

Review article

Phyto-oestrogens, their mechanism of action: current evidence for a role in breast and prostate cancer

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The incidence of hormone-dependent cancers, such as those of the breast and prostate, is much lower in Eastern countries such as China and Japan in comparison with the Western world. Diet is believed to have a major effect on disease risk and one group of compounds, the phyto-oestrogens, which are consumed in large amounts in Asian populations, have been implicated in cancer protection. This view follows the finding that plasma and urinary levels of phyto-oestrogens are much higher in areas where cancer incidence is low in comparison with areas of high cancer incidence. The phyto-oestrogens are comprised of two main groups; the isoflavones and lignans. Of the isoflavones, genistein and daidzein have been the most widely studied. These compounds have been shown to possess anticancer properties; however their precise mechanism of action remains to be elucidated. In comparison, few studies have investigated the effects of lignans in breast and prostate cancer. *In vitro* studies have shown that genistein exerts biphasic effects on cancer cell growth, stimulating growth at low concentrations ($< 10 \mu\text{M}$) and inhibiting growth at high concentrations ($> 10 \mu\text{M}$), which suggests that low phyto-oestrogen levels may stimulate cancer growth *in vivo*. Plasma phyto-oestrogen concentrations of $> 10 \mu\text{M}$ cannot be achieved by dietary intake and therefore the timing of exposure to phyto-oestrogens may be of the utmost importance in determining their chemopreventive effects. The present paper reviews the effects of phyto-oestrogens on breast and prostate cancer *in vivo* and *in vitro* and discusses possible mechanisms of action via which these compounds may exert their effects.

Phyto-oestrogens: Breast cancer: Prostate cancer

Phyto-oestrogens are naturally occurring hormone-like compounds found in plant foods which have a unique diphenolic structure, providing the compounds with exceptional stability (Adlercreutz & Mazur, 1997). Due to their structural similarity to the human female hormone 17- β oestradiol, phyto-oestrogens have the ability to bind to oestrogen receptors (ER; Setchell, 2001), having a greater affinity for ER β than ER α (Kuiper *et al.* 1998). Phyto-oestrogens can therefore act as oestrogen agonists and antagonists competing for oestradiol at the receptor complex (Bingham *et al.* 1998). However, this is not the only mechanism by which phyto-oestrogens exert their effects; many of which may be unrelated to the oestrogenic properties of these compounds.

There are two main classes of phyto-oestrogens; the isoflavones, found predominantly in soya beans (Reinli & Block, 1996) and the lignans, which are found in a wide variety of foods including flaxseed, cereals, fruits and

berries (Thompson *et al.* 1991). The major glycosides found in soya beans are daidzin, genistin and glycitin. These glucose-conjugated compounds are inactive oestrogenically (Miksicek, 1995) but upon consumption are hydrolysed by mammalian enzymes and the gut microflora to form the active aglycone isoflavone compounds daidzein, genistein and glycitein (Fig. 1; Price & Fenwick, 1985; Setchell & Adlercreutz, 1988; Rowland *et al.* 2003). Daidzein is further metabolised by the intestinal microflora to form the oestrogenic compounds equol (an isoflavan) and *O*-desmethylangolensin. There is a wide individual variation in the levels of excretion of phyto-oestrogen metabolites and only approximately 33% of individuals can convert daidzein to equol (Lampe *et al.* 1998; Rowland *et al.* 2000). The major lignans, which occur in the glycosidic form in foods, are matairesinol and seco-isolaricresinol (Fig. 2). These plant precursors are converted to enterolactone and enterodiol respectively

Abbreviations: AR, androgen receptor; ER, oestrogen receptor; HSD, hydroxysteroid dehydrogenase; LH, luteinising hormone; MMP, matrix metalloproteinase; PSA, prostate-specific antigen; PTK, protein tyrosine kinase; SHBG, sex hormone-binding globulin.

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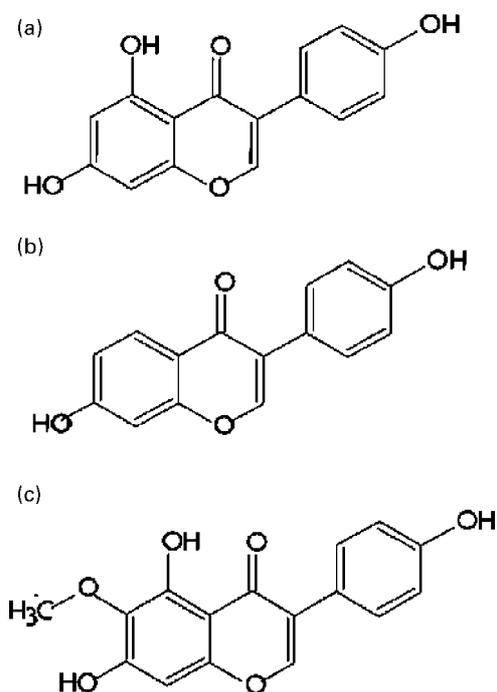


Fig. 1. Structure of the isoflavonoids genistein (a), daidzein (b) and glycitein (c).

by the intestinal bacteria, an action that is inhibited following treatment with antibiotics. Enterodiols can be further converted into enterolactone in the gut (Setchell & Adlercreutz, 1988).

The coumestans comprise another class of phyto-oestrogens, of which coumestrol is the most studied. Coumestrol (Fig. 3), uncommon in the diet, is found in clovers, soya-bean sprouts and in high amounts in mung-bean sprouts (Adlercreutz & Mazur, 1997). This compound has a higher affinity for the ER than the isoflavones, being only ten to twenty times lower than oestradiol (Bingham *et al.* 1998).

As the phyto-oestrogen parent compounds and their metabolites appear to differ in their biological activity and due to the fact that the gut microflora appear to play a crucial role in phyto-oestrogen metabolism (Rowland *et al.* 1999, 2003; Bowey *et al.* 2003), inter-individual metabolism of phyto-oestrogens may have major health implications; for example, for cancer risk.

The oestrogenic effects of phyto-oestrogens first became apparent in the 1940s when sheep became infertile after

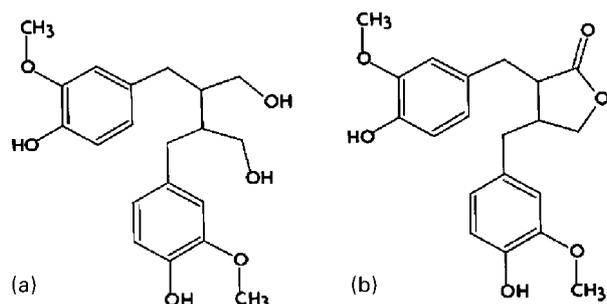


Fig. 2. Structure of the lignans seco-isolariciresinol (a) and matairesinol (b).

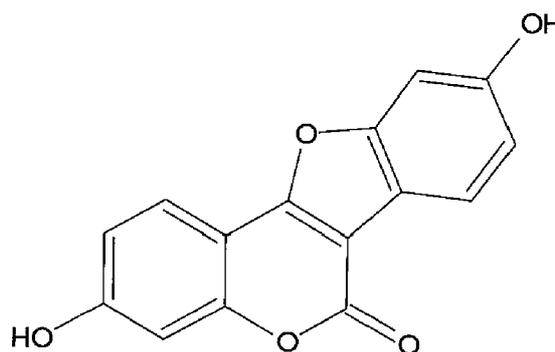


Fig. 3. Structure of the coumestan coumestrol.

grazing on pastures containing clover in Australia (Bennetts *et al.* 1946). This 'clover disease' was later attributed to the rich quantities of formononetin present in the clover which is converted to daidzein in the rumen (Shutt, 1976). Although infertility in captive cheetahs has also been attributed to soya isoflavones in their diet (Setchell *et al.* 1987), no effects of soya intake on fertility have subsequently been reported in commercially bred animals (Barnes, 1998) or in man (Mitchell *et al.* 2001).

The theory that phyto-oestrogens could have a protective effect against cancer due to their similarity in structure to oestrogens was first postulated in the 1980s (Setchell & Adlercreutz, 1988). Subjects with, or at high risk of, hormone-dependent cancers excrete low amounts of isoflavones and lignans (Adlercreutz *et al.* 1982, 1988) whereas subjects living in areas with a low risk excrete high levels (Adlercreutz *et al.* 1986, 1987, 1991, 1992, 1993b, 1995). These findings have prompted a wealth of research into the possible preventive effects of phyto-oestrogens against hormone-dependent cancers. Here we review current findings in relation to the effects of phyto-oestrogens on breast and prostate cancer and discuss possible mechanisms of action by which phyto-oestrogens may exert their effects.

Phyto-oestrogens and breast cancer

Epidemiology

Breast cancer is the most common form of cancer affecting women in the UK with approximately 116 per 100 000 women being affected annually (Ferlay *et al.* 2001). There are a number of hormone-related risk factors for breast cancer; for example, early onset of menarche, late onset of menopause, delayed age of first pregnancy and elevated free oestradiol concentrations in post-menopausal women (Hulka & Moorman, 2001). In addition, environmental factors, especially diet, are also thought to play a major role in cancer risk. This is mainly due to the fact that breast cancer incidence is much higher in Western populations in comparison with Asian populations, a finding which has been associated with the consumption of a traditional low-fat, high-fibre, high-soya diet among Asian populations (Tham *et al.* 1998). Moreover, Asian women living in Asia have approximately 40% lower serum oestrogen levels than Caucasian women living in the USA or Britain (Goldin *et al.* 1986; Key *et al.* 1990).

