

WJCO 5th Anniversary Special Issues (2): Breast cancer

Phytoestrogens and prevention of breast cancer: The contentious debate

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Author contributions: Bilal I, Chowdhury A, Davidson J were MBBS project students in my laboratory and contributed towards collecting and assessing the references and synthesising the available data; Whitehead S wrote the review based on the students assessment.

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Received: December 23, 2013 Revised: April 28, 2014
Accepted: May 16, 2014
Published online: October 10, 2014

Abstract

Phytoestrogens have multiple actions within target cells, including the epigenome, which could be beneficial to the development and progression of breast cancer. In this brief review the action of phytoestrogens on oestrogen receptors, cell signalling pathways, regulation of the cell cycle, apoptosis, steroid synthesis and epigenetic events in relation to breast cancer are discussed. Phytoestrogens can bind weakly to oestrogen receptors (ERs) and some have a preferential affinity for ER β which can inhibit the transcriptional growth-promoting activity of ER α . However only saturating doses of phytoestrogens, stimulating both ER α and β , exert growth inhibitory effects. Such effects on growth may be through phytoestrogens inhibiting cell signalling pathways. Phytoestrogens have also been shown to inhibit cyclin D1 expression but increase the expression of cyclin-dependent kinase inhibitors (p21 and p27) and the tumour suppressor gene p53. Again these effects are only observed at high (> 10) μ mol/L doses of phytoestrogens. Finally the effects of phytoestrogens on breast cancer may be mediated by their ability to

inhibit local oestrogen synthesis and induce epigenetic changes. There are, though, difficulties in reconciling epidemiological and experimental data due to the fact experimental doses, both *in vivo* and *in vitro*, far exceed the circulating concentrations of "free" unbound phytoestrogens measured in women on a high phytoestrogen diet or those taking phytoestrogen supplements.

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Key words: Phytoestrogens; Breast cancer; Cell signalling; Cell cycle; Epigenomics

Core tip: Phytoestrogens have multiple actions within target cells, including the epigenome, which could be beneficial to the development and progression of breast cancer. In this brief review the action of phytoestrogens on oestrogen receptors, cell signalling pathways, regulation of the cell cycle, apoptosis, steroid synthesis and epigenetic events in relation to breast cancer are discussed. The difficulties in interpreting experimental evidence relating to the beneficial effects of phytoestrogens in light of dietary/supplementary intake and bioavailability of ingested phytoestrogens is also addressed.

Bilal I, Chowdhury A, Davidson J, Whitehead S. Phytoestrogens and prevention of breast cancer: The contentious debate. *World J Clin Oncol* 2014; 5(4): 705-712 Available from: URL: <http://www.wjgnet.com/2218-4333/full/v5/i4/705.htm> DOI: <http://dx.doi.org/10.5306/wjco.v5.i4.705>

INTRODUCTION

Phytoestrogens are naturally occurring plant compounds which are structurally similar to oestrogens and can have weak oestrogenic actions. There are six major classes

Table 1 Major classes of phytoestrogens and their common dietary sources

| Flavonoids | Isoflavonoids | Lignans | Coumestans | Stilbenes |
|---|--|---|--------------------------------------|-------------|
| Apigenin Quercetin Narigenin Catechins | Genistein Biochanin A Daidzein → equol | Enterodiol Enterolactone | Coumesterol | Resveratrol |
| Red/yellow fruits and vegetables, tea | Soy beans, soy foods, vegetables | Flaxseed, whole grains, fruit, vegetables | Peas, beans, alfafa, sunflower seeds | Red wine |

of phytoestrogens, all of which have distinct common dietary sources (Table 1) but the most intensively investigated phytoestrogens are the isoflavones and the stilbene, resveratrol. The link between breast cancer and phytoestrogens arose from the early epidemiological evidence showing that the incidence of breast cancer is lower in Asian populations who consume high dietary concentrations of soy products which have a high isoflavonone content^[1,2]. This fuelled the widespread belief that consumption of soy foods reduces the risk of breast cancer and other hormone dependent cancers and led to further research on the protective effects of many other phytoestrogens^[3,4].

