Effects of the Natural Isoflavonoid Genistein on Growth, Signaling Pathways and Gene Expression of Matrix Macromolecules by Breast Cancer Cells

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Abstract: Genistein is a well known protein tyrosine kinase inhibitor. It is structurally similar to 17β-estradiol and exerts antiestrogenic effects. It also affects the signal transduction components Akt, FAK, ErbB-2 and Bcl-2. Key enzymes implicated in cancer invasion are also affected by genistein. A critical evaluation of the effects of genistein on breast cancer growth, signaling and gene expression is presented in this review.

Keywords; genistein, estrogens, estrogen receptor, signaling, breast cancer.

ORIGIN, STRUCTURE AND BIOLOGICAL IMPORTANCE

Phytoestrogens are a group of plant-derived substances that are structurally and/or functionally similar to estradiol [1]. The major classes of phytoestrogens are isoflavones (genistein and daidzein), lignins (e. g., enterodiol and enterolactone), and coumestans [2]. For chemical structures see (Fig. **1**). More than 300 plants are known to possess estrogenic activity. Soy is the major dietary source of phytoestrogens. A high intake of soy-based phytoestrogen may be associated with a lower incidence of breast, endometrial, prostate and colorectal cancer [3]. In October 1999, the Food and Drug Administration approved a health claim for soy and recommends that people should consume 4 servings of 6.25 g of soy protein a day [4].

Genistein (4', 5, 7, -trihydroxyisoflavone) belongs to the isoflavone class of flavonoids and is the predominant isoflavone present in soy. It is a solid substance that is practically insoluble in water. It is a planar molecule with an aromatic A-ring that has a second oxygen atom 11.5 Å from the one in the A ring, and has a molecular weight similar to those of the steroidal estrogens [5] (Fig. **1**).

Genistein shows estrogenic properties in receptor binding assays [6], cell culture [7] and uterine weight assays [8]. Furthermore, genistein inhibits the proliferation of transformed cell lines in cell culture. This effect of genistein on cell growth may be due to the inhibition of topoisomerase II [9, 10], inhibition of protein kinases [11, 12], diacylglycerol synthesis [11], and platelet-activating factor- and epidermal growth factor- induced expression of c-fos [13].

Mentor-Marcel *et al*. [14] demonstrated that in two animal models dietary physiological amounts of genistein can protect against chemically induced and spontaneously developing prostate cancers. Later, in 2002, Lamartienie *et al*. [5] presented evidence that dietary genistein regulates sex steroid receptor, growth factor ligand and receptor mRNA expression. Speculating that these gene products contribute to chemoprevention of prostate cancer by genistein, they suggested that genistein can protect against prostate cancer in men.

It is worth noticing that genistein consumption has been shown to reduce bone loss and slow calcium loss in an animal model of osteoporosis, suggesting a possible beneficial role in preventing osteoporosis in human. Genistein suppresses osteoclast activity by a number of possible mechanisms, including induction of apoptosis, activation of protein tyrosine phosphatase, inhibition of cytokines, changes in intracellular calcium and membrane depolarization, further highlighting the level of complexity in mechanism of estrogens and phytoestrogens in bone turnover [15].

It was found that genistein also exhibits antioxidant properties [16] and was reported to induce differentiation of numerous cell types [17]. High dietary intake of isoflavones may contribute to a low incidence of heart disease in Japanese women. These may be the result from inhibition of low density lipoprotein oxidation by isoflavones. Genistein also appears to improve plasma lipids, resulting in lowered LDL cholesterol, the ratio of total cholesterol to HDL cholesterol, and the ration of LDL to HDL cholesterol, in pre-menopausal women [18].

REGULATORY EFFECTS OF GENISTEIN ON ESTROGENICITY

Genistein has structural similarity to 17β-estradiol (Fig. **1**), but binds to the estrogen receptor with lesser affinity than estrogen itself. Ligand-binding studies show that genistein exhibits a 7- to -30 fold greater binding affinity for estrogen receptor isoform β (ERβ) than the isoform α (ERα). Although genistein binds the ERβ receptor with greater affinity than ERα, it initiates greater gene transcription of ERα as compared to ERβ. Apart the estrogen receptors, genistein can also alter the expression of the progesterone receptor, the androgen receptor and the oxytocin receptor [19].

