Phytoestrogens and breast cancer – promoters or protectors?

Suman Rice and Saffron A Whitehead

Division of Basic Medical Sciences, St George’s University of London, Cranmer Terrace, London SW17 0RE, UK
(Request for offprints should be addressed to S A Whitehead; Email: saffron@sgul.ac.uk)

Abstract

The majority of breast cancers are oestrogen dependent and in postmenopausal women the supply of oestrogens in breast tissue is derived from the peripheral conversion of circulating androgens. There is, however, a paradox concerning the epidemiology of breast cancer and the dietary intake of phytoestrogens that bind weakly to oestrogen receptors and initiate oestrogen-dependent transcription. In Eastern countries, such as Japan, the incidence of breast cancer is approximately one-third that of Western countries whilst their high dietary intake of phytoestrogens, mainly in the form of soy products, can produce circulating levels of phytoestrogens that are known experimentally to have oestrogenic effects. Indeed, their weak oestrogenicity has been used to advantage by herbalist medicine to promote soy products as a natural alternative to conventional hormone replacement therapy (HRT). Such usage could increase in light of recent evidence that long-term HRT usage may be associated with an increased risk of breast cancer with a consequent reduction in prescription rates. So, are phytoestrogens safe as a natural alternative to HRT and could they be promoters or protectors of breast cancer? If they are promoters, then we must assume that it is due to their oestrogenic effect. If they are protectors, then other actions of phytoestrogens, including their ability to inhibit enzymes that are responsible for converting androgens and weak oestrogens into oestradiol, must be considered. This paper addresses these questions by reviewing the actions of phytoestrogens on oestrogen receptors and key enzymes that convert androgens to oestrogens in relation to the growth of breast cancer cells. In addition, it compares the experimental and epidemiological evidence pertinent to the potential beneficial or harmful effects of phytoestrogens in relation to the incidence/progression of breast cancer and their efficacy as natural alternatives to conventional HRT.

Introduction

Breast cancer is not a disease of the modern world and its oldest recorded description was found in an Egyptian seven papyri written between 3000 and 1500 BC. Although the term ‘cancer’ did not exist at that time (the origin of the word being credited to Hippocrates, 460–370 BC), the ‘Edwin Smith’ papyrus described details of eight cases of tumors or ulcers of the breasts that are consistent with modern descriptions of breast cancer (Diamondopoulous 1996). Little is known about the epidemiology of breast cancer until 20th century although, interestingly, in 1713 an Italian doctor and a founder of occupational medicine, Bernardino Ramazzini, reported the virtual absence of cervical cancer and relatively high incidence of breast cancer in nuns (Gallucci 1985). He (prophetically) speculated whether this was in some way related to their celibate life-style. Today, the epidemiology of breast cancer is closely monitored and in both industrialized and developing countries the incidence of breast cancer is steadily rising. It is the commonest form of cancer among women in the US and almost all of Europe and in the US the incidence of breast cancer amongst caucasians rose from 100 cases per 100 000 women in 1983 to over 130 in 2002 (National Cancer Institute, www.cancer.gov/cancertopics/types/breast). The corresponding figures for women in England were under 80 in 1983 to 120 in 2003 (National Statistics online www.statistics.gov.uk).

Apart from age (>50) and a family history of breast cancer, many of the known risk factors for
this disease relate to a woman’s reproductive history, such as early menarche, nulliparity or a late pregnancy, and late menopause as well as prolonged oral contraceptive use and hormone replacement therapy (HRT; McPherson et al. 2000). More recently, dietary factors and exposure to endocrine-disrupting chemicals have been built into the list of risk factors although definitive evidence is still lacking (Mitra et al. 2004). That said, high consumption of phytoestrogens, which are very weak mimics of natural oestrogens, are associated with a lower incidence of breast cancer (Limer & Spiers 2004).

**Phytoestrogens, HRT, and breast cancer**

Phytoestrogens are plant-derived chemicals that have oestrogenic activity, combining with oestrogen receptors and initiating oestrogen-dependent transcription (Kuiper et al. 1998). Their affinities for the oestrogen receptor, however, are at least 1000–10 000 times lower than that of oestradiol and this is an important factor when considering dietary intake of these chemicals and their subsequent circulating concentrations.

There are several different groups of phytoestrogens that are classified according to their chemical structure (see Fig. 1). The most widely studied are the isoflavones, present in high concentrations in soy products and red clover, followed by the flavones and then coumestans (Table 1). Lignans that are widely distributed in fruits and vegetables have been investigated to a more limited extent, despite the fact that the vegetarian diets have been linked with a reduced incidence of breast cancer. More recently, there has been increasing interest in the stilbene, resveratrol found in grape skins and red wine, and 8-prenylnaringenin present in hops and beer (Table 1).

The outcomes of the Million Women Study and the Women’s Health Initiative showing an increased incidence of breast cancer (Beral 2003, Chlebowski et al. 2003) as well as coronary heart disease, stroke, and venous thromboembolism raised alarms about the use of HRT to treat menopausal women (Hickey et al. 2005). These findings were eagerly reported in the media and, together with the clinical evidence, had a negative impact on HRT prescribing. In fact, within months of these publications, there was a significant decrease in HRT prescriptions, particularly the combined conjugated equine oestrogens and progestagen products, and such declines have been reported in several countries (Lawton et al. 2003, Kim et al. 2005, Low Dog 2005, Usher et al. 2006). This could result in an increased use of ‘natural’ plant-derived alternatives to HRT that contain phytoestrogens. This raises two questions: are ‘natural’ alternatives potential promoters of breast cancer or could they actually protect against the growth of breast cancer?

**Oestrogen synthesis in breast cancer**

The majority of breast cancers (~70%) express oestrogen receptors and in these cases oestrogen is considered to promote tumor growth. Hence, treatments subsequent to surgery, radiotherapy, and/or chemotherapy are directed towards reducing oestrogenic effects within breast tissue (Smith & Chua 2006). The incidence rate of breast cancer continues to increase with age despite the loss of ovarian hormones in postmenopausal women. This apparent paradox has been resolved by the fact that extragonadal sites, including breast, brain, muscle, skin, bone, and adipose tissue can synthesize potent androgens and oestrogens from relatively inactive circulating steroid precursors derived from the adrenal cortex and to a much lesser extent the ovaries (Simpson et al. 2005). Indeed, after the menopause, nearly 100% oestrogens are formed in peripheral tissues (Labrie 2003) and exert their effects locally in a paracrine or intracrine manner.