However reconciling experimental evidence, mainly based on high supraphysiological doses of single phytoestrogens, coupled with the limited bioavailability of orally consumed phytoestrogens has raised questions and concerns about the validity of promoting the health benefits of diets rich in phytoestrogens and/or taking dietary supplements^[5-7]. Furthermore phytoestrogens exert a plethora of actions beyond weak oestrogenic effects (Figure 1) and these include antagonist effects oestrogen receptors (ERs), modulation of cell signalling pathways, regulation of the cell cycle, enzyme inhibition, anti-oxidant properties, angiogenesis and epigenetic alterations^[8]. This review will focus on the action of phytoestrogens on oestrogen receptors and cell signalling pathways, their regulation of the cell cycle and apoptosis, inhibition of steroidogenic enzymes and induced epigenetic changes.

DIETARY INTAKE, METABOLISM AND BIOAVAILABILITY OF PHYTOESTROGENS

In foods phytoestrogens are present as mixtures and are usually found as biologically inactive glycoside conjugates containing glucose or carbohydrate moieties. Blood levels can vary widely between individuals depending both on dietary preferences as well the phytoestrogen content of a particular food product resulting from local and/or seasonal variations^[9]. For example Asian diets can result in isoflavone consumption as high as 50 mg/d compared with 1-3 mg/d for individuals eating a typical Western diet although a vegetarian diet or use of supplements can increase dietary intake to levels of an Asian diet^[10-12] and references therein.

In the gut phytoestrogens are broken down by glucosidases to their respective aglycones allowing more effi-

cient absorption, although intestinal bacteria may further metabolise these products. For example the phytoestrogens genistein and daidzein, can be further metabolised to *p*-ethyl phenol and to equol and/or *O*-desmethylangolensin (*O*-DMA) respectively though it should be noted that only 30%-50% of the population can produce equol and approximately 80%-90% *O*-DMA^[10,13]. Thus not only will dietary factors contribute to phytoestrogen intake but also individual variations in metabolism.

Once absorbed the aglycone phytoestrogens are rapidly conjugated to glucuronic acid and to a lesser extent sulphuric acid in the hepatic circulation. They are then de-conjugated prior to excretion with urinary concentrations increasing in parallel to consumption^[14]. There is generally very low levels of biologically active 'free' unconjugated phytoestrogens in the circulation (< 3% of the total) and blood levels are in the ng/mL range or lower^[10,15]. One may then argue what is the relevance of *in vitro* studies showing that only high micromolar doses of unconjugated phytoestrogens can inhibit the growth the breast cancer cells, inhibit oestrogen-dependent gene transcription or inhibit cell signalling pathways?^[16,17]. Similarly *in vivo* studies have only shown that dietary supplements far in excess of those consumed with an Asian diet had any effect on inhibiting experimentally-induced tumour growth and even this data is conflicting^[16,18,19] and references therein.

PHYTOESTROGENS, OESTROGEN RECEPTORS AND CELL SIGNALLING PATHWAYS

Two major oestrogen receptors (ERs) have been identified, ER α and ER β , which are encoded by separate genes and have different tissue distributions and roles in gene regulation^[20]. They also have differential effects in oestrogen-sensitive tissue and in breast tissue ER α activation can stimulate proliferation whilst ER β activation can counteract this proliferative effect. This is thought to be mediated by dimerization of ER β with ER α ^[20,21]. In breast tumours the ratio of ER α to ER β is raised and tumour aggressiveness is increased in those that are ER β negative^[21].

