to 99°C before being quickly chilled to 4°C. The PCR stage was carried out by the addition of reagents (master mix) in Tris/HCl (20 mmol/l) pH 8.4 with KCl (50 mmol/l) and $MgCl_2$ (2.5 mmol/l), 0.5 pmol of sense and antisense primer (for details see below), w-1 detergent (Gibco/BRL) (0.05%) and *taq* DNA polymerase (1 U) in a final volume of 20 µl. A portion of the sense primer (0.005 pmoles/µl) was end-labelled with [^{33}P]. The nucleotides, which were derived entirely from the original reverse transcriptase reaction, were at a final concentration of 0.5 mmol/l each.

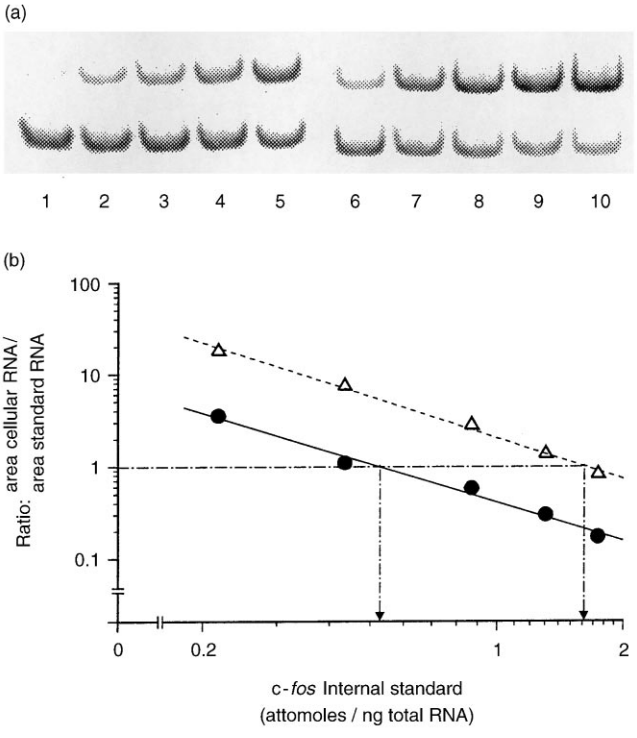

Figure 2. Competitive RT-PCR analysis (a) and its quantitative evaluation by phosphoimage analysis (b) of c-*fos* mRNA induced by EGF (10 ng/ml) in serum-starved MDA-MB-468 cells in the absence (lanes 1–5 in a, open triangles in b) or presence of genistein (100 µmol/l) (lanes 6–10 in a, closed circles in b). In (a) the bottom row represents cellular c-*fos* mRNA, the top row internal standard c-*fos* mRNA, which was added at the following amounts: lanes 1 and 6, 2.5 pg, lanes 2 and 7, 5 pg; lanes 3 and 8, 10 pg; lanes 4 and 9, 15 pg; and lanes 5 and 10, 20 pg. The results are representative of 3 experiments. For details of analysis and synthesis of internal standard c-*fos* mRNA see Materials and Methods.

PCR consisted of 28 cycles at an annealing temperature of 55°C. Nucleotides were denatured at 99°C for 1 min in each cycle except the first, in which it was extended to 5 min; extension time was 1 min at 72°C in each cycle except the last, in which it was extended to 5 min; annealing time was 1 min in each cycle. Each set of reactions included controls in which RNA was omitted. In these incubates PCR products were not detected. After the PCR stage an aliquot (6 µl) of each mixture was analysed on a 8% non-denaturing gel. After drying, the gel was visualised on a phosphorimager screen (Molecular Dynamics, Chesham, U.K.). Expression of each RNA was quantitated by volume analysis using a local background with Image Quant 3.3 software (Molecular Dynamics). To calculate absolute RNA concentrations 5 reactions per RNA were performed using in each the same amount of cellular RNA, but increasing amounts of the internal standard RNA. In Figures 2 and 3 the natural logarithm of the ratio of band intensity of cellular RNA over that of internal standard RNA was plotted against the natural logarithm of amount of internal standard RNA. The amount of internal standard and cellular RNA are equal when this ratio is 1. Care was taken to ensure that the data spanned this ratio. Each sample was analysed in triplicate.