In breast tissues, enzymes are present to convert inactive circulating precursors, such as androstenedione, dehydroepiandrosterenedione (DHEA) and its sulfated form DHEA-S, and oestrone sulfate (E1S) into biologically active androgens and oestrogens (see Fig. 2).

In fact, postmenopausal breast cancer illustrates the importance of local oestrogen biosynthesis. In postmenopausal women, the concentration of 17β-oestradiol (E2) present in breast tumors is at least 20-fold lower than that of oestradiol in peripheral tissues. This reduction is maintained by the presence of enzymes and substrates that are capable of forming oestrogens from relatively inactive precursors. In this manner, breast tumors can synthesize oestrogens by a local biosynthesis process, which is independent of the systemic production of oestrogens.
higher than that in the circulation, but in premenopausal women with carcinomas, this ratio was only 5 (Nakata et al. 2003, Pasqualini & Chetrite 2005).

**Aromatase**

The aromatase enzyme, CYP19, is a key enzyme in the conversion of androgens to oestrogens and over 60% of breast carcinomas express this enzyme (Lipton et al. 1992, Miller 1991) with higher levels of mRNA expression and activity compared with non-malignant tissue (Bulun et al. 1993, Utsumi et al. 1996, Chetrite et al. 2000). It has been reported that aromatase activity is highest in intratumoral stromal cells rather than the epithelial component in breast cancer (Purohit et al. 1995) and, whilst some immunohistochemical studies have supported this observation, others have shown that aromatase was immunolocalized either in both cells types or predominantly in carcinoma cells (see Suzuki et al. 2005a).

**17β-Hydroxysteroid dehydrogenases (HSDs)**

These enzymes catalyze the interconversion of relatively inactive 17β-keto steroids (e.g. androstenedione and oestrone) and active 17β-hydroxysteroids, such as testosterone and oestradiol. To date, 12 isozymes of 17β-HSD have been cloned, but in breast tissue the important isozymes are 17β-HSD1, 5, and 7 (see Fig. 3). Oxidative 17β-HSD2 activity that converts...
testosterone to androstenedione and oestradiol to oestrone (E$_1$) is predominant in normal breast tissue, but the reductive activity of 17$\beta$-HSD1 that drives the reverse reaction (E$_1$ to E$_2$) is dominant in breast cancers (Spiers et al. 1998, Miettinen et al. 1999). 17$\beta$-HSD1 expression has been immunohistochemically detected in breast carcinoma cells of over 55% patients (Sasano et al. 1996, Suzuki et al. 2000) and in postmenopausal breast cancers 17$\beta$-HSD1, mRNA expression was significantly higher than in premenopausal breast cancers as were the intratumoral E$_2$;E$_1$ ratios (Miyoshi et al. 2001). Together, results suggest that 17$\beta$-HSD1 may play an important role in maintaining high intratumoral oestradiol levels in postmenopausal breast cancers. Interestingly, 17$\beta$-HSD type 12, an isofrom of 17$\beta$-HSD3 that is important in producing testosterone in the testis, has recently been characterized. 17$\beta$-HSD12, like type 1, converts oestrone to oestradiol and recent evidence suggests that this isoform is the major oestrogenic 17$\beta$-HSD enzyme in the ovary (Luu-The et al. 2006). It is also highly expressed in mammary tissue though its potential role in breast cancer has not yet been addressed.

**Sulfatase and sulfotransferase**

Steroid sulfatase (STS) is an enzyme that hydrolyzes several steroids, including E$_1$S and DHEA-S. In breast tissue, it catalyzes the biologically inactive oestrone-3-sulfate to oestrone, which can be further metabolized to oestradiol by 17$\beta$-HSD1. STS activity is detected in the majority of breast cancers and activity has been correlated with the level of STS mRNA and protein expression in breast cancer cells (Suzuki et al. 2003, Pasqualini 2004). This is higher in carcinoma tissue compared with normal breast tissue (Chetrite et al. 2000, Utsumi et al. 2000, Chetrite et al. 2005), and both immunohistochemical studies and real-time reverse transcriptase (RT)-PCR have localized STS expression in carcinoma cells but not in intratumoral stromal cells (see Suzuki et al. 2005b). The importance of E$_1$S and STS in postmenopausal breast cancer is highlighted by the following facts: E$_1$S is the most abundant circulating oestrogen in postmenopausal women, it has a considerably longer half-life in plasma compared with oestrone, levels of E$_1$S in breast tumors are considerably higher than in the plasma, and the activity of STS is 50–200 times that of aromatase (Pasqualini et al. 1996, Pasqualini & Chetrite 2005). STS mRNA expression is negatively correlated with the clinical outcome of breast cancer patients (Utsumi et al. 2000, Miyoshi et al. 2003) and it has been proposed that the sulfatase pathway is more important than the aromatase route, since aromatase mRNA expression has no diagnostic value (Reed et al. 2005).

Oestrogen sulfotransferase (EST, SULT1E1) that converts oestrogens to inactive oestrogen sulfates is present in both normal and cancerous breast tissue, and EST immunoreactivity was detected in the carcinoma cells of 44% patients with breast cancer (Suzuki et al. 2003). Despite the fact that the concentration of E$_1$S
Phytoestrogens as enzyme inhibitors

There is growing evidence that phytoestrogens could have a protective effect on the initiation or progression of breast cancer by inhibiting the local production of oestrogens from circulating precursors in breast tissue. Indeed, in vitro experiments have shown that phytoestrogens inhibit the activity of key steroidogenic enzymes involved in the synthesis of oestradiol from circulating androgens and oestrogen sulfate.