The relative binding affinity (RBA) of phytoestrogens to ERs is weak and are in the order of 1000-10000 times less than that of oestradiol although some phytoestrogens

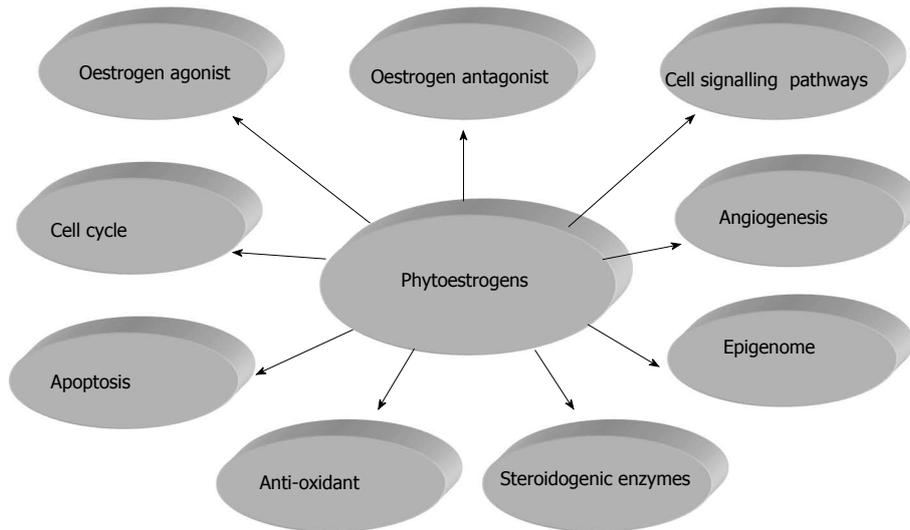


Figure 1 Multiple targets for the action of phytoestrogens.

such as genistein, coumestrol and apigenin have a higher affinity for ER's and their RBAs are in the order of only 10-100 times that of oestradiol^[22]. Interestingly several phytoestrogens such as genistein, daidzein and apigenin have a 9-10 fold increased affinity for ER β than ER α ^[22] and a more recent study showed that after dietary supplementation total genistein and daidzein concentrations were 20-40 fold higher than oestradiol equivalents in breast adipose/glandular tissue^[23]. Thus their ability to preferentially activate ER β and their ability to accumulate in breast tissue may have some clinical significance. That said, the concentrations required to induce apoptosis or at least inhibit cell growth arrest are induced only by over-saturating doses ($\geq 10 \mu\text{mol/L}$) doses of phytoestrogens^[16].

However, phytoestrogens can also act on cell surface oestrogen receptors or interact with growth factor and cytokine signalling pathways (Figure 2). Thus phytoestrogens can modulate the responses to growth factors or activate/inhibit kinases which may alter ligand-independent transcriptional activity of oestrogen receptors or other transcription factors such as AP-1 and NF- κ B^[24]. For example genistein is a tyrosine kinase inhibitor and has been shown to alter the activity/expression of both extracellular regulated kinase (ERK) and the PI-3/Akt pathway as have other phytoestrogens including resveratrol^[25,26]. Long-term treatment of breast cancer cells with dietary levels of genistein (10^{-8} mol/L) have also been shown to down-regulate the expression of Akt^[27]. In context of the growth-repressing effect of ER β and cell signalling, a recent study showed that activation of the MEK 1/2 and PI-3K/Akt pathways inhibited the ER β growth-mediated repression in breast cancer cells^[28]. Thus down regulation of these pathways by long-term dietary phytoestrogens could promote the effectiveness of ER β activation and inhibit proliferation.

PHYTOESTROGENS AS REGULATORS OF THE CELL CYCLE AND APOPTOSIS

Recently much attention has focussed on the action of

phytoestrogens in regulating the expression of proteins regulating the cell cycle and apoptosis (Figure 2). Cyclins are a family of proteins which regulate transition of the cell cycle through the G₁, S, G₂ and metaphase (M) phases and, through coalescing with cyclin-dependent kinases (CDKs), they initiate gene transcription controlling regulation of the cell cycle. Cyclin D1, which regulates the G₁ to S phase of the cell cycle, is the most widely investigated being established as an oncogene and over-expressed in more than 50% breast cancers^[29]. The majority of studies have shown that high concentrations of phytoestrogens ($\geq 10 \mu\text{mol/L}$ range) inhibit the expression of cyclin D1^[30-32] although another study reported a transient increase mimicking the effects of oestradiol^[33] and other studies reported no effects^[34,35].