Construction of the c-fos *internal standard RNA*

The piece of the c-*fos* gene chosen for RT-PCR assay was the region between bases 1610 (numbered from the adenosine of the translation initiation codon) and 1961. The

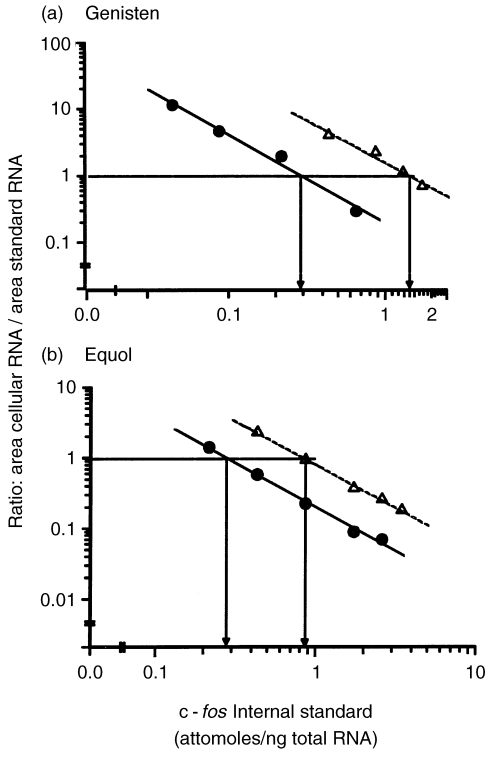

Figure 3. Competitive RT-PCR analysis of c-*fos* mRNA induced by TPA (100 ng/ml) in serum-starved MDA-MB-468 cells in the absence (open triangles) or presence (closed circles) of genistein (a) or equol (b) (both 100 µmol/l). Quantitative evaluation was by phosphoimage analysis. The amounts of internal standard c-*fos* mRNA added were between 2.5 and 20 pg. Results are representative of 3 experiments. For details of analysis and construction of internal standard c-*fos* mRNA see Materials and Methods.

region was amplified using primers 5′-CCGAAGGGAAAG-GAATAAG-3′ (sense strand) and 5′-AAGGGAAGCCACA GACATC-3′ (antisense strand). The internal standard was constructed by duplication of the region between base 1638 (*Pst* I site) and 1821 by PCR of this region using the sense primer described above and an antisense primer spanning bases 1801 to 1820 which had a *Pst* I site added at the 5′ end. Following PCR the product was cut with *Pst* I and inserted into the *Pst* I site of the assay sequence. Thus the internal standard was 74 bp longer than the assay region allowing for inclusion of a second *Pst* I site. The whole construct was contained in the pSP70 vector (Promega) and sequenced using a primer to the SP6 promotor region. The plasmid was linearised using *Cla* I before transcription of sense RNA from the SP6 promotor. Following transcription the size and integrity of the RNA was checked on a denaturing agarose gel and its concentration assessed by determining optical absorbance at 260 nm. The molecular weight of the construct was determined taking into account the additional sequence derived from the vector. The RNA was aliquoted and stored at − 80°C prior to use.

Assay of tyrosine kinase and PKC activities

EGF receptor tyrosine kinase activity was assessed in a MDA-MB-468 cell membrane preparation using the Biotrak EGF-RTK kit (Amersham International plc, Aylesbury, U.K.). The membranes were prepared as described elsewhere [18]. Cytosol for PKC activity measurement was prepared from cells according to Dale and colleagues [19]. Enzyme activity was measured using the PKC kit from Amersham which determines those PKC isoenzymes which are activated by TPA. After enzyme activation by TPA incorporation of the γ-phosphate moiety of ^{32}P ATP into a PKC-specific peptide was measured. Radioactivity was counted by a Packard Tricarb 1500 scintillation counter.

RESULTS

*Effects of isoflavanoids and lignans on EGF- and TPA-induced c-*fos *transcription*

Both EGF and TPA increased c-*fos* mRNA levels measured by competitive RT-PCR from a basal level of 0.026 ± 0.015 attomoles/ng total RNA ($n = 3$) in serum-starved cells to 1.75 ± 0.37 ($n = 7$) and 1.10 ± 0.23 attomoles/ng total RNA ($n = 8$), respectively. These values constitute increases in mRNA levels of 67-fold for EGF and 42-fold for TPA. This elevation is consistent with induction of c-*fos* transcription by EGF and TPA [20]. Figure 2 shows a representative gel (a) and its quantitative evaluation (b) of EGF-induced gene transcription in the absence or presence of genistein. Genistein inhibited EGF-induced c-*fos* transcription, and genistein and equol inhibited TPA-induced gene transcription in a concentration-dependent manner (Figure 3, Table 1). The effect of genistein on the TPA response is consistent with the recent report that it interferes with TPA-induced c-*fos* transcription in mouse skin [21]. Enterodiol and enterolactone at 100 μmol/1 decreased TPA-induced c-*fos* transcription only slightly. None of the lignans affected EGF-induced gene transcription and methyl p-hydroxyphenyllactate did not alter c-*fos* levels after EGF or TPA.