Phytoestrogens and aromatase

Amongst the phytoestrogens, the flavones and flavonones are the most potent inhibitors of aromatase. Early studies showed that apigenin and quercetin were potent inhibitors of aromatase in human placental microsomes and subsequent studies confirmed apigenin as a relatively potent inhibitor of the enzyme, along with chrysin, 7-hydroxyflavone, and hesperetin with IC50s in the order of 0.3–3.0 μM (Le Bail et al. 1998a, Jeong et al. 1999). Isoflavones were inactive (Le Bail et al. 1998a). In the human adrenocortical cell line, H295R, flavones were consistently more potent inhibitors than flavonones with IC50 values for apigenin, 7-hydroxyflavone, and chrysin ranging from 4 to 20 μM. These values, however, were between 100 and 1000 times higher than the IC50 for the steroidal aromatase inhibitor, 4-hydroxyandrostenedione (Sanderson et al. 2004). Apigenin was a potent inhibitor of aromatase activity in primary cultures of human granulosa cells, significant inhibition being observed at 0.1 μM, but only when testosterone was used as the substrate with higher doses being required to inhibit the conversion of androstenedione to oestradiol (Lacey et al. 2005). This could be attributed to the higher affinity of androstenedione compared with testosterone for aromatase (Luu-The et al. 2001).

Overall, the isoflavones have weak inhibitory activity on aromatase in both cell-free and whole-cell preparations (Le Bail et al. 2000, Lacey et al. 2005), although Almstrup et al. (2002) reported that formononetin, biochanin A, and extract of red clover flowers inhibited aromatase activity at low concentrations (<1 μM) but had oestrogenic activity at higher doses. In contrast, genistein has been reported to increase the aromatase activity in H295R cells and isolated rat follicles (Sanderson et al. 2004, Myllymäki et al. 2005), and this was paralleled by an increase in promoter-specific aromatase transcripts (Sanderson et al. 2004).

Similarly, lignans have weak inhibitory activity on aromatase in placental microsomes (Adlercreutz et al. 1993), human pre-adipocytes (Wang et al. 1994), human granulosa luteal cells (Lacey et al. 2005), and MCF-7 cells (Brooks & Thompson 2005) with IC50s being >10 μM, whilst coumestrol inhibited aromatase activity in pre-adipocytes with a Ki value of 1.3 μM (Wang et al. 1994). More recently, Wang et al. (2006) showed that the polyphenol in red wine, resveratrol, inhibited aromatase activity in MCF-7 breast cancer cells stably transfected with CYP19 confirming a previous report that red wine inhibited aromatase activity (Eng et al. 2001). The IC50 of resveratrol was 25 μM and kinetic analysis indicated that both competitive and non-competitive inhibition might be involved. Similarly, flavonoid components of the hop (Humulus lupulus L.) were also shown to inhibit aromatase as well as 20 μl/ml concentrations of different beers and lagers (Monteiro et al. 2006).

The ability of flavones to inhibit aromatase activity rather than the isoflavones has been attributed to the differences in their chemical structure. In a site-directed mutagenesis study, Kao et al. (1998) showed that when the 4'-hydroxyphenol group is attached to C2 (as in the flavones and flavonones), the A and C rings of these phytoestrogens mimic the D and C rings of the androgens substrates. In contrast, when the 4'-hydroxyphenyl group is attached to C3 (as in the isoflavones), there is limited binding with aromatase; although this confirmation increases binding with the oestrogen receptor such that the A and C rings of the phytoestrogen mimic the A and B rings of oestrogen (see Fig. 1).

Phytoestrogens and 17β-HSDs

The isoflavones have been shown to exert inhibitory effects on 17β-HSD type I – the enzyme that converts
oestrone to oestradiol. In both cell-free preparations and T47-D breast cancer cells, genistein and daidzein inhibited the conversion of oestrone to oestradiol between 1 and 10 μM with weaker or no effects of biochanin A (Mäkelä et al. 1995, Le Bail et al. 2000, Brooks & Thompson 2005), whilst in human granulosa luteal cells only high doses of genistein (≥10 μM), but not biochanin A, inhibited 17β-HSD type 1 (Whitehead & Lacey 2003). This contrasts the relatively potent effects of biochanin A on recombinant 17β-HSD type 5 that reduces androstenedione to testosterone (Krazeisen et al. 2001). In MCF-7 and MDA-MB-231 breast cancer cell lines, 100 nM genistein stimulated the oxidation of oestradiol to oestrone and increased the levels of 17β-HSD2, which was confirmed by western blots (Brueggemeier et al. 2001). The flavones have also been reported to inhibit 17β-HSDs and, in this respect, chrysin, apigenin, and naringenin have been shown to inhibit 17β-HSD1 at micromolar doses whilst quercetin was without effect (Mäkelä et al. 1995, Le Bail et al. 2000, Whitehead & Lacey 2003). In contrast, quercetin was a relatively potent inhibitor of 17β-HSD type 5 (Krazeisen et al. 2001). Coumestrol has been reported to inhibit both 17β-HSD types 1 and 5 in purified enzyme preparations (Mäkelä et al. 1995, Krazeisen et al. 2001) and more recent studies on MCF-7 cells showed that both enterolactone and enterodiol inhibited the conversion of oestrone to oestradiol with significant inhibition observed at 10 and 1 μM respectively (Brooks & Thompson 2005).

Thus, there is limited data on the effects of phytoestrogens on the reductive and oxidative reactions of 17β-HSDs, although these may be very important in the generation of oestradiol in oestrogen-producing tissues. In both human granulosa cells and breast cancer cells, the production of oestradiol is several fold higher when androstenedione is used as a substrate compared with testosterone and the conversion of oestrone to oestradiol, which requires only 17β-HSD type 1, is higher than either the conversion of androstenedione or testosterone to oestradiol (Whitehead & Lacey 2003 and unpublished results). Thus, the activity of 17β-HSDs exceeds that of aromatase and may be more important in the local generation of oestradiol from oestrone and E1S than from androgen substrates.