The activity of cyclin/CDK complexes is regulated by CDK inhibitors (CDIs) and thus these proteins can inhibit the cell cycle^[36,37]. The two most widely investigated CDIs are p21^(CIP1/WAF1) and p27^(Kip1) and the expression of p21 is controlled by the tumour suppressor gene p53^[38] which has many other actions as a tumour suppressor including inducing apoptosis^[39]. High doses of phytoestrogens have been shown to increase the expression/activity of p21, p27 and p53^[33,35,40-43] which parallel changes in the reduction of cyclin D1. Such effects have been seen with high doses of phytoestrogens but a microarray analysis showed that low levels of genistein (1 and 5 $\mu\text{mol/L}$) that stimulated growth of MCF-7 cells had no effect on the expression of p53 target genes such as p21^(CIP1/WAF1) and only at a higher pharmacological dose of genistein (25 $\mu\text{mol/L}$) cell growth was inhibited and increased the expression of p53 target genes. This would result in increased apoptosis and decreased proliferation^[44].

The pro-apoptotic protein, Bax, is regulated positively by p53 whilst the anti-apoptotic protein, Bcl-2, is negatively regulated by p53^[45]. Both *in vivo*^[46-48] and *in vitro* studies^[33,49-52] have shown that phytoestrogens can stimulate apoptosis and increase the Bax/Bcl-2 ratio but evidence as to whether this effect is due to increased or decreased activity of ERK1/2 is controversial^[49-52].

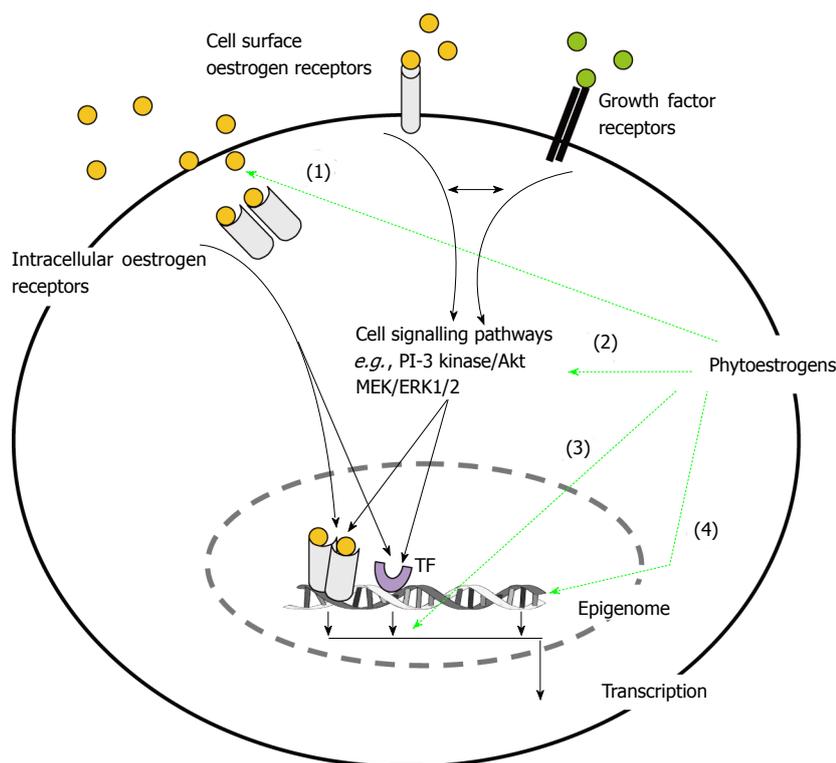


Figure 2 Different ways in which phytoestrogens may alter gene transcription. (1) Acting as an oestrogen agonist/antagonist and the transcriptional activity of oestrogen receptors; (2) Modulating cell signalling pathways which can be activated by cell surface oestrogen receptors or growth factor receptors and ultimately activate gene transcription by activating oestrogen receptors or other transcription factors (TF); (3) Inhibiting/stimulating transcription of genes regulating apoptosis and the cell cycle; and (4) Epigenetic alterations in DNA, histone proteins and RNA to alter transcription/translation of proteins.