Effects of isoflavanoids and lignans on kinase activities

These results suggested that genistein and equol interfere similarly with events leading to TPA-induced c-*fos* transcription, but differentially with pathways culminating in EGF-stimulated c-*fos* transcription. This difference could result from differential abilities to inhibit tyrosine kinases and/or PKC. Genistein inhibited EGF receptor-linked tyrosine kinase obtained from MDA-MB-468 cells with an IC_{50} of 60 μmol/l (mean of 2 experiments), which is 24-fold higher than the value reported in the literature for enzyme from A431 cell membranes (2.6 μmol/l) [8]. In contrast, equol, enterolactone, enterodiol, NDGA and methyl p-hydroxyphenyllactate (at 100 μmol/l) did not affect EGF receptor tyrosine kinase activity. None of the agents at concentrations of up to 100 μmol/l altered PKC activity.

As equol inhibited TPA-mediated c-*fos* transcription but not PKC, we evaluated our experimental design as to its capacity to discriminate between PKC and tyrosine kinase inhibition. Three well-characterised selective kinase inhibitors, GF 109203X, which is selective for PKC [22] and lavendustin-A and CGP 52411, which are specific for EGF receptor-linked protein tyrosine kinases [23, 24], were investigated. GF 109203X inhibited TPA-induced c-*fos* transcription by 43% ($n = 2$) at 1 μmol/l and by 69 ± 13% ($n = 3$) at 10 μmol/l. It did not affect EGF-mediated c-*fos* transcription at 1 μmol/l and inhibited it only slightly (< 10%) at 10 μmol/l. Lavendustin-A (50 μmol/l) reduced EGF-induced c-*fos* transcription by 23% ($n = 2$), but had no effect on the TPA response. CGP 52411 at 1 μmol/l inhibited TPA-induced c-*fos* transcription by 83% ($n = 2$), but surprisingly not the

Table 1. *Effects of isoflavonoids and lignans on c-*fos *levels induced by EGF or TPA in MDA-MB* 468 *cells*

Compound	Conc. (μM)	% Inhibition of transcription relative to control*			
		EGF		TPA	
Genistein	10	0	(3)†	41	(2)
	50	35 ± 11	(3)	55	(2)
	100	63 ± 6	(3)	75 ± 9	(3)
Equol	50	0	(3)	42 ± 5	(3)
	100	0	(3)	67 ± 18	(3)
Enterolactone	100	0	(3)	11	(2)
Enterodiol	100	0	(3)	18 ± 5	(3)
NDGA	100	0	(2)	0	(2)
Methyl p-hydroxyphenyllactate	100	0	(2)	0	(2)

*Values are the mean of 2, or the mean ± S.D. of 3 experiments. c-*fos* mRNA was determined by RT-PCR analysis. For calculation of c-*fos* mRNA levels 5 different amounts of internal c-*fos* standard were used. For details see Materials and Methods. †Number of experiments in parentheses.

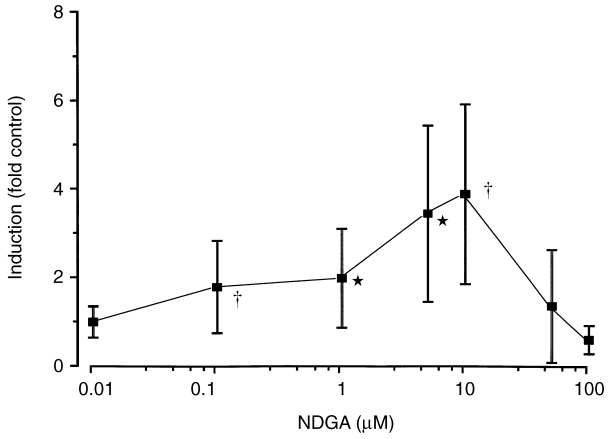

Figure 4. Effect of NDGA on c-*fos* mRNA levels in serum-starved MDA-MB-468 cells. c-*fos* mRNA was analysed by competitive RT-PCR after incubation of quiescent cells with NDGA for 30 min and quantitated by phosphoimage analysis as described under Materials and Methods. *The amount of c-*fos* mRNA measured was significantly higher than that in cells without NDGA $P<0.05$, †$P<0.01$, paired *t*-test. Results are the mean ±S.D. of 3 experiments.

response triggered by EGF. At 10 μmol/l it abolished both TPA- and EGF-induced c-*fos* transcription equally.