**Phytoestrogens, oestrone sulfatase and sulfotransferase**

Oestrone sulfatase (ETS) that converts E1S to oestrone may be more important for generating oestradiol in breast tissue compared with the aromatase pathway (Kirk et al. 2001, Reed et al. 2005). Despite this, comparatively few studies have investigated the effects of phytoestrogens on either ETS or oestrogen sulfotransferase (EST/SULT1E1), the latter catalyzing the sulfation of oestrone. Early studies reported that quercetin, genistein, daidzein, and other flavonoids inhibited hepatic ETS, with quercetin being the most potent phytoestrogen (Huang et al. 1997). In contrast, daidzein was reported to have no effect on ETS although sulfoconjugates of this phytoestrogen inhibited ETS (Wong & Keung 1997, Harris et al. 2004). The effects of several dietary flavonoids, including quercetin, genistein, daidzein, and equol, on sulfotransferases have also been investigated and they inhibited hepatic oestrogen/androgen sulfotransferase with IC50s in the nanomolar range (Ghazali & Waring 1999, Mesia-Vela & Kauffman 2003, Harris et al. 2004). Although the reported inhibitory potency of these compounds varied between studies, taken together the evidence shows that phytoestrogens have an overall inhibitory activity on enzymes involved in the interconversion of E1S and oestrone. In addition, dietary flavonoids can be sulfated by several human sulfotransferases, including oestrogen sulfatase (Harris et al. 2004). This sulfation may further influence the bioavailability of endogenous oestrogens and alter the ratio of active oestrogens and inactive oestrogen sulfates.

**Oestrogen receptors and breast cancer**

Two oestrogen receptors (ERs) coded on different genes have been identified – ERα and ERβ – although numerous mRNA splice variants exist for these receptors in both diseased and normal tissue (Moore et al. 1998, Flouriot et al. 2000, Deroo & Korach 2006). Like other steroid receptors, they are transcription factors and have a similar domain structure (Fig. 4). The N-terminal A/B domains are completely distinct between the two receptors, the DNA-binding C domain is highly conserved, whilst the C-terminal E/F domains are similar, sharing about 50% homology. That said, the ligand-binding cavity in the E domain is highly conserved in the two receptors (Kuiper et al. 1996, Mosselman et al. 1996).

There are two activation domains in the oestrogen receptors, an N-terminal activation function (AF-1) and a C-terminal activation function (AF-2) and these regions act synergistically to recruit co-activators or co-repressors and thus regulate gene transcription. The AF-1 region of the receptor is involved in protein–protein interactions (e.g. phosphorylation) and can activate transcriptional activity of target genes in the
absence of oestrogen or other oestrogenic ligands (Onate et al. 1998, Tremblay et al. 1999). Co-activator binding to AF-1 leads to either partial or no activation of ERα, whilst full activation of this receptor requires recruitment of co-activators to both AF-1 and AF-2 regions (Tzukerman et al. 1994, Benecke et al. 2000). This requires binding of oestrogenic ligands (see Bardin et al. 2004, Koehler et al. 2005). The transcriptional activity of AF-1 in ERβ is weak or negligible compared with ERα whilst that of AF-2 is similar in both receptors (Barkhem et al. 1998, Cowley & Parker 1999, Delaunay et al. 2000). Thus, when only AF-2 activity is required, the transcriptional activities of the two receptors are similar but when both AF-1 and AF-2 are active, then the activity of ERα greatly exceeds that of ERβ (McInerney et al. 1998, Cowley & Parker 1999).

In addition to the classical mechanism of activated oestrogen receptors regulating gene transcription through binding to the consensus oestrogen response element (ERE) on the DNA, they can also regulate transcription by binding to other promoter elements on the DNA, such as AP-1-binding sites (Webb et al. 1995), cAMP response element (Sabbah et al. 1999), and SF1-response element (Vanacker et al. 1999) to which other transcription factors bind. Interestingly, there is also some evidence that the potency of the two different ERs on non-ERE-binding sites versus ERE-binding sites differs (Paech et al. 1997, Cowley & Parker 1999). For example, ERβ is more potent overall than ERα on AP-1-binding sites whilst the contrary occurs on EREs.

There is a distinct tissue expression of ERα and ERβ and of their co-regulators (Mueller & Korach 2001) and thus oestrogenic ligands will have different effect in different tissues. In addition, selective oestrogen receptor modulators (SERMs), such as tamoxifen and raloxifen can exhibit tissue-specific oestrogenic activity. Thus, they are both antagonists in breast tissue but agonists in bone, while only raloxifen is a pure antagonist in the uterus (Yamamoto et al. 2003). This is because the binding of a SERM results in
specific conformational change in the receptor and this determines which co-activators or co-repressors are recruited (Shang & Brown 2002). Another factor influencing selectivity of ER ligands is that the specific co-regulators recruited also depend on whether the activated oestrogen receptors bind to the ERE promoter or other transcription-binding sites (Klinge et al. 2004, Koehler et al. 2005). Finally, different ligands to these two receptors will attract different co-activators (McKenna et al. 1999, Moggs & Orphanides 2001). Of these co-activators, steroid receptor co-activator 1 (SRC1) has been widely investigated. Interestingly, studies have shown that genistein binding to ER\(_{β}\) enhanced interactions between SRC1 and SRC3 but had minimal effects on other transcription factors (Wong et al. 2001). Thus, phytoestrogens may not only bind to ERs but may also modulate the recruitment of specific co-activators through which specific gene transcription could be achieved.

A comparison of the relative binding affinities of the two receptor subtypes showed that oestriadiol binds with equal affinity and yet tamoxifen and the pure anti-oestrogen, ICI 164,384, bind with twice the affinity to ER\(_{β}\), diethylstilbestrol with half the affinity, and phytoestrogens with up to five times the affinity for ER\(_{β}\) (Kuiper et al. 1997). However, the two oestrogen receptors may either form homo- or heterodimers and thus their biological activity may depend on the specific dimerization of the receptors.