PHYTOESTROGENS AS INHIBITORS OF OESTROGEN SYNTHESIS

Low doses of phytoestrogens are generally found to stimulate growth of breast cancer cells with only high supraphysiological doses inhibiting growth and with evidence that they regulate the expression of proteins involved in controlling the cell cycle and apoptosis as opposed to their action as weak oestrogen agonists/antagonists at ER α and ER β . There is, however, evidence that phytoestrogens can also inhibit steroid synthesis and this may be particularly significant in relation to the local production of oestrogens in breast tissue^[53] and references therein. Fatty tissues are a major storage site for phytoestrogens^[13] and the most abundant cells surrounding breast cancer cells are mature adipocytes and progenitors which may be a key component of breast cancer progression by locally affecting breast cancer cell behaviour^[54].

Approximately 60%-70% breast cancers express oestrogen receptors and these are considered to promote tumour growth. Hence initial treatments are directed towards reducing oestrogenic effects by inhibitors of oestrogen receptors and inhibitors of aromatase which converts androgens to active oestrogens^[55]. The incidence of breast cancer increases with age despite the loss of ovarian hormones in post-menopausal women. Peripheral tissue can convert circulating androgens (dehydroepiandrosterone/DHEA) and oestrone sulphate into 17 β -oestradiol (E₂) and in post-menopausal women with breast cancer concentrations of E₂ in the tumour is at least 20-fold higher than in the circulation^[56].

Flavones and isoflavones are the most potent phytoestrogens that inhibit aromatase and the IC₅₀ values are in

the order of 0.1-10 μ mol/L which is more than 100 times higher than the IC₅₀ value for the steroidal inhibitor, 4-hydroxyandrostendione^[53,57]. Isoflavones are generally weak inhibitors of aromatase but like other phytoestrogens can inhibit 17 β -hydroxysteroid dehydrogenase (HSD) type 1 which reduces oestrone (E₁) to E₂ and 17 β -HSD type 5 that converts androstenedione to testosterone, which can subsequently be converted to E₂ by aromatase^[53] and references therein. More recently certain phytoestrogens have been shown to alter the activation of breast cancer-associated aromatase promoters^[58]. Overall, however, the inhibition of these enzymes by unconjugated phytoestrogens are in the order of 1-10 μ mol/L whilst total circulating phytoestrogens are in the low nanomolar range except in vegetarians or those eating a high soy diet where concentrations of 100 nM to 1 μ mol/L may be achieved^[59]. Thus there is another discrepancy between experimental results and levels of phytoestrogens achieved by dietary means. However our study on human granulosa cells showed that low dose mixtures of three isoflavones in the nM range inhibited expression and activity of aromatase though a similar inhibition was only achieved with a single phytoestrogen at 100 times the dosage^[60]. Further studies are required to investigate mixtures of phytoestrogens as occurs through dietary means.

EPIGENETIC MODULATION BY PHYTOESTROGENS

Over the last decade there has been an explosion in the number of studies concerning epigenetic changes and the development and progression of breast cancer^[61] and not surprisingly these have included studies on the ability

of phytoestrogens to alter the epigenome which could be useful in the prevention of cancer^[61-63]. In fact studies have indicated that early childhood exposure to phytoestrogens could protect against breast cancer in later life^[62] and references therein and this could involve epigenetic events (Figure 2). Epigenetic changes are defined as heritable changes in gene expression which do not involve mutations of DNA nucleotide sequences. They include DNA methylation, histone acetylation and microRNAs (miRNAs).