The protection against breast cancer conferred on Asian women is however lost upon immigration and exposure to Western lifestyles within a few generations (Ziegler *et al.* 1993), further linking lifestyle with breast cancer risk. These studies suggest that early exposure to phyto-oestrogens is extremely important in order to gain from their cancer-preventive effects. Most of the epidemiology and dietary intervention studies have been conducted with soya or flaxseed and not phyto-oestrogens *per se* (Table 1). Results from studies investigating the effects of these compounds on breast cancer risk have however been conflicting. High intakes of soya proteins and total soya products were found to be associated with a decreased risk of breast cancer in premenopausal Singapore Chinese women in a case-control study carried out by Lee *et al.* (1991). Furthermore Wu *et al.* (1996) found increased tofu consumption to be associated with a decreased breast cancer risk in a case-control study of pre- and post-menopausal Asian-American women. In contrast, Yuan *et al.* (1995) found no protective effect against breast cancer with a high intake of soya protein in a case-control study in two Chinese populations. Furthermore, phyto-oestrogen levels consumed by non-Asian US women have been shown to have little effect on breast cancer risk for both pre- and post-menopausal women (Horn-Ross *et al.* 2001). In a soya-feeding (154 mg, SE 8.4 mg, total isoflavones consumed/d) intervention study, circulating levels of 17 β -oestradiol were found to be reduced by 25% in premenopausal women, implicating a protective effect against breast cancer (Lu *et al.* 2000). Flaxseed supplementation significantly increased the urinary 2-hydroxyoestrogen:16 α -hydroxyoestrone ratio in premenopausal women in a cross-over study, implicating a decreased breast cancer risk (Haggans *et al.* 2000). Furthermore, serum enterolactone concentration has been shown to correlate inversely with breast cancer risk in a case-control study in both pre- and post-menopausal Finnish women (Pietinen *et al.* 2001). In a short-term soya intervention study in premenopausal women with breast disease, McMichael-Phillips *et al.* (1998) found a significant correlation between soya intake and Ki67 and thymidine-labelling index in normal breast tissue indicating that

soya may induce the proliferation of breast tissue. These findings were not substantiated when the study was expanded, although apo D levels were significantly lowered and expression of the oestrogen-responsive gene, pS2, was increased in nipple aspirate suggesting that short-term dietary soya may exert a weak oestrogenic effect on the breast (Hargreaves *et al.* 1999).

Animal studies

In animal studies, both soya and purified phyto-oestrogen diets have been fed, thus providing specific information on the activity of phyto-oestrogens. The majority of animal studies have shown that phyto-oestrogens can protect against chemically induced mammary cancer (Table 2). Isoflavones have been implicated as the active components of soya, since protection against tumour formation is not evident when isoflavone-free soya protein is fed (Barnes *et al.* 1990). What has become increasingly apparent is that the time of exposure to the test compound is of the utmost importance. For example, rats treated with genistein neonatally or prepubertally have a longer latency before the appearance of chemically induced mammary tumours and a marked reduction in tumour number, whereas rats treated after 35 d of age have smaller alterations in breast cancer risk (Barnes, 1997). These findings suggest that early exposure to soyabean products is vital in breast cancer prevention and may explain why protection against breast cancer is lost in Asian immigrants after a few generations. Lamartiniere *et al.* (1995) have postulated that genistein may exert its chemopreventive effects in animal models by enhancing mammary cell maturation and lobular-alveolar development, thus reducing cell proliferation in the mammary gland. In contrast Hsieh *et al.* (1998) have demonstrated that dietary genistein (750 parts per million) fed to 28-d-old athymic ovariectomised mice stimulates mammary gland growth without maturation and also stimulates the growth of MCF-7 oestrogen-dependent tumours. The concentration of total plasma genistein (free and conjugated forms) employed by Hsieh *et al.* (1998) following genistein supplementation was 2.1 μ M (0.24 μ M in free form), a concentration which

Table 1. Studies on phyto-oestrogen intake and breast cancer in women

Study type	Population	Diet or compound	Findings	Reference
Case-control	Premenopausal Singapore Chinese women	Soya protein, total, soya products	Decreased risk of breast cancer	Lee <i>et al.</i> (1991)
Case-control	Asian-American women	Tofu	Decreased risk of breast cancer	Wu <i>et al.</i> (1996)
Case-control	Two Chinese populations	Soya protein	No protective effect	Yuan <i>et al.</i> (1995)
Case-control	Finnish women	Enterolactone	Inverse correlation between serum enterolactone and breast cancer	Pietinen <i>et al.</i> (2001)
Cross-over	Premenopausal women	Flaxseed	Increased urinary 2-hydroxyoestrogen:16 α -hydroxyoestrone	Haggans <i>et al.</i> (2000)
Intervention	Premenopausal women	Isoflavones (154 \pm 8.4 mg/d)	Circulating levels 17 β -oestradiol decreased by 25%	Lu <i>et al.</i> (2000)
Intervention	Premenopausal women	Soya (45 mg isoflavones/d)	Increased Ki67, thymidine labelling index	McMichael-Phillips <i>et al.</i> (1998)
			Decreased apo D, increased pS2 (nipple aspirate)	Hargreaves <i>et al.</i> (1999)

Table 2. Effects of flaxseed, soya and soya isoflavones on chemically induced mammary cancer in laboratory animals

Animal	Product tested	Cancer-inducing agent	Finding	Reference
SD rats	Soya protein isolate	DMBA	No effect	Carroll (1975)
SD rats	Soya protein isolate	DMBA	No effect	Hsueh & Park (1990)
SD rats	Soyabean chips, soya protein isolate	MNU, DMBA	Protective	Barnes <i>et al.</i> (1990)
SD rats	Flaxseed flour	DMBA	Protective	Thompson & Serraino (1990), Serraino & Thompson (1992)
Rats	Genistein, daidzein	MNU	Genistein decreased tumour multiplicity	Constantinou <i>et al.</i> (1996)
SD CD rats	Genistein	DMBA	Protective	Murrill <i>et al.</i> (1996)
SD CD rats	Genistein	DMBA	Protective	Fritz <i>et al.</i> (1998)
CD and Crj rats	Soyabeans, miso biochanin A	MNU	Protective	Gotoh <i>et al.</i> (1998)
SD rats	Soya protein isolate	DMBA	Protective	Hakkak <i>et al.</i> (2000)
SD rats	Fermented soya milk	PhIP	Protective	Ohta <i>et al.</i> (2000)
F344 rats	Soyabean protein, soyabean hypocotyls	MNU	Protective	Zaizen <i>et al.</i> (2000)
Rats	SOYSELECT soya extract	DMBA	Slowed tumour development	Gallo <i>et al.</i> (2001)
SD rats	Genistein	DMBA	Protective	Lamartiniere <i>et al.</i> (1998, 2002a); Lamartiniere (2000)
Rats	Daidzein	DMBA	No effect	Lamartiniere <i>et al.</i> (2002b)

SD, Spague–Dawley; DMBA, dimethylbenz[a]anthracene; MNU, *N*-methyl-*N'*-nitrosourea; PhIP, 2-amino-1-methyl-6-phenylimidazo[4,5-*b*]pyridine.

these workers have shown to stimulate MCF-7 cell proliferation *in vitro*. It is therefore clear that both the dose and time of exposure are critical factors in determining the effects of phyto-oestrogens on breast cancer risk.

Although both epidemiological and *in vivo* studies have demonstrated a protective role of phyto-oestrogens against breast cancer, their precise mechanisms of action remain to be elucidated and can only be determined at the cellular level by *in vitro* studies.

In vitro studies

Proliferation. Many *in vitro* studies have examined the effects of phyto-oestrogens on the proliferation of both ER(+) (mainly MCF-7) and ER(−) breast cancer cell lines (Table 3). Genistein has been the most intensively studied compound, probably due to its abundance in soya foods and because it has been shown to have anticancer properties in animal models. Genistein exerts biphasic effects on the proliferation of ER(+) cell lines, stimulating growth at concentrations up to 10 μ M (Wang & Kurzer, 1997; Zava & Duwe, 1997; Hsieh *et al.* 1998; Le Bail *et al.* 2000) and potentially inhibiting cell proliferation > 10 μ M (Peterson & Barnes, 1991; Monti & Sinha, 1994; Pagliacci *et al.* 1994; Wang & Kurzer, 1997; Zava & Duwe, 1997; Le Bail *et al.* 2000). The lignan metabolite enterolactone has also been shown to exert similar biphasic effects on the proliferation of MCF-7 cells (Welshons *et al.* 1987; Mousavi & Adlercreutz, 1992). Phyto-oestrogenic compounds are unable to stimulate the growth of ER(−) cell lines but, as for ER(+) cell lines, inhibit cell proliferation at high concentrations (> 10 μ M). Zava & Duwe (1997) have shown that stimulation of the ER(+) cell lines MCF-7 and T47D by genistein and equol correlates with the binding affinities of these compounds to the ER. These studies suggest differential mechanisms of action for phyto-oestrogens on cell proliferation; at low

concentrations they appear to act via an ER-mediated mechanism whereas at higher concentrations a different mechanism of action is exerted on the cells as both ER(+) and ER(−) cell growth is inhibited. The intracellular phyto-oestrogen concentration following treatment is unknown; however, it is evident that media phyto-oestrogen concentration is important in cell growth response.