There are further complications in unraveling the diverse actions of activated oestrogen receptors (Fig. 5). First, growth factor-stimulated protein-kinase signaling pathways can modulate ER transcription by phosphorylating ER or ER-associated co-regulators and affecting subcellular localization of co-factors (Tremblay et al. 1999, Watanabe et al. 2001). Thus, several steroid responses are functionally linked to c-Src or tyrosine kinase receptors that can induce ligand-independent stimulation of steroid receptor-mediated transcription (Shupnik 2004). On the other hand, membrane-associated ERs which may be activated by growth factors, can interact with cell signaling pathways, such as the mitogen-activated protein extracellular kinase/extracellular signal-regulated kinase (MEK/ERK) and phosphatidylinositol

**Figure 5** Activation and interaction of oestrogen receptors with cell-signaling pathways and inhibition by phytoestrogens. Growth factors activate cell-signaling pathways, (1) such as ERks and PI3K/AKT (3). They also activate membrane-associated ERs (2). In turn activated kinases phosphorylate and activate oestrogen receptors (4). Activated cytosolic ERs may also modulate the activity of cell-signaling pathways (dotted lines). Genistein is a potent tyrosine kinase inhibitor and inhibits signal transduction of growth factors. Apigenin inhibits PI3K and further inhibits cell signaling.
kinase (PI3K) pathways and thus initiate non-genomic effects (Razandi et al. 2004, Levin 2005, Marino et al. 2006). Both steroids and growth factors stimulate proliferation of steroid-dependent tumor cells and interaction of these pathways can occur at several levels. This is important in relation to the possible effects of phytoestrogens because although these compounds are all weakly oestrogenic, some are also inhibitors of cell signaling pathways (see below).

Whilst activation of ERα is known to promote growth in breast tumors, the specific functions of ERβ are less well understood, although in cell lines activation of ERβ inhibits cell proliferation. The ratio of ERα:ERβ is increased in carcinogenesis (Rutherford et al. 2000, Skliris et al. 2003) and expression of ERβ was associated with low breast tumor aggressiveness and improved disease-free survival rates compared with those with ERβ-negative tumors. One theory for the effects of ERβ on the growth of breast tumors is that it may dimerize with ERα and silence its activation functions and that loss of ERβ in cancer cells could signal the stage of oestrogen-dependent tumor progression (Hall & McDonnell 1999, Pettersson et al. 2000, Lindberg et al. 2003). This may be significant as a possible protective effect of phytoestrogens on breast cancer since many phytoestrogens have a stronger affinity for ERβ than ERα (Kuiper et al. 1998).

Whilst several variants of the full-length ERα have been identified, as well as a short isoform that lacks the N-terminal AF-1 region (Flouriot et al. 2000), ERβ isoforms are comparatively more abundant and, in human breast tissue, the expression levels of the different isoforms are higher than that of the wild-type ERβ (Saji et al. 2002, Fuqua et al. 2003). The long ERβ1 isoform is now considered to be the wild type (Xu et al. 2003), although the original ERβ clone encoded a shorter protein with a truncated N-terminal region (Kuiper et al. 1996). Therefore, most studies investigating the affinity of phytoestrogens for ERβ were carried out on the short form of this receptor (Kuiper et al. 1998).

The ERβ gene yields other splice variants (numbered 2–5) that encode proteins with different C-terminal amino acids, and in breast tumors total ERβ mRNA is significantly lower than in normal breast tissue (Girault et al. 2004, Park et al. 2006). Several studies have been undertaken to determine whether there is an altered expression pattern of the different isoforms of ERβ – notably ERβ1, ERβ2 (ERβc), and ERβ5 – in breast cancer, but results have not been consistent (Saji et al. 2002, Zhao et al. 2003, Davies et al. 2004, Esslimani-Sahla et al. 2005, Park et al. 2006). All ERβ isoforms can inhibit the transcriptional activity of ERα on ERE-containing reporters, but only human (h)ERβ1 can bind the oestrogen ligand and has transcriptional activity on its own (Peng et al. 2003).

Whilst phytoestrogens induce a stronger transactivation of ERβ compared with ERα (though not compared with oestradiol) and can superstimulate ER activation against a background of oestradiol (Harris et al. 2005), no studies have addressed the interaction of phytoestrogens with the various isoforms of ERβ. A recent study, however, investigated whether or not phytoestrogens could modulate the expression of ERβ isoforms (Cappelletti et al. 2006). In breast cancer cell lines, both oestradiol and genistein upregulated total ERβ, particularly ERβ2, whilst quercetin was without effect. Through modulation of ERβ expression, genistein inhibited oestrogen-induced cell growth – perhaps by dimerizing with and hence silencing the growth-promoting effects of ERα? Therefore, some phytoestrogens may increase the expression of ERβ isoforms and this could be important in limiting the oestrogenic stimulation of growth. Clearly, studies are required to investigate the interaction of phytoestrogens with different ERβ isoforms and their modulation of ERβ expression.

**Phytoestrogens and the growth of breast cancer cells in vitro and in vivo**

**In vitro studies**

The growth of oestrogen-responsive breast cancer cell lines, notably MCF-7 cells, has been used to test the oestrogenicity of various phytoestrogens and xenoestrogens – the E-Screen – which measures metabolically active cells by their ability to cleave the yellow tetrazolium salt, MTT, to purple formazan crystals (Soto et al. 1995). This has subsequently been widely used in conjunction with other assays, such as the luciferase-reporter-gene assay, competitive binding assays, viability studies, DNA synthesis, and induction of oestrogen-responsive genes (Guttendorf & Westendorf 2001).

The growth-promoting effects of genistein and other isoflavones in soy products and extracts of red clover, such as daidzein and biochanin A and a metabolite of daidzein, equol, have been most widely investigated, presumably due to the epidemiological evidence linking high soy diets with a lower incidence of breast cancer (Bouker & Hilakivi-Clarke 2000). Overall, studies have shown that low doses (≤10 µM) of genistein, biochanin A, and daidzein stimulate in vitro growth of MCF-7 and T-47D cells, but not the...