DNA methylation occurs on cytosine in the cytosine-phosphate-guanine (CpG) dinucleotide sequence of genomic DNA, a reaction catalysed by DNA methyl transferases (DNMTs). CpG dinucleotide rich regions (known as CpG islands) are found in the promoter region of approximately 60% of all human genes and, whilst most CpG islands are unmethylated in normal cells, they become hypermethylated in cancerous cells leading to gene suppression, including the tumour-suppressing genes^[61]. Along with DNMTs are the methyl-CpG-binding domain family of proteins which bind to a methylated gene and can inhibit transcriptional activity by altering chromatin structure. Chromatin structure can also be modified by histone acetylation which is catalysed by histone acetylase (HAT) and results in a more open structure of chromatin allowing access for transcription factors to DNA. The reverse occurs when histone proteins become deacetylated and this reaction is catalysed by histone deacetylases (HDACs). Histones may also be methylated by histone methyl transferases (HMT's) and generally methylation causes gene transcription to be switched off. The most recent participant of the epigenetic field are the miRNAs, small non-coding RNAs that inhibit protein expression of target genes by binding to the 3'-untranslated region of mRNA causing degradation or inhibition of mRNA of the target gene^[61,62] and references therein.

The most widely studied dietary components in relation to epigenetic changes are the tea polyphenols, epicatechins and epigallocatechins (EGCCs), the isoflavones, genistein and diadzein, resveratrol and curcumin and all have been well reviewed recently^[63-66]. Relatively few studies have been directed towards epigenetic changes in breast cancer models and results have been inconclusive^[61,62].

Recent studies have shown that 20-40 $\mu\text{mol/L}$ genistein stimulated expression of the tumour suppressing genes, *p21^{WAF1}* and *p16^{INK4a}*, in breast cancer cells and that this was associated with a small reduction in the activity of HDACs but a large increase in the activity of HMTs^[67]. The same group also showed that genistein can reactivate ER α expression in ER^{ve} breast cancer cells and that this effect was associated with increased markers of histone acetylation in the ER α promoter region and decreased activity of HDAC and DNMT^[68]. Another study showed that $\mu\text{mol/L}$ doses of genistein and diadzein "might reverse" DNA hypermethylation in breast cancer cells thus restoring expression of the oncosuppressor genes BRCA1 and BRCA2^[69]. In biopsies of human

breast tissue specific DNMT transcripts were increased in cells taken from the tumourous tissue compared to adjacent normal breast tissue and parallel studies showed that treatment of breast cancer cells lines with genistein, resveratrol, curcumin and EGCC also reduced the mRNA of the same DNMTs^[70]. Whilst all these studies have been performed acutely with high doses of single phytoestrogens, we showed that long-term treatment with 10 nmol/L genistein down-regulated the expression of acetylated histone3, cyclin D1 and procaspase 9 and reduced the growth promoting effects of E₂ and epidermal growth factor^[71].

It is clear that both nutrition and exposure to phytoestrogens and other phytochemicals can have dramatic effects on epigenetic events and that these may become heritable through transgenerational mechanisms. Thus their impact on both disease and the health of future generations needs to be carefully considered.

CONCLUSION

Phytoestrogens have multiple targets within cells and whilst acute studies with supraphysiological doses of these compounds indicate that they may inhibit the development and progression of breast cancer, lower doses have been shown to promote the growth of breast cancer cells *in vitro* and experimentally induced tumours *in vivo*. More studies utilizing long-term exposure to lower doses and mixtures of phytoestrogens are required to demonstrate unequivocally that dietary supplements do have beneficial rather than detrimental effects on breast cancer. However their multiple targets in breast cancer cells and their ability to modulate epigenetic events associated with breast cancer and prevention may lead to new, non-toxic therapeutic approaches through development of highly specific and long-acting analogues of phytoestrogens.

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P- Reviewer: Petmitr S, Shen J **S- Editor:** Wen LL **L- Editor:** A
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