*Direct effect of isoflavonoids and lignans on c-*fos *mRNA*

Neither genistein, equol, enterodiol, enterolactone nor methyl p-hydroxyphenyllactate on their own altered c-*fos* mRNA levels, but NDGA did. When serum-starved cells were incubated with 10 μmol/l NDGA, c-*fos* mRNA levels were increased up to 4-fold over control (Figure 4). 100 μmol/l NDGA abolished the stimulatory effect exerted at lower concentrations, whilst EGF- and TPA-induced c-*fos* transcription was not decreased.

Effect of isoflavonoids and lignans on colony formation

All six phenols interfered with the formation of MDA-MB-468 cell colonies (Table 2). NDGA was the most potent inhibitor, with an IC_{50} of 2.6 μmol/l. Of the two isoflavonoids genistein was the more efficient growth inhibitor (IC_{50} 9.6 μmol/l). The lignans were less potent with IC_{50} values of between 20 and 50 μmol/l. The rank order of growth-inhibitory potency was NDGA > genistein ≫ enterodiol > enterolactone > methyl p-hydroxyphenyllactate > equol.

Table 2. Inhibition of colony formation of MDA-MB-468 cells by isoflavonoids and lignans

Compound	IC_{50}★ (μM)
Genistein	9.6
Equol	139.5
NDGA	2.6
Enterolactone	41
Enterodiol	19.8
Methyl p-hydroxyphenyllactate	90.6

★The IC_{50} values were calculated from one experiment, representative of 2, using four different concentrations, each data analysed in triplicate. The S.D. of each data point was < 19%.

DISCUSSION

The results shown above delineate considerable differences between six isoflavonoids and lignans in ability to modulate c-*fos* transcription in MDA-MB-468 cells. These differences can be summarised as follows:

(1) Of these agents only genistein inhibited EGF-mediated c-*fos* transcription.
(2) Both isoflavonoids inhibited TPA-induced c-*fos* transcription with comparable potency.
(3) Enterodiol and enterolactone were only weak inhibitors of TPA-induced c-*fos* transcription.
(4) Methyl p-hydroxyphenyllactate did not affect c-*fos* transcription.
(5) NDGA on its own at low concentrations increased c-*fos* mRNA levels.

Interference by genistein of EGF-induced c-*fos* transcription might reflect events which contribute to its antimitogenic efficacy. The discrepancy between genistein and equol in potency as inhibitors of EGF-induced gene transcription correlates with a 15-fold difference between them in ability to arrest cell colony formation. Whereas genistein has frequently been reported to inhibit the proliferation of a variety of cancer cells when present in the 10^{-5}–10^{-4} molar concentration range [2, 9, 10], information on the growth-arresting properties of equol is scarce. There is an almost 10-fold difference in IC_{50} values for the effects of genistein on MDA cell growth (9.6 μmol/l) on the one side and on EGF-induced c-*fos* transcription (between 50 and 100 μmol/l) on the other, which militates against a causal link between both events. Similarly, the lignans NDGA and enterodiol did not affect EGF-induced c-*fos* transcription, although they were strong growth inhibitors. Therefore interference with pathways other than those stimulated by EGF seem to be primarily responsible for the growth-arresting potential of the isoflavonoids and lignans. Like genistein, NDGA has been shown to inhibit the proliferation of several tumour cell types at concentrations similar to those which inhibited MDA-MB-468 cell growth here [25]. It is a strong inhibitor of lipoxygenase A_2 [26, 27] and this property could conceivably contribute to its antiproliferative effect.