Of the flavones, apigenin and quercetin have been the most widely studied, and reports generally show that both compounds inhibit E\textsubscript{2}-induced DNA synthesis and proliferation of ER-positive and ER-negative breast cancer cells (Wang & Kurzer 1998, Collins-Burow et al. 2000, Yin et al. 2001). There is some debate as to whether these effects are mediated through the ER (van der Woude et al. 2005) or through an ER-independent mechanism (Collins-Burow et al. 2000). Coumestrol, like genistein, has a bi-phasic effect on cell growth with increased proliferation at low doses and anti-proliferative activity at high doses (\(\geq 10\ \mu M\); Wang & Kurzer 1998, Dixon & Shaikh 1999, Schmidt et al. 2005). Lignans have also been investigated for their effects on cell growth. At low doses enterolactone stimulated but at concentrations above 10 \(\mu M\), it inhibited proliferation of MCF-7 cells (Wang & Kurzer 1997, Saarinen et al. 2005).

Resveratrol has been shown to have chemopreventive activity. Jang et al. (1997) reported that resveratrol inhibited a number of events associated with the initiation, promotion, and progression of cancer. Numerous other in \textit{vitro} studies have confirmed these findings (Bhat & Pezzuto 2002, Kim et al. 2004, Le Corre et al. 2005, Lanzilli et al. 2006), although Matsumura et al. (2005) failed to show any inhibitory effect of resveratrol on the growth of MCF-7 cells except in the presence of oestradiol. In contrast, other studies have shown the resveratrol can have a bi-phasic effect on cells growth in both ER\textsubscript{z}-positive and ER\textsubscript{z}-negative cell lines (Basly et al. 2000). Finally, recent studies have shown that the effects of prenylnaringenin on MCF-7 cell growth were similar to genistein, in that low doses (\(10^{-8}-10^{-6}\ M\)) stimulated growth with a sharp decline occurring at \(10^{-5}\ M\) (Matsumura et al. 2005).

The mechanisms through which phytoestrogens may stimulate/inhibit growth of ER-positive breast cancer cells are somewhat controversial, although it is generally assumed that the growth-stimulatory properties of phytoestrogens are mediated through their ability to bind to oestrogen receptors (Matsumura et al. 2005). Such receptor binding may stimulate events in the G\textsubscript{1} to S phase entry in the cell cycle (Foster et al. 2001) or, like oestrogens, inhibit apoptosis (Schmidt et al. 2005). Growth inhibitory effects of high doses of phytoestrogens may involve inhibition of cell-cycle progression, inhibition of growth factor cell signaling pathways (e.g. tyrosine kinase and/or PI3K) or antagonism of endogenous oestrogens. It is the authors’ view that high concentrations of certain phytoestrogens are simply cytotoxic, as has been expressed by others (Maggiolini et al. 2001), since concentrations of certain phytoestrogens, such as genistein and prenylnaringenin show little or no dose inhibitory effects on cell growth with a steep reduction being observed at 10 \(\mu M\) and above (Matsumura et al. 2005).

It is beyond the scope of this review to address the evidence relating to the mechanisms of cell growth/inhibition of breast cancer cells \textit{in vitro}, but it is interesting to note that phytoestrogens bind more strongly to ER\textsubscript{z} than ER\textsubscript{\textbeta} (Kuiper et al. 1998). In a recent study on MCF-7 cells transiently transfected with ER\textsubscript{z} or ER\textsubscript{\textbeta}, only coumestrol and genistein were shown to bind dose-dependently with ER\textsubscript{z} with an EC\textsubscript{50} of \(10^{-6}\) and \(5 \times 10^{-7}\ M\) respectively (Harris et al. 2005). Other phytoestrogens tested showed very weak binding and usually only at the highest doses tested (\(10^{-6}\) and \(10^{-5}\ M\)) such that IC\textsubscript{50}, in the dose range tested, could not be determined. All phytoestrogens investigated including daidzein, equol, apigenin, quercetin, and narigenin bound to ER\textsubscript{\textbeta} with IC\textsubscript{50} in the range of \(10^{-9}-10^{-6}\ M\), genistein being most potent in this respect (\(3 \times 10^{-9}\ M\)). In light of these data, it is tempting to speculate why genistein and coumestrol stimulate cell growth and DNA synthesis (Wang & Kurzer 1998). Unlike other phytoestrogens, they can activate the growth-promoting effects of ER\textsubscript{z}, but the caveat exists that the IC\textsubscript{50}s, for the ER\textsubscript{\textbeta} is 100 times higher than the IC\textsubscript{50}s for the ER\textsubscript{\textbeta} and thus difficult to reconcile with the evidence that activation of the ER\textsubscript{\textbeta} inhibits growth and might silence ER\textsubscript{z} activation through dimerization (see above). However, the inability of apigenin and quercetin to bind to ER\textsubscript{\textbeta}, except at very high doses, could explain why these flavones only exert growth-inhibitory effects (Miodini et al. 1999, Yin et al. 2001) although other cellular mechanisms may exist (see below). In this respect, it is interesting to note that resveratrol binds with comparable affinity to both receptor subtypes, though with 7000-fold lower affinity than oestradiol (Bowers et al. 2000), and, whilst the growth-promoting activity of resveratrol at low doses appears to be dependent on ER\textsubscript{\textbeta}, growth-inhibitory effects may not be mediated by antagonizing this receptor (Basly et al. 2000).

\textbf{In \textit{vivo} studies}

The effects of phytoestrogens on mammary tumors \textit{in vivo} have been investigated in chemically induced
(7,12-dimethylbenz(a)anthracene (DMBA) or N-methyl-N-nitrosomethylurea (NMU)) models of mammary carcinogenesis, nude mice xenografted with breast cancer cell lines and more recently in mouse mammary tumour virus (MMTV)-wt-erbB-2/neu transgenic mice. Genistein has been the most widely investigated phytoestrogen, presumably because of the use of soy extracts as an alternative to conventional HRT and because of the comparatively low incidence of breast cancer in Eastern countries, such as Japan and China, where high concentrations of dietary soy products are consumed.