In addition to directly binding to the ER, genistein may indirectly affect estrogenicity through inhibition of the

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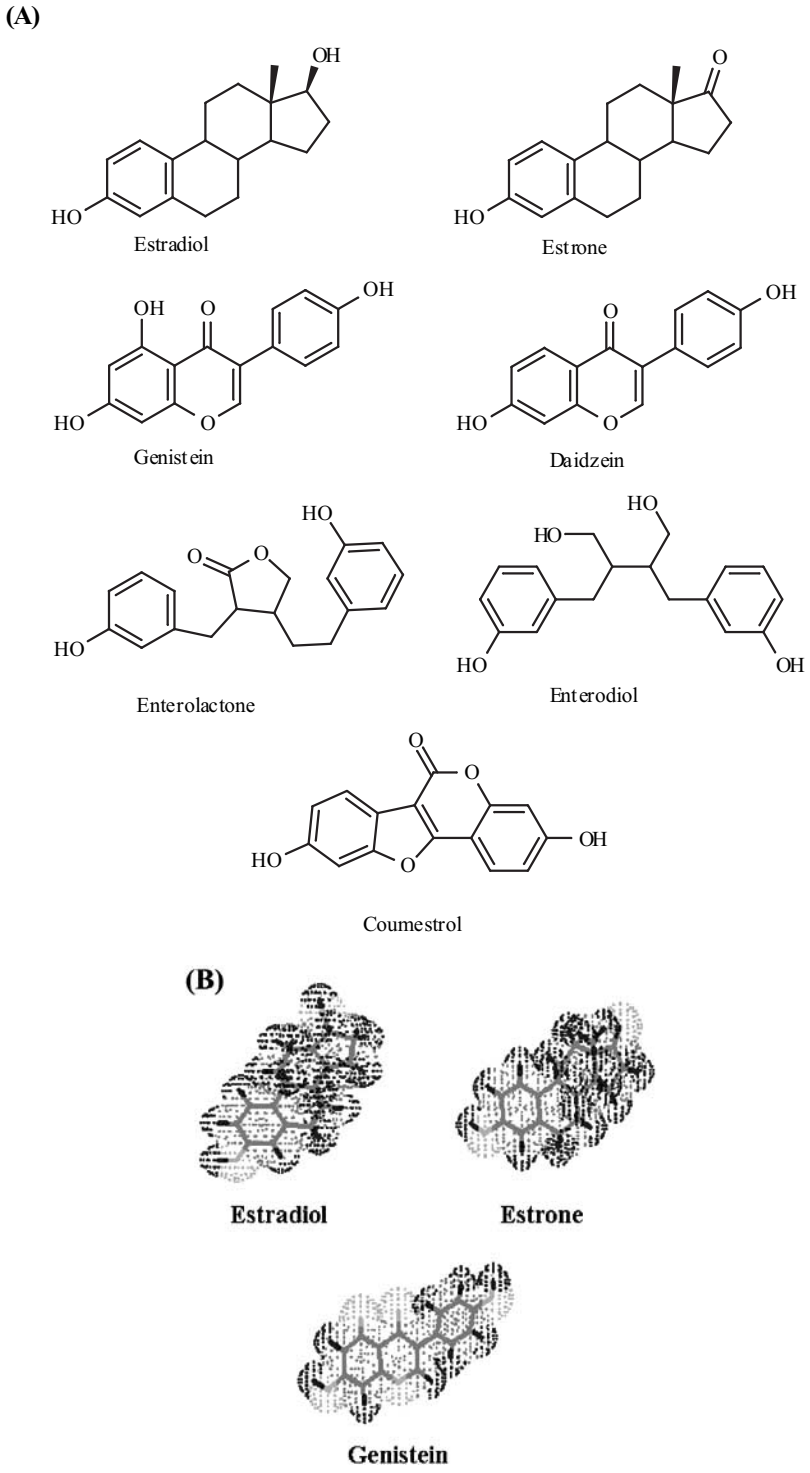