Cell cycle and apoptosis. Pagliacci *et al.* (1994) have shown that 10 μ M-genistein causes a reversible G₂/M arrest in MCF-7 cell-cycle progression whereas doses \geq 50 μ M result in a marked fall in S-phase cell percentage associated with a persistent arrest in the G₂/M phase. In addition, exposure of MCF-7 cells to genistein for > 48 h induced apoptosis. Similar effects have been reported by others (Choi *et al.* 1998; Constantinou *et al.* 1998; Hu *et al.* 2001). Genistein also exerts similar effects on the cell cycle of ER(−) MDA-MB-231 and MDA-MB-468 cells (Choi *et al.* 1998; Fioravanti *et al.* 1998; Balabhadra-pathruni *et al.* 2000; Cappelletti *et al.* 2000). Furthermore, genistein blocks G₂/M cell-cycle progression in non-neoplastic human mammary epithelial cells (MCF-10F; Frey *et al.* 2001). G₂/M cell-cycle arrest induced by genistein in breast cancer cells is associated with an increased expression of the cell-cycle inhibitor p21^{WAF1}/CIP1 followed by an increase in apoptosis (Shao *et al.* 1998a; Li *et al.* 1999). Shao *et al.* (1998a) found that these effects were induced similarly in a variety of breast cancer cell lines upon genistein exposure irrespective of the cell line's ER and p53 status, suggesting that genistein's mechanism of action is modulated via ER- and p53-independent mechanisms. Furthermore, the induction of apoptosis induced by genistein in MDA-MB-231 and MDA-MB-435 cells is associated with the up regulation of Bax and p21^{WAF1} and the down regulation of p53 and Bcl-2, an anti-apoptotic protein regulated by the ER (Li *et al.* 1999). Similar effects have been seen following the exposure of MDA-MB-231 cells to oestradiol (Nomoto

Table 3. Effect of phyto-oestrogens on breast cancer cell proliferation *in vitro*

Phyto-oestrogen tested	Concentration	Cell line	Effect on proliferation	Reference
Daidzein and equol	10 ⁻⁸ –10 ⁻⁵ M	MCF-7	↑	Sathyamoorthy & Wang (1997)
Genistein	IC ₅₀ 6.5–12 µg/ml	MCF-7, MCF-7-D-40,	↓	Peterson & Barnes (1991)
daidzein,	IC ₅₀ 20–34 µg/ml	MDA-468		
biochanin A				
Genistein	IC ₅₀ 7–37 µM	MCF-7/WT, MCF-7/ADR ^R , MDA-231	↓	Monti & Sinha (1994)
Genistein	IC ₅₀ 40 µM	MCF-7	↓	Pagliacci <i>et al.</i> (1994)
Enterolactone	0.5–2 µM	MCF-7	↑	Mousavi
	> 10 µM		↓	& Adlercreutz (1992)
Genistein	10–100 nM	MCF-7	↑	Hsieh <i>et al.</i> (1998)
Daidzein, equol,	10 nM–10 µM	MCF-7	↑	Welshons <i>et al.</i> (1987); Schmitt <i>et al.</i> (2001)
O-DMA				
Enterolactone	1–10 µM	MCF-7, T47D	↑	Welshons <i>et al.</i> (1987)
Equol	0.1–1 µM		↑	
Genistein	1 nM–10 µM	MCF-7	↑	Zava & Duwe (1997)
	> 10 µM	MCF-7	↓	
	10 nM–10 µM	T47D	↑	
	20 µM	T47D	↓	
	10 nM–1 µM	MDA-468	Little or no effect	
	> 1 µM	MDA-468	↓	
Genistein, coumestrol,	0.1–10 µM	MCF-7	↑	Wang & Kurzer (1997)
biochanin A, enterolactone	10–100 µM	MDA-MB-231, MCF-7	↓ ↓	

↑, Increased proliferation; ↓, decreased proliferation.

et al. 2002) suggesting that some of the mechanisms of action of phyto-oestrogens may be similar to those of oestradiol. In MCF-7 cells, genistein exposure has been shown to induce apoptosis in conjunction with the expression of both Bcl-2 and Bax (Leung & Wang, 2000). Cells were also found to be under stress following genistein exposure, with *c-jun* N-terminus kinase and stress-activated protein kinase activity being directly proportional to genistein concentration. As the excess Bcl-2 protein may be involved in neutralising the up regulated Bax, the stress pathway may play an important role in inducing cell death since persistent elevated stress-activated protein kinase activity has been linked to apoptosis. Thus, the anti-tumour effects of genistein may be modulated by the compound's ability to arrest two critical points in the control of the cell cycle and by the induction of apoptosis.

Invasion and metastasis. The development of clinical metastasis is a significant cause of morbidity and mortality from cancer. An important step in the process of metastasis is that of tumour invasion and any agent that can inhibit this process may have potential therapeutic value. Using the well-established matrigel invasion assay, genistein has been shown to inhibit the invasion of MCF-7 cells (Shao *et al.* 1998b) and also the ER(–) cell lines MDA-MB-231 and MDA-MB-468 (Shao *et al.* 1998c). This inhibition is independent of ER and p53 status and is characterised by the down regulation of the 92 kDa type IV collagenase matrix metalloproteinase (MMP)-9 and up regulation of tissue inhibitor of metalloproteinase-1. MMP-9 is one of several MMP associated with breast tumour progression (Balduyck *et al.* 2000); for example, high levels of MMP-2 and MMP-11 (stromelysin-3) have been found to correlate with a poor prognosis in breast-cancer patients (Duffy *et al.*

2000). Balduyck *et al.* (2000) have found that MMP-9 is specifically up regulated in MDA-MB-231 cells following cell contact with matrigel and consequently may play a key role in the invasiveness of cells through the basement membrane. Therefore any agent that can inhibit these proteinases would be of value in preventing breast cancer and averting metastasis. Furthermore, genistein has been shown to inhibit the invasion of the murine cell line 410.4 (a highly metastatic subline of BALB/c mammary carcinoma) with an EC₅₀ of approximately 1 µM (Scholar & Toews, 1994). Daidzein was also able to inhibit invasion, yet at a much higher concentration (370 µM). Scholar & Toews (1994) have postulated that the ability of genistein to inhibit tumour cell invasion is due to its potent inhibitory action on tyrosine kinases and have supported this theory with preliminary studies which demonstrate that other tyrosine kinase inhibitors, for example, methyl 2,5-dihydroxycinnamate and herbimycin, also inhibit tumour invasion. The effects of other phyto-oestrogens on mammary cancer cell invasion have not been studied.

Angiogenesis

Tumours require a blood supply to develop and grow. They take over existing blood vessels and stimulate the production of new vessels from these; a process termed angiogenesis. Phyto-oestrogens can inhibit angiogenesis, both *in vitro* and *in vivo*. Fotsis *et al.* (1995) have shown that genistein can inhibit the proliferation and *in vitro* angiogenesis of vascular endothelial cells at half-maximal concentrations of 5 and 150 µM respectively. Subsequently structurally related flavonoids, for example, 3-hydroxyflavone, have been found to potentially inhibit angiogenesis in

tumour cells at half-maximal concentrations, seen in plasma, in the low micromolar range (Fotsis *et al.* 1998). Furthermore the ability of genistein to inhibit capillary formation *in vivo* has been demonstrated in both mouse xenografts of various cancer cells (Shao *et al.* 1998b; Zhou *et al.* 1998) and in animal models of experimentally induced angiogenesis (Hayashi *et al.* 1997; Kruse *et al.* 1997). Shoa *et al.* (1998b) were the first to demonstrate that genistein can inhibit angiogenesis *in vivo* utilising MDA-MB-231 xenografts in nude mice. In rats, genistein, administered as an eye drop (5 mg/ml), prevented extensive neovascularisation of the cornea induced by chemical cauterisation (Hayashi *et al.* 1997). The expression of vascular endothelial growth factor mRNA is increased in breast cancer and vascular endothelial growth factor is also thought to play a key role in promoting tumour angiogenesis (Yoshiji *et al.* 1996). Dabrosin *et al.* (2002) have recently shown that the extracellular vascular endothelial growth factor levels from large tumours in nude mice exposed to a flaxseed diet were reduced in comparison with those of mice fed a basal diet suggesting that lignans also have anti-angiogenic properties. These findings may explain one of the mechanisms of action by which phyto-oestrogens exert their protective effects against cancer metastasis, as the angiogenic process is a key mechanism in tumour growth, progression and metastatic dissemination.