As interception of TPA-stimulated c-*fos* transcription might reflect antitumour promotional activity, our results suggest that genistein and equol might be more potent antipromotors than enterodiol and enterolactone and that NDGA and methyl p-hydroxyphenyllactate lack this type of antipromotional activity. Inhibition of TPA-induced c-*fos* transcription has previously been demonstrated for genistein [21] but not for the other agents studied here. Blockade of growth factor- or tumour promoter-induced c-*fos* transcription is the result of inhibition of one or more steps in the cascade of events initiated by interaction with the EGF receptor or with PKC. EGF receptor-linked tyrosine kinase and PKC, respectively, are early electors in these cascades. Their inhibition is a potential mechanism via which isoflavonoids and lignans might exert effects on c-*fos*. The differential abilities of the kinase inhibitors lavendustin-A, CGP 52144 and GF 109203X to impair c-*fos* transcription suggest that in MDA-MB-468 cells it is controlled by at least two pathways, one involving protein tyrosine kinase and one encompassing PKC. This interpretation is consistent with a previous report in which differential inhibition of growth factor- and TPA-induced c-*fos* transcription by staurosporine and its PKC-specific analogue CGP 41251 was correlated

with differences in the selectivity of kinase inhibition [28]. The inhibition by genistein of EGF-elicited c-*fos* transcription (with an IC_{50} of between 50 and 100 μmol/l) described above is probably related to its ability to inhibit MDA MB-468 cell EGF receptor tyrosine kinase (IC_{50} 60 μmol/l). However there does not appear to be a mechanistic link between genistein-induced inhibition of EGF receptor-linked tyrosine kinase on the one side and cell growth inhibition on the other, which was characterised by an IC_{50} of 9.6 μmol/l. This conclusion is consistent with the recent finding according to which concentrations of genistein which arrested human breast cancer cell growth failed to cause gross inhibition of protein tyrosine phosphorylation [29]. Inhibition of TPA-induced c-*fos* transcription by genistein, equol and, to minor extent, by enterodiol and enterolactone is clearly not the consequence of PKC inhibition. PKC transduces signals via the mitogen-activated protein kinase pathway by activation of raf [30]. Whether genistein and equol inhibit constituents of this pathway remains to be investigated.

NDGA was the most intriguing of the three lignans under study. It is a selective inhibitor of platelet-derived growth factor (PDGF) receptor phosphorylation in Swiss 3T3 cells and of PDGF receptor tyrosine kinase [31]. We show here that NDGA at low concentrations increases c-*fos* mRNA levels, a characteristic not shared by its lignan cogeners enterodiol and enterolactone. This effect of NDGA must be mediated by events downstream of, or distal to, the EGF receptor level, as it did not activate EGF receptor tyrosine kinase or stimulate EGF receptor phosphorylation, nor did it display affinity for the EGF receptor as detected by competition with EGF for binding to the receptor (results not shown). NDGA has recently been shown to increase c-*fos* mRNA levels in human-derived fetal lung fibroblasts and this finding has been postulated to be the corollary of its antioxidant properties [32].

The intake of genistein in ardent soy consumers in Asia has been reported to be approximately 185 μmoles per day [33]. Assuming that this dose is distributed evenly across the whole body, the consequent maximal genistein concentration is unlikely to exceed 3–4 μM [2]. Humans who received 50 mg genistein orally had peak serum concentrations of just below 1 μM [33]. These figures are considerably below the levels which alter c-*fos* transcription. Thus, genistein ingested in the diet is unlikely to elicit this effect. However, certain tissues might be exposed to higher levels of isoflavonoids or lignans than the figures quoted above would indicate, as the polyphenols can accumulate and occur as conjugates inaccessible to analytical determination by gas chromatography—mass spectrometry [34].

The importance of phorbol ester antagonism and cell growth arrest as antipromotional mechanisms needs to be substantiated. Likewise the question has to be addressed, whether genistein, equol, enterolactone and enterodiol exert their phorbol ester antagonism via the same or different mechanisms. The results described above were obtained in only one cell type and, therefore, they have to be interpreted cautiously with reference to chemoprevention. Nevertheless, they hint at important differences between genistein and equol. If ability to antagonise phorbol ester-mediated effects is a pivotal determinant of chemopreventive activity, equol might be of similar chemopreventive efficacy as genistein, whereas enterolactone, enterodiol and NDGA should be much less potent. Alternatively, if phorbol ester antagonism paired with strong antimitogenic activity are prerequisites of optimal chemopreventive activity of this type of agent, genistein would rank high above the other phenols investigated here and would be the most suitable of these agents as candidate for cancer intervention trials.

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