Fig. (1). (A), Chemical structures of the two human estrogens: estradiol and estrone and of the phytoestrogens: genistein, daidzein, entorolactone, enterodiol and coumestol. (B), 3D structures of genistein, estradiol and estrone showing that the three molecules exhibit similar space filling characteristics, which allow them to interact with mammalian estrogen receptors and to exert hormonal effects.

cytochrome P450 enzyme CYP1A1. It has recently been shown [20] that genistein is a non competitive inhibitor of the CYP1A1 enzyme, which apart from playing a role in the metabolism of carcinogens, is responsible for the metabolic degradation of 17β-estradiol. Thus, it is possible that genistein-mediated inhibition of estradiol degradation could result in higher levels of circulating estradiol and thus elevated ER activity. All these suggest that genistein inhibits estrogenicity acting with an antiestrogenic fashion through its inhibition of estrogen-metabolizing enzymes [20, 21].

Genistein does not always induce proliferation of ER-positive cells. Studies have shown that genistein exhibits an

antiproliferative effect in human mammary and uterine tissues [22, 23]. These data could be interpreted to indicate that genistein is acting as a classical antiestrogen; i.e., it competitively inhibits estrogen's binding to the ER and transactivation of estrogen-responsive genes.

ACTION OF GENISTEIN ON SIGNAL TRANSDUCTION COMPONENTS

At the level of signal transduction, it has been shown that genistein has the ability to inhibit protein tyrosine kinases (PTK), particularly the autophosphorylation and therefore activation of the epidermal growth factor receptor (EGFR). This effect has been attributed to the competition of genistein with ATP rather than with the protein substrate [11] and indicated the genistein antiproliferative effect as a result of interference in the tyrosine kinase cascade activated by mitogens. EGFR contains a PTK domain, which is phosphorylated upon activation and dimerization of the receptor (Fig. **2**). This results in signaling events to downstream effector molecules, ultimately leading to an inhibition of apoptosis. It has been postulated that genistein's ability to inhibit the phosphorylation of EGFR (or other potentially oncogenic proteins) may be an important mechanism mediating its observed antiproliferative effects in breast cancer. McIntyre *et al*. [24] demonstrated that the antiproliferative effects of genistein are not directly associated with a reduction in EGF tyrosine kinase activity, but with the down-regulation in EGFR and subsequent decrease in normal mammary epithelial cell mitogenic-responsiveness to EGF stimulation. In support of this, a number of studies have demonstrated genistein's ability to inhibit both PTK and the proliferation of a number of ER-positive and ER-negative breast cancer cell lines [22, 25]. An indication that genistein-induced inhibition of PTK may be independent of ER-mediated functions was shown by Schultze-Mosgau *et al*. [25]. The authors demonstrated that pharmacologic doses of genistein inhibit the PTK-dependent transcription of c-fos and subsequent cellular proliferation in an ER-human breast cancer cell line. Thus, the antiproliferative effects appear to be due to an inhibition of PTK rather than an inhibition of ER signaling.

Genistein's ability to inhibit tyrosine phosphorylation not only allows for an inhibition of a proliferation of cancer cells, but it may also lead to an inhibition of metastasis. It has been suggested that tyrosine phosphorylation of membrane proteins plays a critical role in the mediation of degradation of the extracellular matrix, thus allowing for cellular invasion [26].

Genistein has been shown to decrease transcriptional production and block tyrosine kinase-mediated export of some receptors [27, 28]. It can also inhibit the binding capability in hormone receptors, and the phosphorylated activation of growth factor receptors by both mitogens and radiation [29, 30]. Disabling receptor tyrosine kinase function can be crucial because it prevents the eventual activation of particular oncomessengers known as Ras proteins. Ras proteins stimulate protoongogene transcription and cellular proliferation *via* an initiation of a