Both genistein and soy protein have been shown to dose-dependently stimulate growth of implanted MCF-7 xenografts (Hsieh et al. 1998, Allred et al. 2001), whilst a more recent study reported that a soy extract did not stimulate tumor growth in either MCF-7 or MDA-MB-231 xenografts and, when used in association with 17β-oestradiol, it displayed anti-oestrogenic activity (Gallo et al. 2006). In contrast, Shao et al. (1998) reported that genistein inhibited growth of these ER-positive and ER-negative xenografts and stimulated apoptosis. Similar anti-tumor effects have also been seen in chemically induced mammary carcinogenesis (Barnes et al. 1990, Lamartiniere et al. 1998, Constantinou et al. 2001) and nude mice (Constantinou et al. 1998), although Santell et al. (2000) found no growth-inhibitory effects of genistein in vivo, even when relatively high circulating plasma concentrations were achieved. In MMTV/neu transgenic mice, there were no significant differences in the tumor latency between low- and high-dose isoflavone diets nor in the percentage of disease-free mice at 60 weeks. As anticipated, tamoxifen inhibited tumor growth but tamoxifen in conjunction with a low (but not high) dose isoflavone diet significantly reduced the preventative effect of this oestrogen antagonist (Liu et al. 2005). In reviewing the evidence of genistein on breast cancer growth in vivo, De Lemos (2001) concluded that this phytoestrogen stimulated growth at low concentrations, but at high concentrations inhibited tumor growth. Few in vivo studies have been carried out on other phytoestrogens although anti-tumor effects of apigenin, quercetin, and enterolactone have been reported (Caltagirone et al. 2000, Chen et al. 2004, Saarinen et al. 2005).

Animal models have also been used to look at the cancer chemoprotective effects of resveratrol. Resveratrol increased tumor latency and reduced the number of tumors in an NMU-induced mammary cancer model (Bhat et al. 2001), and more recently long-term administration of resveratrol (since birth) was shown to increase the differentiation of lobular structures in the mammary gland and reduce proliferative activity, whilst reducing numbers and increasing the latency of DMBA-induced tumors (Whitsett et al. 2006). Along the same lines, resveratrol reduced tumor growth and angiogenesis but increased apoptosis in ERα negative/ERβ positive MDA-MB-231 xenografts in vivo (Garvin et al. 2006).

**Human studies**

Several studies have investigated the effects of soy consumption on cell proliferation or biomarkers of cell proliferation in breast tissue. Hargreaves et al. (1999) investigated the effect of 60 g/day soy (48 mg total isoflavones) administered for 14 days on breast tissue of premenopausal women. Whilst there was no effect on oestrogen receptor status, proliferation, apoptosis, or mitosis in breast epithelial cells, the levels of apolipoprotein D were significantly lowered and expression of the oestrogen-responsive gene pS2 was increased in nipple aspirate (P ≤ 0.002), suggesting a weak oestrogenic effect.

In another study, a dietary supplement of 45 mg total isoflavones/day for 14 days significantly increased the thymidine-labeling index and immunocytochemical staining of Ki67 (both biomarkers of cell proliferation) in biopsies of normal breast tissue obtained from women previously diagnosed with either benign or malignant breast disease (McMichael-Phillips et al. 1998). These data suggest that short-term dietary soy supplementation can induce proliferation in breast tissue of premenopausal women with breast disease. In contrast, soy consumption has been associated with a reduction of mammographic density (Atkinson & Bingham 2002, Jakes et al. 2002), whilst Maskarinec & Meng (2001) reported a positive correlation between self-reported soy food intakes and percentage breast density in women living in Hawaii. Increased mammographic density has been associated with a four- to sixfold increased risk of breast cancer (Atkinson et al. 1999). Recent studies on cynomolgus monkeys showed that 12-month dietary soy supplements (equivalent to 129 mg/day) had no effect on the premenopausal breast (Wood et al. 2006a), whilst high doses of soy isoflavones (240 mg/day) selectively antagonized oestrogen-induced changes in the postmenopausal primate breast (Wood et al. 2006b). Taken together, the overall evidence shows no consistent effects of dietary phytoestrogens on indicators of cell proliferation in normal human breast tissue, although phytoestrogens may increase proliferation in existing breast cancer.

**Other actions of phytoestrogens**

Whilst this review has focused on the direct action of phytoestrogens on oestrogen synthesis and...
oestrogen receptors in relation to breast cancer, other actions have been ascribed to these compounds that may act independently of the ER or indirectly impinge on ER signaling. Phytoestrogens have been shown to increase the levels of human sex hormone binding globulin that will consequently reduce the concentration of circulating free ‘active’ hormones (Brezinski et al. 1997, Adlercreutz et al. 1998, Berrino et al. 2001). Such an effect could be significant in the progression of breast cancer although local synthesis is considered more important than circulating concentrations of hormones. Highly reactive oxygen have been shown to play a role in the development of cancer and several studies have shown that phytoestrogens can act as anti-oxidants, although the concentrations at which anti-oxidant activity is observed are unlikely to be reached through dietary means (Wei et al. 1995, Arora et al. 1998, Harper et al. 1999, Wilson et al. 2002).

Some phytoestrogens are inhibitors of cell-signaling pathways. For example, genistein is an inhibitor of protein tyrosine kinase (Akiyama et al. 1987), whilst apigenin and quercetin are inhibitors of the phosphatidylinositol kinase (PI3K) pathway (Nguyen et al. 2004). Resveratrol has been reported to inhibit Src tyrosine kinase and blocks Stat 3 activation in malignant cells (Kotha et al. 2006). Both MEK/ERK 1/2 and PI3K/AKT pathways can be activated by growth factors and thus phytoestrogens may modulate such control of breast tumor growth (Limer & Speirs 2004, Weldon et al. 2005).

In addition, activated oestrogen receptors have also been shown to interact with these cell-signaling pathways (Moggs & Orphanides 2001, Bardin et al. 2004), and a recent study has shown that resveratrol modulated the PI3K pathway through an ERα-dependent mechanism (Pozo-Guisado et al. 2004). Therefore, the cellular actions of phytoestrogens may be complex. Although some kinetic studies show that phytoestrogens may bind competitively with steroid substrates to steroidogenic enzymes, other evidence shows they can alter enzyme expression (Whitehead & Rice 2006). Indeed, recent real-time RT-PCR studies in our laboratory have shown that certain phytoestrogens and low-dose mixtures of phytoestrogens are potent inhibitors of aromatase expression (Rice et al. 2006). Therefore, further experiments are required to elucidate the actions of phytoestrogens on cell-signaling pathways rather than their ability to bind to oestrogen receptors.