Possible mechanisms of action of phyto-oestrogens in breast cancer

Oestrogen-dependent mechanisms: oestrogen receptor α - and oestrogen receptor β -mediated mechanisms. Phyto-oestrogens appear to exert biphasic effects on breast cancer cell growth *in vitro* implicating differential mechanisms of action. As highlighted earlier (p. 516), at low concentrations (1–10 μM), cell proliferation is stimulated in ER(+) cell lines only, suggesting that the phyto-oestrogens are acting via the ER. This idea is strengthened by the finding that phyto-oestrogens can induce pS2 expression in MCF-7 cells. Both the ER α and ER β forms could be involved in the stimulation of cell proliferation by phyto-oestrogens. At low concentrations genistein and quercetin have been shown to be full agonists for ER α as well as ER β (Maggiolini *et al.* 2001). It is possible therefore that phyto-oestrogens may exert their effects at the cellular level via a similar mechanism of action to that of oestradiol. It is thought that the binding of oestradiol to the ER results in a conformational change, which enables binding of the oestradiol-ER complex to the oestrogen response element on the DNA. Activation of the oestrogen response element may induce the expression of the growth-related proto-oncogene *c-fos* (Burg *et al.* 1989), forming a *c-fos*-*c-jun* heterodimer that activates the activation protein-1 site, leading to cell proliferation (Wang & Kurzer, 1997). In contrast, at higher concentrations (>10 μM) cell growth is inhibited in both ER(+) and ER(-) cell lines demonstrating that growth inhibition is not mediated via the ER but instead possibly via anti-tyrosine phosphorylation and/or via inhibition of cell-cycle progression. Wang *et al.* (1996), finding that tamoxifen can inhibit pS2 expression induced by genistein

at high concentrations, suggest that the ER mechanism may still be active at high phyto-oestrogen concentrations. These findings are difficult to relate to human studies, as the concentration required for the inhibition of breast cancer cell proliferation *in vitro* would rarely be achieved *in vivo*. Serum phyto-oestrogen levels can be >5 μM in adults consuming a high-soya diet (Morton *et al.* 1994; Xu *et al.* 1994, 1995), or in infants fed exclusively with soya-based formula (Setchell *et al.* 1997). Plasma genistein levels in Japanese men may exceed 2 μM (Adlercreutz *et al.* 1993b). These levels would appear to be tumour-growth stimulatory; however, this is not consistent with the apparent protective effect of the high-soya Asian diet and results should be interpreted with caution, as the concentrations of phyto-oestrogens found in tissues are unknown.

Oestrogen-dependent mechanisms: effects on endogenous hormones and growth factors. The intracellular concentration of oestradiol plays a key role in regulating hormone action and a further mechanism of action via which phyto-oestrogens may influence cell behaviour is by altering the metabolism and availability of endogenous oestrogens, which drive the growth, and development, of hormone-dependent tumours. Enzymes involved in the metabolism and biosynthesis of oestrogen are shown in Fig. 4. Phyto-oestrogens can potentially inhibit sulfotransferases that sulfate both oestrogenic steroids and dietary pro-carcinogens (Kirk *et al.* 2001), thus preventing their activation. In post-menopausal breast tumours, circulating steroid sulfates are thought to provide a major source of oestradiol. Phyto-oestrogens have been shown to inhibit some of the key enzymes involved in oestrogen biosynthesis and metabolism such as aromatase, 17 β - and 3 β -hydroxysteroid dehydrogenase (HSD). Flavones that are hydroxylated at the 7-position on the A ring have been found to be the most potent aromatase inhibitors (Kirk *et al.* 2001); however, lignans can also exert similar effects. Enterolactone (Adlercreutz *et al.* 1993a) and enterodiol, as well as coumestrol (Wang *et al.* 1994), can inhibit aromatase activity *in vitro* in human pre-adipocytes and it has been suggested that these compounds may compete with the natural substrate androstenedione for the enzyme. Coumestrol and apigenin are the most potent inhibitors of 17 β -HSD activity (IC₅₀ 0.2, 0.3 μM) having the ability to inhibit the reduction of oestrone to 17 β -oestradiol by this enzyme which is expressed in both normal and malignant breast tissue (Makela *et al.* 1995). Genistein, daidzein and biochanin A have also been found to inhibit 17 β -HSD activity, though at higher concentrations, i.e. IC₅₀ 1–10 μM . These isoflavones can also inhibit 3 β -HSD activity, but at a higher concentration than is required for 17 β -HSD inhibition (Le Bail *et al.* 2000). The phyto-oestrogen concentration required for inhibition of these enzymes may be achieved physiologically from a phyto-oestrogen-enriched diet and therefore these mechanisms of action may play a key role in the chemopreventive properties of these compounds.

Observational studies have led to the suggestion that phyto-oestrogens may be able to reduce the proportion of free oestrogens circulating in the plasma by stimulating sex hormone-binding globulin (SHBG) levels (Adlercreutz *et al.* 1987). Intervention studies, however, have shown no

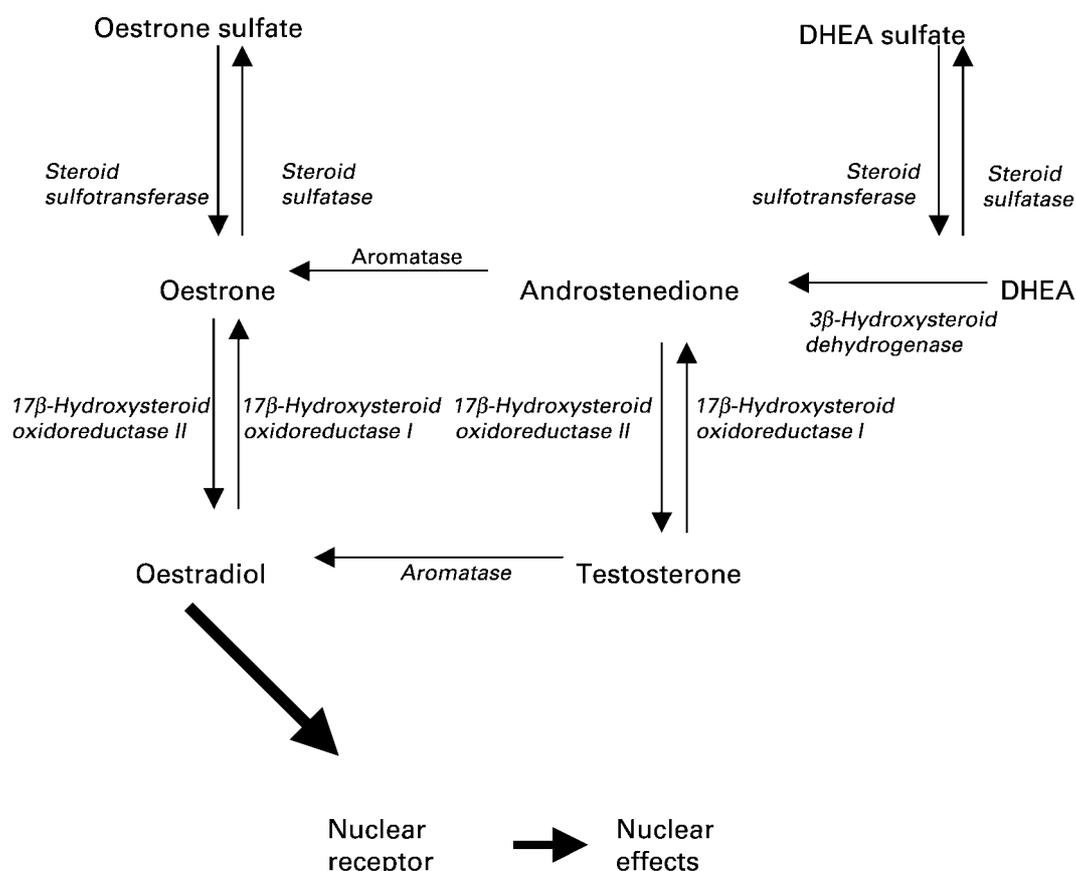


Fig. 4. Enzymes involved in oestrogen biosynthesis and metabolism. DHEA, dehydroepiandrosterone. (Adapted from Kirk *et al.* 2001.)

effect of soya beans on SHBG levels in pre- or post-menopausal women (Cassidy *et al.* 1994, 1995; Baird *et al.* 1995; Petrakis *et al.* 1996). Similarly no effects were evident in premenopausal women receiving lignans (Phipps *et al.* 1993). Furthermore, in a case-control study Murkies *et al.* (2000) found no differences between SHBG levels in post-menopausal breast-cancer patients in comparison with controls.

Other theories as to the mechanism of action of phyto-oestrogens *in vivo* include those concerning their effects on hormone levels. Cassidy *et al.* (1994) found that a soya-protein diet (45 mg isoflavones/d) administered to premenopausal women for 1 month increased the length of the follicular phase of the menstrual cycle and/or delayed menstruation. In addition, it was reported that mid-cycle surges of luteinising hormone (LH) and follicle-stimulating hormone were suppressed. These results suggest that phyto-oestrogens present in soya may protect against breast cancer since an increase in menstrual cycle length would result in a decreased lifetime exposure to oestrogens. Additionally, a longer follicular phase would be protective as the mitotic activity of the breast is thought to be four times higher during the luteal phase in comparison with the follicular phase (Treolar *et al.* 1970; Ferguson & Anderson, 1981). These findings have been supported by others (Kumar *et al.* 2002); however, Maskarinec *et al.* (2002) found no significant effect on hormone levels in premenopausal women following supplementation with

isoflavones (100 mg/d) alone, suggesting that other components in soya may contribute to these effects.