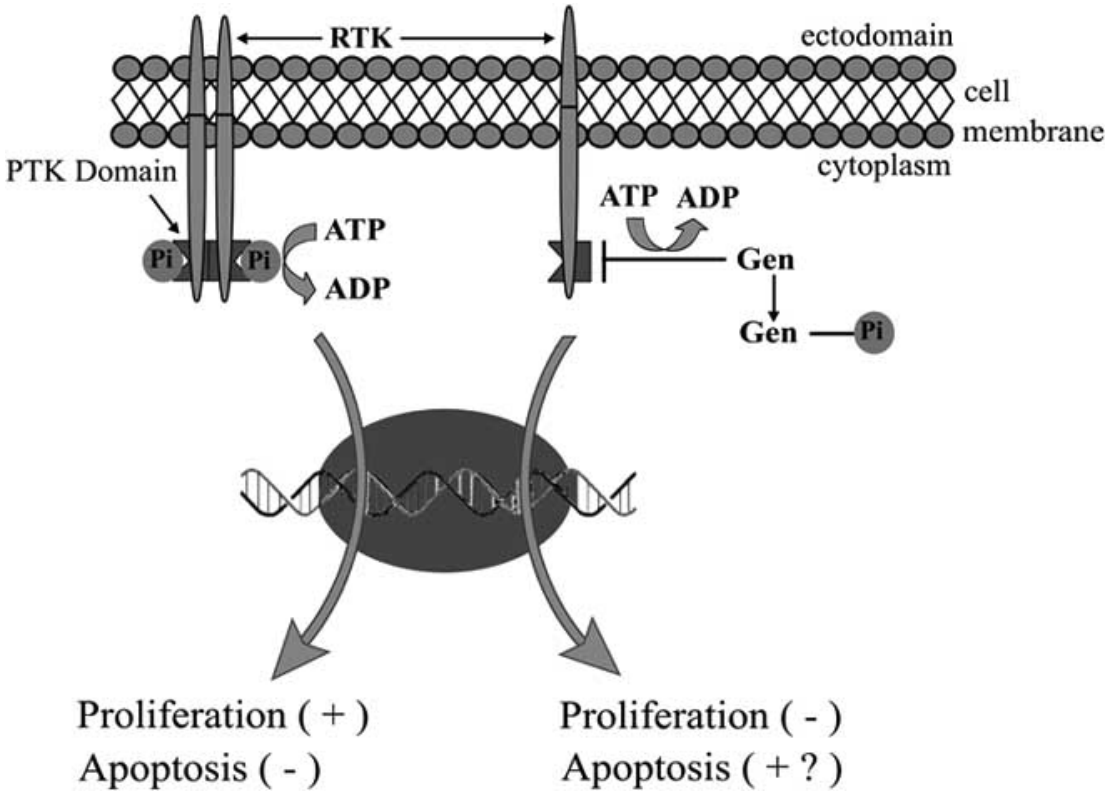

Fig. (2). Schematic representation showing the antiproliferative effect of genistein as a result of its interference with the phosphorylation of the cytoplasmic PTK domain of the tyrosine kinase receptor. Receptor is activated upon ligand binding and dimerization is occured, whereas in the presence of genistein PTK phosphorylation and receptor activation are inhibited. (+?): genistein possibly causes apoptosis, but this was not yet established.

serine/threonine phosphorylation cascade [31]. Genistein, also decreases NF-kappa B activation caused by various DNA-damaging agents via blocking the nuclear translocation of NF-kappa B, prohibiting DNA-binding and preventing NF-kappa B activation in prostate cancer cells [32].

EFFECTS OF GENISTEIN ON SIGNALING OF BREAST CANCER CELLS

Estradiol has been shown to affect the tyrosine phosphorylation status of key signaling intermediates, such as c-Src and focal adhesion kinases (FAK) in both ER-positive and ER-negative breast cancer cell lines [33]. Recent studies have shown that MAPK signaling not only affects gene transcription leading to tumorigenesis, but also may promote cancer cell invasion [34, 35]. Therefore, estradiol signaling to MAPK cascades may be relevant for breast cancer malignancy. Brownson *et al*. [36] demonstrated that genistein, in addition to its effect as a tyrosine kinase inhibitor and an antiestrogen via ER, may also have ER-independent effects on breast cancer cells. With a similar panel of ER-positive and ER-negative breast cancer cell lines, the antiproliferative effects of genistein were shown to be dependent on estradiol, thus leading the authors to conclude that antiestrogenic effects of genistein were only operative in ER-positive breast cancer cells [37]. However, in other studies it has been suggested that genistein can inhibit growth and induce apoptosis in the ER-negative highly metastatic MDA-MB-435 breast cancer cells via down-regulation of ErbB-2 and Bcl-2 and decreased matrix metalloproteinase activity [38]. Brownson *et al*. [36] suggested that genistein may exert additional effects on breast cancer cell survival, proliferation and invasion via activation of Akt and FAK. The effects of genistein on breast cancer cell signaling are summarized in (Fig. **3**).

