MULTI-TARGETED THERAPY OF CANCER BY GENISTEIN

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Abstract

Soy isoflavones have been identified as dietary components having an important role in reducing the incidence of breast and prostate cancers in Asian countries. Genistein, the predominant isoflavone found in soy products, has been shown to inhibit the carcinogenesis in animal models. There is a growing body of experimental evidence showing that the inhibition of human cancer cell growth by genistein is mediated via the modulation of genes that are related to the control of cell cycle and apoptosis. It has been shown that genistein inhibits the activation of NF-κB and Akt signaling pathways, both of which are known to maintain a homeostatic balance between cell survival and apoptosis. Moreover, genistein antagonizes estrogen- and androgen-mediated signaling pathways in the processes of carcinogenesis. Furthermore, genistein has been found to have antioxidant properties, and shown to be a potent inhibitor of angiogenesis and metastasis. Taken together, both in vivo and in vitro studies have clearly shown that genistein, one of the major soy isoflavones, is a promising agent for cancer chemoprevention and further suggest that it could be an adjunct to cancer therapy by virtue of its effects on reversing radioresistance and chemoresistance. In this review, we attempt to provide evidence for these preventive and therapeutic effects of genistein in a succinct manner highlighting comprehensive state-of-the-art knowledge regarding its multi-targeted biological and molecular effects in cancer cells.

Keywords

Genistein; Akt; NF-κB; chemoprevention; chemosensitization; cancer therapy

1. Introduction

Laboratory research backed by epidemiological studies emancipating from the last few decades have provided convincing evidence that isoflavones in soy rich foods contribute to relatively lower rates of prostate and breast cancers in Asian countries such as China and Japan than in Western population. Genistein (4,5,7-trihydroxyisoflavone) has been identified as the predominant isoflavone in soybean enriched foods which comprises a significant portion of the Asian diet, and provides 10% of the total per capita protein intake in Japan and China. A recent study among women in Shanghai, China found that plasma isoflavone concentration were inversely associated with the risk of non-proliferative and proliferative benign fibrocystic conditions as well as breast cancer [1]. In parallel, relatively high levels of soy isoflavones
have been found in the serum, urine and prostatic fluid of Asian men who consume a soy rich diet possibly contributing in lowering the incidence of prostate cancer [2;3]. Since these initial findings, several subsequent reports have been published documenting decreased risk of localized prostate cancer associated with soy product and isoflavone consumption [4]. High consumption of soymilk has also been associated with reduced risk of cancer [5]. These facts provide an excellent opportunity for primary prevention of the most common cancers worldwide. Furthermore, isoflavone sensitizes the effect of radiotherapy and cytotoxic chemotherapeutic drugs used in variety of cancers, thus opening avenues for devising novel therapeutic options.

We attempt herein to summarize the known inhibitory effects of genistein on cancer cells and provide a comprehensive review on the multi-targeted molecular mechanism(s) underlying the chemopreventive and therapeutic actions of genistein.

1.1. Molecular structure, estrogenic activity and metabolism of genistein

The isoflavone genistein was originally identified as having a close similarity in structure to estrogens and harboring weak estrogenic activity and, as such, was labeled as a phytoestrogen. The basic structural feature of isoflavone compounds is the flavone nucleus, which is composed of 2 benzene rings linked through a heterocyclic pyrane ring. Because of its structural similarity to 17β-estradiol, genistein has been shown to compete with 17β-estradiol in ER binding assays. Kuiper et al. [6] reported that the binding affinity of genistein for ER-α was 4%, and for ER-β was 87%, compared to estradiol. Thus, by interaction with estrogen receptor, genistein blocks the binding of more potent estrogens at the same time and affects estrogen metabolism, thereby exerting a potential favorable role in the prevention of hormone related cancers.

After intake and ingestion, genistein along with other isoflavones is conjugated with glycoside and metabolized by the enzymes of the intestine. It has been proposed that in humans, genistein is metabolized to dihydrogenistein and 6′-hydroxy-O-desmethylangolensin. Genistein and their metabolites have been detected in plasma, prostatic fluid, breast aspirate and cyst fluid, urine, and feces [2;3;7;8]. Adlercreutz et al. [2] have found that the plasma level of genistein in people having a soy rich diet was 1–5 μM after metabolism and excretion. A recent report from India also revealed an adequate circulating level of genistein after a single dose of soy extract [9]. Another study targeted Phase I pharmacokinetic and pharmacodynamic analysis following administration of unconjugated soy isoflavones (containing 43% and 90% genistein respectively), to individuals with cancer found plasma concentration of genistein supposedly associated with antimetastatic activity in vitro [10]. Genistein is relatively hydrophobic and expected to be taken up by cells without previous cleavage and does not have to be biologically active to exert its inhibitory effects on cancer cell growth [11]. However, cancer cell-specific concentration of genistein in human population has not been determined.