An alternative mechanism for growth inhibition by genistein was postulated by Kim *et al.* (1998) who suggested that genistein had the ability to modulate transforming growth factor β -1-signalling pathways *in vitro*. These findings were strengthened by preliminary studies in patients with hereditary haemorrhagic telangiectasia, a genetic disorder involving mutations in proteins that regulate transforming growth factor β receptor complex formation and signalling. Following 1 week of consuming soya-based beverages, several patients experienced a dramatic attenuation of their symptoms.

Oestrogen-dependent mechanisms: pS2 expression. pS2 is a 6.7 kDa protein secreted by MCF-7 cells in response to oestrogens (Prud'homme *et al.* 1990) and therefore serves as a marker for oestrogen-like activity. Genistein (1–50 μ M) has been shown to increase pS2 levels in the growth medium of MCF-7 cells (Zava & Duwe, 1997; Hsieh *et al.* 1998), as has daidzein, equol (1 μ M) (Sathyamoorthy *et al.* 1994; Zava & Duwe, 1997) and enterolactone (1 μ M). Equol was 100-fold more oestrogenic than its parent compound daidzein in stimulating pS2 mRNA expression. Enterolactone (1 μ M) induced a weak response whereas enterodiol did not exert any effect (Sathyamoorthy *et al.* 1994). Several studies have shown that the anti-oestrogen tamoxifen inhibits pS2 expression stimulated by genistein, daidzein and equol (Wang *et al.*

1996; Sathyamoorthy & Wang, 1997), suggesting that these compounds are stimulating pS2 expression via an ER-mediated mechanism. Genistein has also been found to promote the transcription of another oestrogen-regulated gene, cathepsin-D, in MCF-7 cells (Fioravanti *et al.* 1998; Miodini *et al.* 1999), a finding which may have implications for the effects of genistein on tumour promotion since the overexpression of cathepsin-D has been implicated in invasion and metastasis in breast cancer (Rocheffort, 1998).

Oestrogen-independent mechanisms: protein tyrosine kinase and topo-isomerase II inhibition. In addition to their actions as partial oestrogen agonists or antagonists (Kuiper *et al.* 1997; Barkhem *et al.* 1998), many different mechanisms of action have been proposed to explain the chemopreventive effects of phyto-oestrogens. Genistein in particular exerts a wide range of biological activities suggesting that its mechanism of chemoprevention is pleiotropic in nature. Genistein is a potent inhibitor of protein tyrosine kinase (PTK) activity (Akiyama *et al.* 1987), especially that of the epidermal growth factor receptor. Permanently increased levels of tyrosine phosphorylation are implicated in many cancers, with approximately 50% of known oncogenes encoding for either membrane-bound receptors with tyrosine kinase activity or intracellular proteins undergoing or catalysing tyrosine phosphorylation (Polkowski & Mazurek, 2000). It is improbable, however, that this is a major mechanism behind the chemopreventive effects of genistein since the inhibition of PTK in whole cells *in vitro* is only apparent at relatively high genistein concentrations and it has been reported that in several cell systems in which genistein inhibits growth, no effect on tyrosine phosphorylation was found (Kim *et al.* 1998). Furthermore, daidzein, which is not a PTK inhibitor (Scholar & Toews, 1994), exerts similar effects to genistein in many studies. Genistein has also been shown to inhibit topo-isomerase II (Markovits *et al.* 1989), the nuclear enzyme responsible for maintaining DNA structure. Upon genistein exposure DNA strand breakage is induced, which may explain why cultured cells undergo apoptosis and growth inhibition and differentiation. Interestingly some of the most potent anti-tumour agents currently in use are topo-isomerase II inhibitors; for example, anthracyclines and epipodophyllotoxins.

Oestrogen-independent mechanisms: free radical scavengers. Genistein and flavonoids such as quercetin can also act as free radical scavengers and thus prevent DNA damage (Wei *et al.* 1993), with genistein having the ability to stimulate several antioxidant enzymes such as catalase, superoxide dismutase, glutathione peroxidase and reductase (Adlercreutz, 2002). Sierens *et al.* (2001) have reported that genistein and equol offer protection against H₂O₂-induced DNA damage in human lymphocytes at physiological concentrations, as assessed using the Comet assay. Furthermore, this protection was greater than that offered by 17 β -oestradiol, tamoxifen and the known antioxidant vitamins ascorbic acid and α -tocopherol. Wiseman *et al.* (2000) have shown that soya phyto-oestrogens prevent lipid oxidation *in vivo* in human subjects. Other workers, however, have postulated that the circulating levels of phyto-oestrogens in the

blood are too low to significantly contribute to the removal of free radicals from the human body (Beatty *et al.* 2000).

Oestrogen-independent mechanisms: metastasis. The anti-invasive properties of genistein appear to be mediated via the transcriptional down regulation of MMP-9 and by the up regulation of tissue inhibitor of metalloproteinase-1. Shao *et al.* (1998b) have demonstrated that genistein (20 μ g/ml) causes a reduced ratio of *c-jun:c-fos* proteins in MCF-7 and MDA-MB-231 cells, due to an increase in *c-fos* expression. They have postulated that an excess of *c-fos* could interfere with *jun-jun* homodimers and *jun-fos* heterodimer formation accounting for the reduced activation protein-1-mediated transcription of MMP-9. Furthermore, genistein was also found to regulate the expression of these molecules *in vivo* in MCF-7 and MDA-MB-231 xenografts. Strong differences in MMP expression and release have been found between highly invasive breast cancer cell lines in comparison with only slightly invasive cells (Balduyck *et al.* 2000), highlighting the importance of MMP expression in the metastatic process. Further investigations into the effects of phyto-oestrogens on MMP are therefore needed.

Phyto-oestrogens and prostate cancer

Epidemiology

After lung cancer, prostate cancer is the most common form of cancer affecting men in the UK with approximately 74 per 100 000 being affected annually (Ferlay *et al.* 2001). Although the incidence of latent and non-infiltrative prostate carcinomas is similar in the Western world in comparison with Asian populations, prostate cancer mortality is profoundly higher in the West (Yatani *et al.* 1982). As with breast cancer, environmental factors, especially diet, have been implicated in disease risk (Giovannucci *et al.* 1995; Clinton & Giovannucci, 1998; Kolonel *et al.* 1999; Cohen *et al.* 2000); for example, in a case-control study conducted in China, Lee *et al.* (1998) reported that dietary fat intake was associated with an increased risk for prostate cancer. The importance of environmental influences on cancer risk is further emphasised by migration studies; for example, the age-adjusted incidence rate of prostate cancer in Japanese men living in Hawaii is approximately ten times that in Japan (Severson *et al.* 1989). Other risk factors associated with prostate cancer include age, race, polymorphic repeats in the androgen receptor (AR) gene (Stanford *et al.* 1997) and high circulating levels of androgens and insulin-like growth factor (Signorello *et al.* 1999).

Isoflavonoid levels have been found to be higher in men living in Eastern countries, where prostate cancer mortality is low, in comparison with those living in the West; for example, plasma isoflavonoid levels in Japanese men have been shown to be 7–110 times higher than those of Finnish men (Adlercreutz *et al.* 1993b). In addition, lignan levels in the prostatic fluid in populations at low risk of prostate cancer are higher in comparison with those populations at increased risk (Morton *et al.* 1997). However, Stattin *et al.* (2002) found no protective effect of high circulating enterolactone levels against prostate cancer in a Nordic nested case-control study. Only one

study has specifically investigated the effects of isoflavones on prostate cancer risk. Strom *et al.* (1999) reported an inverse association between daidzein and coumestrol intake and prostate cancer risk in a case-control study in American men. Genistein also showed a slightly protective effect.

In a prospective study in Japanese men living in Hawaii, tofu (derived from soya beans) consumption was associated with a decreased risk of prostate cancer (Severson *et al.* 1989). An additional prospective study in Californian Adventist men showed that the frequent consumption of soya milk was associated with a 70% reduction of the risk of prostate cancer (Jacobsen *et al.* 1998). In contrast Urban *et al.* (2001) found no protective effect of soya beverages in lowering levels of the serum prostate cancer biomarker, prostate-specific antigen (PSA), in elderly men. The exposure period, however, was short (6 weeks), which may explain why no significant decreases in PSA levels were found. Probably the most promising study has been conducted by Hussain *et al.* (2002). Their pilot study demonstrated that isoflavone supplements (100 mg twice daily for a minimum of 3 months) decreased the linear rise in PSA levels in men with treated but uncontrolled prostate cancer (both hormone-sensitive and hormone-refractory). The potential chemoprotective properties of phyto-oestrogens have been highlighted in a case reported by Stephens (1997). A 66-year-old male, with an elevated PSA level of 13.1 µg/l, self-administered a phyto-oestrogen supplement (160 mg), derived from red clover, daily for 1 week before radical prostatectomy for moderately high-grade adenocarcinoma. The resected specimen showed prominent apoptosis suggestive of tumour regression. Furthermore, no adverse side effects were reported.