IN VITRO ANTICANCER EFFECTS OF GENISTEIN ON BREAST CANCER CELLS AND CLINICAL TRIALS

Genistein inhibits the growth of a wide range of cancer cells *in vitro* including both hormone-dependent and -independent breast cancer cells [3, 29, 39, 40]. At high concentrations (50 and 100 μM), it significantly arrest the cells at G_2/M phase of the cell cycle [41]. Flow cytometric results obtained from cells exposed to high concentrations of genistein showed an increase in the late S-phase compartment in MCF-7 cells and an accumulation in the G_2/M phase in HBL-100 and MDA-MB-231 cells. Apoptosis was observed in MCF-7 and MDA-MB-231 cells [42]. Upadhyay *et al*. [43] demonstrated that genistein causes a greater degree of G_2/M arrest and induces apoptosis in malignant cell lines as compared to normal mammary epithelial cells.

Shao *et al*. [44] showed that genistein exerts a pleiotrofic effect in ER-positive (MCF-7) and ER-negative (MDA-MB-231) epithelial breast cancer cells. Santell *et al*. [45] reported that in ER-negative MDA-MB-231 breast cancer cells, genistein inhibits cell proliferation by ~50% at the concentration of ~20 μmol/L. Genistein at 10 μmol/L had

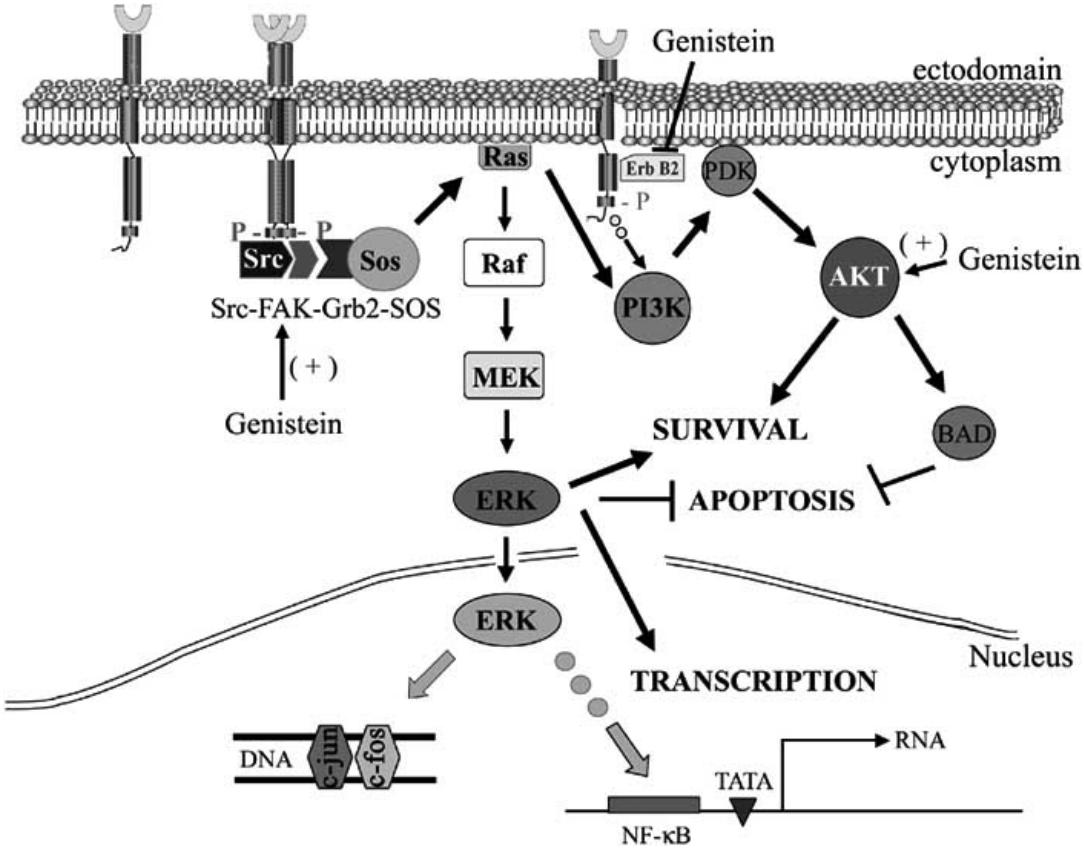

Fig. (3). Effects of genistein on the various signaling pathways. Genistein acts through the Ras pathway activating FAK, while it interferes with ErbB-2 and Akt pathways.

no apparent effect on cell proliferation, whereas 20, 40 and 80 μmol/L resulted in a dose-dependent decline in cell proliferation. At concentrations < 10 μM cell growth of ER-positive MCF-7 cancer cells is stimulated by genistein [23, 42], whereas genistein does not stimulate the growth of ER-negative