Epidemiology of breast cancer in relation to dietary phytoestrogens

In the early 1980s, a possible link between isoflavones and lignans and the prevention of breast cancer was noted and this led to numerous studies to evaluate this hypothesis.

The epidemiology of breast cancer in relation to dietary intake of phytoestrogens has been adequately reviewed (Adlercreutz 2003, Ziegler 2004) and the current opinion, mainly based on studies of immigrant populations, suggests that early exposure to relatively high concentrations of soy phytoestrogens may have a protective effect on breast cancer in later life (Adlercreutz 2003). Animal studies support this proposal (Lamartiniere 2000) as does a recent study in Japanese women (Shu et al. 2001). However, results from epidemiologic studies are mixed, even from studies in Asian populations (Yamamoto et al. 2003).

Evidence relating to a high dietary intake of lignans, particularly in vegetarian diets, and the incidence of breast cancer is more controversial (Adlercreutz 2003). Some studies have shown a modest reduction in breast cancer in women with a high intake of lignans (McCann et al. 2004, Ziegler 2004), whilst another study showed that higher enterolactone excretion was associated with a non-significant increase in breast cancer risk (den Tonkelaar et al. 2001). In contrast, it was reported that both low (below the 12.5th percentile) and high (above the 87.5th percentile) plasma enterolactone concentrations were associated with an increased incidence of breast cancer (Hulten et al. 2002). It should be noted that the metabolism of plant lignans to mammalian lignans in the gut is dependent on diet. An increase in dietary fat decreased urinary excretion of lignans whilst whole grain fiber increased the production of enterolactone (see Adlercreutz 2003).

There is still no conclusive evidence that a high dietary intake of phytoestrogens and the reduced incidence of breast cancer are directly related or whether phytoestrogens are simply biomarkers of a healthy diet and lifestyle. Despite the evidence that some phytoestrogens at low doses can promote the growth of breast cancer cell lines and increase biomarkers of cellular proliferation in human breast cells after a short-term diet rich in phytoestrogens, current evidence would suggest that a high dietary intake of phytoestrogens does not increase the risk of breast cancer.
Efficacy of phytoestrogens as alternatives to HRT

Many botanical preparations are sold to alleviate menopausal symptoms, but the most widely used are extracts of soy or red clover that contain isoflavones or black cohosh (BC; Actaea racemosa; Wuttke et al. 2003, Beck et al. 2005). The latter, containing triterpene glycosides and aromatic acids, is not strictly a phytoestrogen, and whilst the ‘active’ components of extracts of BC have not yet been identified, total extracts of BC do not bind to oestrogen receptors or show direct classical oestrogenic effects (Viereck et al. 2005).

Hot flushes/flashes and night sweats are the most frequent symptoms of the menopause and the most common reason for women to seek symptomatic relief. Therefore, most studies investigating the efficacy of phytoestrogens as alternatives to HRT have specifically focused on the incidence, frequency, and intensity of hot flushes. Unfortunately, most studies to date have been on a small scale and of short-term duration and the majority was not randomized, double-blind or even placebo-controlled trials (Low Dog 2005, Speeroff 2005). The topic has been adequately reviewed and overall evidence shows that phytoestrogen extracts of soy and red clover have little or no effect; at best only 10% reduction of symptoms beyond that of the placebo effect (Kurzer 2003, Geller & Studee 2005). Some studies have shown that black cohosh reduced symptoms, primarily hot flushes and mood disorders (Geller & Studee 2005, Viereck et al. 2005), but recent studies have shown no significant effects of this extract on hot flushes (Newton et al. 2005, Verhoeven et al. 2005).

Conclusion

Equating epidemiological evidence with experimental evidence is fraught with problems. Epidemiological evidence suggests that exposure to relatively high concentrations of phytoestrogens during development or early life may be important in programming an individual’s risk of developing cancer in late life. This could explain why the reduced risk of certain cancers observed among migrants increases with subsequent generations. Alternatively, this may simply reflect changing life-styles and dietary habits unrelated to phytoestrogen intake.

The question of dietary intake versus experimental doses at which phytoestrogens may exert effects is an important consideration. Studies have shown that the average daily intake of phytoestrogens in some Eastern populations is around 30 mg/day compared with Western societies in which the dietary intake is <10 mg/day or much lower. Reported circulating concentrations of various phytoestrogens range from nanomolar to low micromolar concentrations and yet, with the exception of in vitro studies on the growth of ER-positive breast cancer cell lines, most experiments have shown that relatively high concentrations of phytoestrogens, in the micromolar range, are required to exert any pharmacological effects, i.e. at concentrations higher than those likely to be achieved as a result of dietary exposure. It is not known, however, whether or not phytoestrogens accumulate in tissues and whether they accumulate in their conjugated forms, as they mainly exist in the circulation, or in their free active forms.

Short-term dietary supplementation has been shown to have proliferative effects on breast tissue in premenopausal women with breast tumors (not in women without breast disease) and animal studies have provided conflicting data as to whether phytoestrogens stimulate or inhibit chemically induced tumors or tumor implants. Generally, high concentrations of phytoestrogens are required to inhibit specific steroidogenic enzymes and hence local production of oestrogens that could be important in oestrogen-dependent breast cancers. Therefore, there is experimental evidence for both a promotional and a protective effect of phytoestrogens on breast cancer, but at the present time it is impossible to reconcile dietary/supplement exposure with epidemiological and experimental studies. Of major concern is that phytoestrogen supplements are over-the-counter drugs and women who do not find relief of menopausal symptoms with recommended dosages may simply up the dose of such ‘natural’ alternatives and achieve circulating concentrations of these compounds that may have deleterious effects on their health. Further work is required to determine the cellular actions of phytoestrogens beyond the oestrogen receptor and the effects of combinations of different phytoestrogens during long-term exposure.

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