No investigations into the direct effects of lignans on prostate cancer have been conducted. However, a cohort study of Adventist men (Mills *et al.* 1989) and a few case-control studies (Graham *et al.* 1983; Ewings & Bowie, 1996; Key *et al.* 1997) have identified the consumption of lignan-rich foods such as lentils, beans and peas as associated with a decreased risk of prostate cancer. Furthermore, a pilot study has suggested that a flaxseed-supplemented, fat-restricted diet may protect against prostate cancer (Demark-Wahnefried *et al.* 2001).

Evidence is emerging of the beneficial role of soya in prostate cancer (Messina, 2003); however, few human studies have investigated the specific effects of phyto-oestrogens on prostate cancer risk and although isoflavone and lignan intake would appear to protect against prostate cancer (Table 4), a firm link remains to be established.

Animal studies

Studies in animal models of prostate cancer have also demonstrated a protective effect of phyto-oestrogens against prostate cancer development. Onozawa *et al.* (1999) investigated the effects of a diet containing an isoflavone mixture (genistein, 74%; daidzein, 21%) on the development of adenocarcinomas in the prostate and seminal vesicles of F344 rats induced by 3,2'-dimethyl-4-aminobiphenyl and testosterone propionate. Rats fed a diet containing the soya-bean isoflavone mixture (100 and 400 parts per million) developed significantly fewer adenocarcinomas than controls (Fig. 5). In Lobund-Wistar rats, the spontaneous development of prostate-seminal vesicle complex cancers were significantly prevented in rats consuming a soya protein isolate-isoflavone diet (Pollard & Wolter, 2000).

Table 4. Studies on phyto-oestrogen intake and prostate cancer in human subjects

Study type	Population	Diet or compound	Findings	Reference
Prospective	7999 Japanese men	Rice, tofu	Decreased risk of prostate cancer	Severson <i>et al.</i> (1989)
Prospective	225 Adventist men with prostate cancer	Soya milk	Decreased risk of prostate cancer	Jacobsen <i>et al.</i> (1998)
Pilot	Twenty-five prostate cancer patients awaiting prostatectomy	Flaxseed-supplemented, (30 g/d) fat-restricted diet	Decreased total serum cholesterol, total testosterone and free androgen index, decreased proliferative rates, increased apoptosis	Demark-Wahnefried <i>et al.</i> (2001)
Nested case-control	794 men with prostate cancer, 2550 controls	Serum enterolactone	No protective effect	Stattin <i>et al.</i> (2002)
Case-control	Eighty-three prostate cancer patients and 107 controls	Coumestrol, daidzein, genistein	Inversely associated with prostate cancer risk	Strom <i>et al.</i> (1999)
Randomised double-blind	Thirty-four men >55 years old with PSA >4 ng/ml	Soya beverages	Decreased serum cholesterol, no effect on serum PSA and p105erB-2	Urban <i>et al.</i> (2001)
	Male with high-grade, adenocarcinoma	160 mg Phyto-oestrogen tablets daily for 1 week	Increased apoptosis	Stephens (1997)

PSA, prostate-specific antigen.

Dietary soya has also been shown to play a protective role against the development of prostatitis in rats (Sharma *et al.* 1992). In a transgenic mouse model of prostate cancer (TRAMP), the percentage of mice that developed adenocarcinomas was reduced in a dose-dependent manner in those mice fed a diet supplemented with genistein (0, 250 and 500 mg/kg) in comparison with controls (Mentor-Marcel *et al.* 2001).

Genistin and daidzin have been shown to reduce the incidence of ventral prostate carcinomas in F344 rats induced by 3,2'-dimethyl-4-aminobiphenyl (Kato *et al.* 2000). However, invasive carcinomas which developed in the anterior prostate and seminal vesicles with testosterone propionate were unaffected by the isoflavones suggesting that phyto-oestrogens offer protection only during the early stages of prostate cancer development. This theory is supported by the work of Bylund *et al.* (2000) who reported that in mice, in which LNCaP cells were transplanted subcutaneously, feeding a rye-bran or soya-protein diet decreased tumour development in comparison with those mice fed control diets. Similar findings have been reported by others (Landstrom *et al.* 1998). In the study of Bylund *et al.* (2000), tumours that grew to a palpable size were smaller and secreted less PSA than in control groups. Once tumours became palpable however, their growth rates were similar in all groups, suggesting that the beneficial effects of consuming a rye or soya diet are exerted during the early stages of tumour development.

In rats, genistein failed to inhibit significantly the growth of subcutaneously implanted MAT-LyLu cells, a hormone-refractory prostate cancer cell line (Naik *et al.* 1994). It is improbable that phyto-oestrogens only protect against androgen-responsive prostate cancer as Kato *et al.* (2000) have shown that genistein and daidzein inhibit the growth of the androgen-independent rat prostate cell line PLS10 *in vitro*; however, a few rat prostate studies have implicated the involvement of endogenous hormones and receptors in response to phyto-oestrogens. Fritz *et al.* (2002) have demonstrated that genistein administered in the diet of Sprague-Dawley rats (from conception or in adults) down regulated the expression of the AR, ER- α

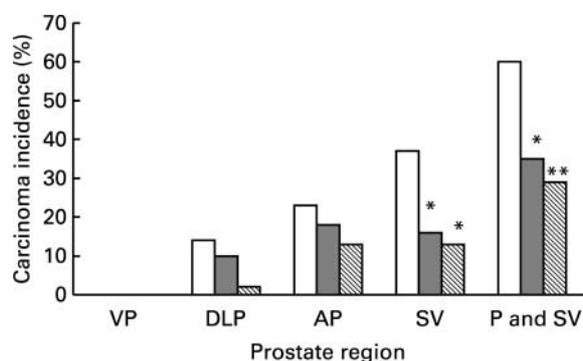


Fig. 5. Effects of dietary soyabean isoflavone mixture on carcinoma incidence in the prostate (P) and seminal vesicles (SV) of F344 rats. (□), Control diet; (■), 100 parts per million isoflavone diet; (▨), 400 parts per million isoflavone diet; VP, ventral prostate; DLP, dorso-lateral prostate; AP, anterior prostate. Incidence of carcinoma was significantly different to that in the control group: * $P < 0.05$, ** $P < 0.01$ (χ^2 test). (Adapted from Onozawa *et al.* 1999.)

and ER- β in the dorsolateral prostate at concentrations similar to that of human subjects consuming a soya diet. Furthermore, Weber *et al.* (2001) have shown that plasma testosterone and androstenedione levels were significantly lower in rats fed a phyto-oestrogen-rich diet for 5 weeks in comparison with those rats fed a phyto-oestrogen-free diet. In addition, prostate weight was also reduced. These findings suggest that human consumers eating a phyto-oestrogen-rich diet may be protected against prostate cancer due to the down regulation of sex steroid receptor expression.

In vitro studies

Proliferation. Studies have shown that both isoflavones and lignans inhibit the growth of androgen-dependent and -independent prostate cancer cell lines (Table 5). With the exception of one study (Mitchell *et al.* 2000) however, the concentration required for growth inhibition exceeds that which would be achieved from dietary intake. Unlike the biphasic effects which phyto-oestrogens exert on breast cancer cell growth, most studies have reported the growth inhibition of prostate cancer cells by phyto-oestrogens to be dose dependent. In addition, the presence of the AR does not appear to influence growth inhibition; however phyto-oestrogens may act via this pathway to invoke other effects on prostate cell growth such as stimulation. In LNCaP cells the AR is mutated which relaxes the specificity of the AR and allows other ligands, such as phyto-oestrogens, to bind (Schuurmans *et al.* 1990). Maggiolini *et al.* (2002) have reported that genistein and quercetin can activate the AR mutant T877A in LNCaP cells inducing growth stimulation. At high concentrations, however, the compounds exert a cytotoxic effect on cell growth independent of AR expression. Indeed, most *in vitro* studies show that phyto-oestrogens exert similar effects on androgen-dependent and -independent cell lines (Davis *et al.* 2000; Mitchell *et al.* 2000); for example, Mitchell *et al.* (2000) reported similar effects on growth inhibition exerted by genistein (100 μM) on LNCaP and PC-3 cells. Furthermore, the inhibition of prostate cancer cell growth by genistein *in vitro* appears to occur in an oestrogen-independent manner (Kyle *et al.* 1997). The finding that the inhibition of prostate cancer cell growth occurs regardless of hormone responsiveness is of particular importance when identifying possible chemotherapeutic agents, as a major clinical problem in prostate cancer treatment is the conversion of androgen-sensitive tumours to a hormone-refractory state. Hempstock *et al.* (1998) investigated the effects of combined phyto-oestrogens (biochanin A, genistein, daidzein, genistin and nordihydroguaiaretic acid) on prostate cancer cell proliferation, as this best mimics exposure through diet. No marked synergism among the five compounds was evident in inhibiting cell proliferation; however a synergistic effect on cell metabolic activity was observed.