breast cancer cells *in vitro* [23, 45]. This biphasic effect is attributed to the fact that genistein exerts estrogen-like effects at lower concentrations exerting non-estrogen receptor-mediated effects, for example, inhibition of the activity of one or more cellular molecules that control cell signaling, growth and death [46].

Our group has shown that genistein affects both cell proliferation and the synthesis of the key matrix macromolecules glycosaminoglycans and proteoglycans by human malignant mesothelioma cell lines [47, 48]. Furthermore, genistein has been shown to affect the expression of enzymes, such as metalloproteinases (MMPs) and their endogenous inhibitors (TIMPs), which are of crucial importance for breast cancer cell invasion [49]. Genistein inhibition *in vitro* was characterized by down-regulation of MMP-9 and -2 and up-regulation of TIMP-1 and this was demonstrated in nude mouse xenografts [44]. This was in accordance with the effect of genistein *in vitro* to reduce the invasiveness of breast cancer cells [38, 50, 51] (Fig. **4A**). We have recently shown [51] that the addition of genistein resulted in down-regulation of the transcription of all MMPs genes in MDA-MB-231 and most of MMPs in MCF-7 cells (Fig. **4B**). We have also suggested that the regulation of the different MMPs depends on the concentration of genistein that is supplied and as it has been earlier proposed, the effect of genistein on synthesis of matrix macromolecules at 35 μM in estrogen receptor-positive cell lines (MCF-7 and MCF-12A) may well be associated with the estrogen receptors, whereas at 100 μM may well be related with the inhibition of PTK pathways [52].