Genistein (1.25–10 $\mu\text{g/ml}$) has also been found to inhibit the growth of both benign prostatic hypertrophy and prostate cancer tissue in histoculture in a dose-dependent manner as measured by the inhibition of [^3H]thymidine incorporation. Geller *et al.* (1998) obtained specimens from transurethral resection and radical retropubic prostatectomy

Table 5. Effect of phyto-oestrogens on prostate cancer cell proliferation *in vitro*

Phyto-oestrogen tested	Concentration	Cell line	Effect on proliferation	Reference
Genistein	0–50 μM	PC-3, LNCaP, DU-145	↓	Santibanez <i>et al.</i> (1997)
Genistein	IC ₅₀ 35–110 μM	PC-3, DU-145, ND1, LNCaP, AWA31, JCA1	↓ (to an intermediate degree)	Rokhlin & Cohen (1995)
Daidzein, biochanin A, genistin, genistein, nordihydroguaiaretic acid	IC ₅₀ 19– > 100 μM	PC-3, DU-145	↓	Hempstock <i>et al.</i> (1998)
Genistein biochanin A	IC ₅₀ 8–27 $\mu\text{g/ml}$ (serum stimulated) IC ₅₀ 4.3–15 $\mu\text{g/ml}$ (EGF stimulated)	LNCaP, DU-145	↓	Peterson & Barnes (1993)
Genistein, daidzein, coumestrol, equol	0.01–100 μM	PC-3, LNCaP	↓	Mitchell <i>et al.</i> (2000)
Genistein, daidzein, Genistein	3.1–100 μM 0.0001–1 mg/ml	PLS10 MAT-LyLu, PC-3	↓ ↓	Kato <i>et al.</i> (2000) Naik <i>et al.</i> (1994)
Enterolactone	10–100 μM	DU-145, PC-3, LNCaP	↓	Lin <i>et al.</i> (2001)
Enterodiol		PC-3, LNCaP	↓	
Genistein	0.1–50 mM	LNCaP, VeCaP	↓	Davis <i>et al.</i> (2000)

↓, Decreased proliferation; EGF, epidermal growth factor.

and compared genistein-treated histocultures with 5α -dihydrotestosterone-treated controls. [³H]thymidine incorporation in benign prostatic hypertrophy and cancer histocultures from the same specimens were similarly inhibited by genistein.

Few studies have investigated the effects of lignans on prostate cancer. Lin *et al.* (2001) investigated the effects of enterolactone and enterodiol on the growth of PC-3, DU-145 and LNCaP cells. Enterolactone significantly inhibited the growth of all cell lines (IC₅₀ for LNCaP cells, 57 μM) whereas enterodiol, which was a less potent inhibitor, only inhibited PC-3 and LNCaP (IC₅₀ 100 μM) cell growth.

Invasion. To our knowledge only one study has investigated the effects of genistein on the invasive capacity of prostate cancer cell lines *in vitro*. Santibanez *et al.* (1997) found that genistein (30 μM) was able to inhibit (3-fold) the invasion of PC-3 cells through matrigel. This inhibition of invasion did not result as a secondary effect of genistein on cell proliferation. These findings agree with those of Naik *et al.* (1994) who showed that genistein inhibits cell proliferation of the hormone-refractory prostate cell line MAT-LyLu *in vitro*, but had only a modest anti-invasive effect *in vivo*. Rather, Santibanez *et al.* (1997) have postulated that invasion is inhibited via genistein inhibiting the tyrosine phosphorylation of membrane-bound proteins in transformed cells, preventing the formation of invadopodia which permit cellular contact and degradation of the extracellular matrix (Mueller *et al.* 1992).

Effects on prostate-specific antigen production. PSA is a 33 kDa protein produced mainly by the prostatic epithelium and the epithelial lining of the periurethral glands (Polascik *et al.* 1999). Serum PSA is the only accepted biomarker used to detect and monitor prostate cancer. Davis *et al.* (2000) have demonstrated that genistein (0.1–5 μM) decreases PSA secretion in LNCaP cells. Much higher concentrations (10–50 μM), however, are required to inhibit secretion in VeCaP cells, which express PSA in an androgen-independent manner. Therefore the

effects of genistein on PSA expression and prostate cancer cell growth may be regulated via differential mechanisms. The effect of genistein on intracellular PSA protein levels was also investigated and levels correlated with decreased levels of secreted PSA. This reduction in PSA protein expression also correlated with decreased PSA mRNA levels. Furthermore Rosenberg Zand *et al.* (2002) found that seventeen flavonoids, including genistein and biochanin A, inhibited dihydrotestosterone-induced PSA production in PC-3 cells transfected with the human AR cDNA, PC-3(AR)₂. Biochanin A has also been shown to inhibit PSA production in LNCaP cells, a finding associated with the induction of UDP-glucuronyl transferase, an enzyme responsible for metabolising testosterone to inactive products (Sun *et al.* 1998).

Cell-cycle and apoptosis. Shen *et al.* (2000) have reported that genistein, at physiological concentrations (<20 μM), induces a G₁ cell-cycle block in LNCaP cells and increases expression of the cell-cycle regulators p27^{KIP1} and p21^{WAF1} (mRNA and protein). Apoptosis, however, was only induced when genistein was present at high concentrations (>20 μM). Interestingly, expression of the apoptotic regulators *bcl-2* and *bax* were unaffected by genistein (0–40 μM), as assessed by PCR analysis. Davis *et al.* (1998) also reported that genistein (50 μM) increased p21^{WAF1} protein levels in LNCaP cells as determined by Western blotting. Genistein (60 μM) has also been found to arrest the cell cycle at the G₂/M phase in LNCaP cells (Kobayashi *et al.* 2002), a finding which was associated with the suppression of cyclin B expression. Furthermore, genistein induced the cyclin-dependent kinase inhibitor p21 in a p53-independent manner. Zhou *et al.* (1999) also reported that genistein, at 50 μM , but not 10 μM , significantly affected cell-cycle progression by arresting LNCaP cells at the G₂/M phase. Furthermore, genistein (50 μM) induced a 2-fold increase in DNA fragmentation, a marker of apoptosis. Kyle *et al.* (1997) found that genistein (50 μM) induced apoptosis in PC3-M prostate cancer cells without affecting cell-cycle transition.

Furthermore, apoptosis was preceded by the suppression of the PTK, focal adhesion kinase.

Angiogenesis

Zhou *et al.* (1999) have reported that soya phytochemicals exert anti-angiogenic effects on human prostate cancer cells implanted in mice. LNCaP cells were subcutaneously inoculated in severe combined immune deficient (SCID) mice who were then fed either a control diet (containing no isoflavones) or a diet containing various amounts of isoflavones (341–2120 mg/kg diet) for 21 d. An *in vivo* assessment of prostate tumour microvessel density as a biomarker of tumour angiogenesis showed a reduced vascularity in the mice fed soya products. A significant increase in tumour apoptosis was also reported. A more potent effect was demonstrated with a soya phytochemical concentrate (which contained more isoflavones) compared with a soya-protein isolate. Insulin-like growth factor-1 is associated with enhanced angiogenesis (Nakao-Hayashi *et al.* 1992) and is positively associated with prostate cancer risk in man (Chan *et al.* 1998; Wolk *et al.* 1998). In Zhou's study (1999), in mice fed a diet containing 20% soya-protein isolate with 1% soya-phytochemical concentrate, serum insulin-like growth-1 levels were significantly lower than in the control group. These findings suggest that soya isoflavones may, in part, inhibit angiogenesis by reducing circulating concentrations of critical growth factors.

Possible mechanisms of action of phyto-oestrogens in prostate cancer

Many of the mechanisms of action postulated for the role of phyto-oestrogens in prostate cancer are shared with those of breast cancer (see earlier; pp. 518–520) and will therefore not be discussed again. Some differential mechanisms of action of phyto-oestrogens on breast and prostate cancer are however apparent.

Hormone-dependent mechanisms. 5 α -Dihydrotestosterone, the main prostatic androgen, is synthesised by the 5 α -reductase enzyme from testosterone. The prostate is unable to develop, grow or function in its absence (Griffiths *et al.* 1991). It has been postulated that phyto-oestrogens may offer protection against prostate cancer due to their effects on androgen activity. Information on the effects of phyto-oestrogens on male hormone levels is however limited.

In a randomised cross-over study in which forty-two men consumed 150 g lean meat or 290 g tofu (about 70 mg isoflavones) daily for 4 weeks, Habito *et al.* (2000) found no difference in the blood levels of oestradiol, testosterone, dihydrotestosterone and androstenediol glucuronide between the two diets. A slight reduction in androgen activity was, however, reported following the consumption of tofu. Nagata *et al.* (2001) performed a parallel-arm study in which thirty-four men consumed soya milk (about 48 mg isoflavones) daily for 2 months. Although oestrone levels tended to decrease in the soya-milk group, no differences in the blood levels of oestradiol, total and free testosterone and SHBG were found. Furthermore, Allen *et al.* (2001) found no association between soya-milk intake and the serum concentrations of testosterone, free testosterone,

androstenediol glucuronide, SHBG or LH in British men. These findings were supported by those of Mitchell *et al.* (2001) who reported that an isoflavone supplement (40 mg/d) did not affect serum levels of testosterone, follicle-stimulating hormone, LH or oestradiol. One cross-sectional study has investigated the relationship between soya-product intake and reproductive hormones in Japanese men (Nagata *et al.* 2000). An inverse association was found between soya-food consumption and serum oestradiol concentrations. Furthermore, significant associations were reported for serum oestrone and total testosterone levels.