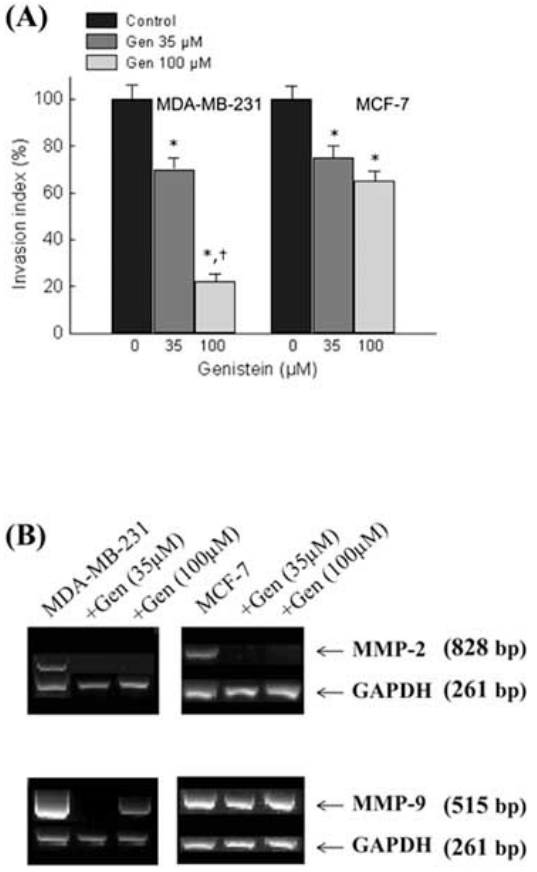

Fig. (4). (A), Effects of genistein at 35 and 100 μM on the invasion of MDA-MB-231 and MCF-7 cells. The results are expressed as the average ± SD of three experiments in triplicate. Statistically significant differences among the genistein-treated and control cells are shown by (*) and between the two genistein-treated groups (35 and 100 μM) by (†) ($p \leq 0.01$) (B), RT-PCR analyses showing the effect of genistein on the expression of MMPs in two cell lines (MDA-MB-231 and MCF-7). Modified by Kousidou, O.Ch.; Mitropoulou, T.N.; Roussidis, A.E.; Kletsas, D., Theocharis, A.D.; Karamanos, N.K. *Int. J. Oncol*., **2005**, *26*, 1101 [Ref. 51].

At 50 and 100 μM, genistein also up-regulate the expression of HSP 105 kDa, protein kinase (Y-linked, PRKY) and thymidine kinase 1, while the expression of SRF, Era, disabled (*Drosophila*) homolog 2, recombination activating gene 1, replication factor C and fms-related tyrosine kinase 1 were down-regulated [53].

An early epidemiological study of Singapore Chinese women that included 420 healthy controls and 200 with histologically confirmed breast cancer indicated that soy consumption was directly correlated with reduced risk of cancer [54], and the effects appeared to be dietary than genetic. Similar observations have been reported in many studies undertaken up the present day. In a 1 year pilot study, consumption of a commercial protein soy isolate was associated with effects on the breast that could be considered adverse [55]. Two week dietary soy supplementation produced a weak estrogenic response on the normal breast in premenopausal women [56]. Several studies in Asian population have found beneficial effects only in premenopausal women [54, 57] or in a population recently menopausal [58]. Other studies have shown that phytoestrogen consumption has no effect on endogenous estradiol levels [59, 60], whereas some others have shown a decrease in serum estradiol [61, 62] and the levels of 16α-(OH)E (16α-hydroxyestrone or 16α-hydroxyestradiol), estrogen metabolites that have been associated with increased breast cancer risk.

CONCLUDING REMARKS

Based on *in vitro* and *in vivo* data, it has been shown that genistein can act both as an estrogen and an antiproliferative agent. Its structural similarity to 17β-estradiol allows this isoflavonoid to mimic the pharmacology of the natural steroid and to compete with 17β-estradiol for binding to the ER, but only when present in a 1,000- to 10,000-fold molar excess as compared to that of the steroid. In signal transduction, the ability of genistein to inhibit protein tyrosine kinases has been attributed to its competition with ATP rather than with the protein substrate and this may be attributed to the phosphorylation of the available -OH groups rather than phosphorylation of PTK. In addition to its effect as a tyrosine kinase inhibitor and an antiestrogen via ER, genistein may also have ER-independent effects on breast cancer cells, through signaling

molecules, such as ErbB-2, Bcl-2 and activation of signaling cascades, such as Akt and FAK.

Although, genistein's consumption seems to be of great importance, modulation of genistein's intake must be occurred whether cancer is hormone-dependent or not. Further studies must be done before the true scope of genistein's action can be understood. Genistein continues to be an active area of research interest and, therefore, more clinical trials will open a new era and answer these fundamental questions.

ABBREVIATIONS

ER	=	Estrogen receptor
ERα	=	Estrogen receptor alpha
ERβ	=	Estrogen receptor beta
PTK	=	Protein tyrosine kinase
MAPK	=	Mitogen activated protein kinase
FAK	=	Focal adhesion kinase
NFκB	=	Nuclear factor κB
EGF	=	Epidermal growth factor
MMPs	=	Metalloproteinases
TIMPs	=	Tissue inhibitors of metalloproteinases
HSP	=	Heat shock protein

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