In a pilot study in men with prostate cancer awaiting prostatectomy, a low-fat (<20% energy), flaxseed-supplemented (30 g/d) diet resulted in a significant decrease in total testosterone and free androgen index levels (Demark-Wahnefried *et al.* 2001). Total serum cholesterol levels were also significantly reduced. In rats fed an isoflavone-rich diet (600 μ g/g), plasma levels of testosterone and androstenedione were significantly reduced compared with animals fed a phyto-oestrogen-free diet. No significant differences were, however, found between plasma LH and oestradiol levels or prostate 5 α -reductase enzyme activity. In contrast Evans *et al.* (1995) demonstrated that biochanin A, genistein and enterolactone inhibited 5 α -reductase activity in prostate tissue homogenates. These compounds were also found to be potent inhibitors of 17 β -HSD isoenzyme activity which converts testosterone to androstenedione (see Fig. 4). Sun *et al.* (1998) reported that biochanin A significantly decreased testosterone-stimulated PSA release in LNCaP cells, possibly due to an increase in UDP-glucuronosyltransferase activity, resulting in the stimulation of the intracellular glucuronidation of testosterone.

In rats, exposure to dietary genistein (25–1000 mg/kg diet) from conception resulted in the down regulation of the AR and ER- α and ER- β mRNA expression in the dorsolateral prostate in a dose-dependent manner (Fritz *et al.* 2002). Furthermore, AR, ER- α and ER- β mRNA expression were reduced in adult rats who were fed genistein (250 or 1000 mg/kg diet) for 2 weeks. These findings suggest that the lower incidence of prostate cancer in populations consuming high levels of dietary phyto-oestrogens may in part be due to the down regulation of sex steroid receptor expression.

Insufficient studies have been performed to allow a conclusion to be reached as to the effects of phyto-oestrogens on hormones involved in prostate cancer. Results from current studies are inconsistent, although the possibility that phyto-oestrogens may exert protective effects on prostate cancer partly by altering hormone activity cannot be ruled out.

Hormone-independent mechanisms. Phyto-oestrogens appear to exert their effects on prostate cell growth via differing mechanisms of action; for example, Mitchell *et al.* (2000) reported that genistein (<10 μ M) induced DNA strand breakage in LNCaP and PC-3 cells whereas daidzein, which like genistein inhibited the growth of LNCaP and PC-3 cells, had no effect on DNA damage at concentrations up to 500 μ M. Zhou *et al.* (1999) also reported that daidzein exerted a less potent effect on the inhibition of the proliferation of LNCaP and DU145 cells

than genistein. Hempstock *et al.* (1998) have implicated that growth inhibition may occur via a cytostatic mechanism of action rather than a cytotoxic mechanism due to their findings that phyto-oestrogens had a more marked effect on the growth inhibition of four prostate cell lines (including two derived from normal epithelium) than on the inhibition of cell metabolic activity. Peterson & Barnes (1993) have reported that although genistein and biochanin A inhibited the growth of both serum and epidermal growth factor-stimulated LNCaP and DU-145 cells, neither compound (10 µg/ml) inhibited tyrosine autophosphorylation of the epidermal growth factor receptor. Therefore the mechanism of action of biochanin A and genistein is not dependent on the inhibition of epidermal growth factor receptor activation. Rather, these authors have postulated that inhibition is dependent on a more distal event in the epidermal growth factor receptor-mediated signal transduction cascade. Indeed genistein has been shown to inhibit other cytoplasmic tyrosine kinases such as src (Akiyama *et al.* 1987) and p21^{bc_r-ab₁} (Honma *et al.* 1990), demonstrating that genistein can inhibit tyrosine phosphorylation events distal to membrane-bound growth factor receptors.

Apoptosis is only induced in prostate cancer cells when phyto-oestrogens are present at supraphysiological concentrations (> 20 µM). A variety of factors appear to contribute to the induction of apoptosis by phyto-oestrogens in prostate cancer; for example, genistein induced apoptosis in LNCaP and PC-3 cells by inactivating the transcription factor, NF-κB (Davis *et al.* 1999) whereas Kyle *et al.* (1997) reported a suppression of the PTK, focal adhesion kinase, before apoptosis. Davis *et al.* (1999) also reported that genistein had the ability to abrogate NF-κB activation by DNA-damaging agents (H₂O₂, TNF-α).

p21^{WAF1} Protein expression has been associated with prostate cancer cell proliferation and patient survival (Aaltomaa *et al.* 1999). The findings of Shen *et al.* (2000) and Davis *et al.* (1998) that genistein can induce p21^{WAF1} expression in prostate cancer cells therefore supports genistein's potential role as a chemopreventive agent.

Bergan *et al.* (1996) have demonstrated that genistein can increase prostate cancer cell adhesion to solid matrix supports at low micromolar levels. Thus genistein could protect against prostate cancer by increasing cell adhesion, thereby preventing cellular detachment, a significant step in the process of metastasis.

Hormone-independent mechanisms: microarray analysis. The effects of phyto-oestrogens on gene expression in prostate cancer can be assessed using cDNA microarray analysis. Of the few studies that have been conducted using this relatively novel technique, it is evident that phyto-oestrogens affect the expression of genes involved in tumour biology and this method may provide an invaluable insight into the effects of phyto-oestrogens at the molecular level. Li & Sarkar (2002) have investigated the effects of genistein on the expression of 12 558 known genes in PC-3 prostate cancer cells. In total, 832 genes were affected by genistein treatment. Of the genes implicated in tumour angiogenesis, invasion and metastasis, eleven were found to be down regulated and two were up regulated. Biochanin A has also been shown to alter gene expression in LNCaP cells (Rice *et al.* 2002).

Following treatment, twenty-nine genes were down regulated and eleven were up regulated suggesting that multiple pathways of action are involved. Furthermore, genistein down regulated many genes in LNCaP cells including apoptosis inhibitor (survivin), DNA topo-isomerase II, cell division cycle 6 and mitogen-activated protein kinase 6 (Suzuki *et al.* 2002). The glutathione peroxidase-1 gene expression level was the most up regulated implicating its involvement in the mechanism of action of genistein in prostate cancer cells.

Conclusion

What is apparent from phyto-oestrogen research is that these compounds do not appear to act via a single mechanism of action; rather their effects are pleiotropic in nature. Structurally related phyto-oestrogens have discrete target sites and mechanisms of action in inhibiting the growth of cancer cells and phyto-oestrogens with similar structures may not produce an identical biological response. It is therefore important to assess phyto-oestrogens on an individual basis in addition to investigating their synergistic effects, as exposure to multiple phyto-oestrogenic compounds would occur following the consumption of a phyto-oestrogen-enriched diet. A further factor complicating the assessment of the role of phyto-oestrogens in cancer aetiology is the different biological activities of various metabolites and the individual variation in metabolic profile.

Genistein has been the most studied compound to date, probably due to its well-documented chemopreventive properties; however it is evident that many of the other iso-flavones and lignans possess similar anticancer properties and so deserve further study.

It is extremely difficult to draw any definite conclusions as to the effects of phyto-oestrogens on breast and prostate cancer risk. Low levels of phyto-oestrogenic compounds would appear to exert oestrogenic effects, thus stimulating the growth of cancer cells, and perhaps tumours already undergoing treatment. This is evident from a study demonstrating that low-dose genistein was able to inhibit the therapeutic effects of tamoxifen in a post-menopausal model of breast cancer (Jones *et al.* 2002). Furthermore, the high levels of phyto-oestrogens needed to inhibit cancer cell growth would rarely be achieved in the UK diet. Some of the serum phyto-oestrogen levels reported for Japanese men and women would also appear to be tumour stimulatory suggesting that the timing of exposure to phyto-oestrogenic compounds may be of the utmost importance. The low incidence of breast and prostate cancer in Asian populations may be attributed to their lifetime exposure to phyto-oestrogens. Furthermore, phyto-oestrogen concentrations in cell-culture media, to a certain extent, would mimic those found in the plasma. It is probable that cells in media have better access to phyto-oestrogens in the culture media in comparison with *in vivo* cells in plasma. This would suggest that even higher plasma levels would be required to inhibit breast tumour cell growth *in vivo*. In addition, tissue phyto-oestrogen concentrations are unknown and may differ markedly between Asian and Western populations. Inter-individual

variation in gut microflora may also be implicated in cancer risk as evidenced by the inability of certain individuals to produce active phyto-oestrogenic metabolites.

It is also possible that phyto-oestrogens are acting in synergy with other compounds to exert their overall effects. For example, there are many other components of soya, in addition to isoflavones, which may contribute to an anticancer effect: a low content of methionine, and a high content of phytic acid, saponins and sterols.

Clearly more research is needed before a definite conclusion concerning the chemoprotective properties of phyto-oestrogens can be drawn. With developing technology such as DNA microarrays and proteomics the precise mechanisms of action and target sites of phyto-oestrogens, along with their specific role in tumour development, may be elucidated.

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