1.2. Biological effects of genistein

Many important biological effects of genistein consumption have been elucidated with respect to its anticancer properties. Nevertheless, genistein has many other important health benefits, such as lowering the incidence of cardiovascular diseases [12], prevention of osteoporosis, and attenuation of postmenopausal problems [13]. Furthermore, it has been reported that genistein decreases body mass and fat tissue, accompanied by a decreased appetite. After ingestion of dietary genistein, alterations in concentrations of hormones such as insulin, leptin, thyroid hormones, adrenocorticotropic hormone, cortisol and corticosterone were observed. Additionally, genistein intake is also associated with altered expression of genes engaged in lipid metabolism and disturbed glucose transport into cells affecting lipolysis, lipogenesis and altered ATP synthesis. These metabolic and hormonal changes have been succinctly summarized by Szkudelska and Nogowski in a review article [14].
Genistein is a known inhibitor of protein-tyrosine kinase (PTK), which may attenuate the growth of cancer cells by inhibiting PTK-mediated signaling mechanisms [15]. Sakla et al recently reported that genistein inhibits the protooncogene HER-2 protein tyrosine phosphorylation in breast cancer cells as well as delaying tumor onset in transgenic mice that overexpress the HER-2 gene. This data supports its potential anticancer role in chemotherapy of breast cancer [16]. However, effects independent of this activity have also been demonstrated [17]. For example, genistein also inhibits topoisomerase I and II [18], 5α-reductase [19] and protein histidine kinase [20], all of which may contribute to the antiproliferative or pro-apoptotic effects of genistein. It has been found that soy isoflavones, including genistein, have antioxidant effects and protects cells against reactive oxygen species by scavenging free radicals, inhibiting the expression of stress response related genes, thereby reducing carcinogenesis [21;22]. Genistein has been shown to inhibit the growth of both estrogen and androgen receptor positive and negative breast and prostate cancer cells in vitro, respectively, and showed inhibitory effect on estrogen-stimulated growth of breast cancer cells [23;24]. Furthermore, we have found that genistein is a powerful inhibitor of NF-κB and Akt signaling pathways, both of which are important for cell survival [25]. These effects of genistein are believed to be involved in the induction of apoptotic processes in genistein-treated cells. Collectively, the knowledge regarding the effects of genistein on cancer cells is rapidly growing although it is clear that genistein is a powerful agent whose utilization for the prevention and/or treatment of cancer is likely be forthcoming.

2. Molecular targets and effects of genistein in vitro

Extensive experiments have concluded that genistein functions as a promising chemopreventive agent that inhibits carcinogenesis. Additionally, genistein has been shown to inhibit the growth of various cancer cells through the modulation of genes that are intimately related to the regulation of cell cycle and programmed cell death (apoptosis). Table-1 briefly summarizes multiple molecular targets of genistein action. Genistein also intervenes in several cellular transduction signaling pathways inhibiting carcinogenesis and may also be involved in the regulation of gene activity by modulating epigenetic events such as DNA methylation and/or histone acetylation directly or through the estrogen receptor dependent process [26]. Genistein can also up-regulate mRNA expression of the BRCA1 gene during mammary tumorigenesis, which is frequently inactivated by epigenetic events in breast cancer [27]. Moreover, it has been demonstrated that the angiogenesis and metastasis could also be inhibited by genistein, implying the pleiotropic effects of genistein on the inhibition of carcinogenesis and cancer cell growth.

2.1. Effects on cell cycle regulation

Experiments have shown that genistein inhibits the growth of several cancer cells including leukemia, lymphoma, ovarian, cervical, leiomyoma, melanoma, neuroblastoma, gastric, pancreatic, breast, and prostate cancer cells [23;24;28–31]. The growth inhibition of cancer cells could be due to cell cycle arrest, which ultimately results in cessation of cell proliferation. It has been demonstrated that genistein induces a G2/M cell cycle arrest in breast cancer, gastric adenocarcinoma and melanoma cells [31;32]. We also showed that genistein induces a G2/M cell cycle arrest in PC3 and LNCaP prostate cancer cells; H460 and H322 non small cell lung cancer cells; MDA-MB-231 and MCF-10CA1a breast cancer cells [33;34]. Genistein also causes G2/M arrest in normal MCF10A breast epithelial cells [35]. However, the effect of genistein was more pronounced in malignant cells compared to normal cells. Thus, it is generally accepted that genistein can cause G2/M cell cycle arrest, but a report has shown that genistein could also arrest mouse fibroblast and melanoma cells at G0/G1 phase of the cell cycle [36]. These data suggests that genistein induces either G2/M or G0/G1 cell cycle arrest, depending on cell lines.
Cell cycle progression is known to be tightly regulated by different cyclins, cyclin dependent kinases (CDKs) and cyclin dependent kinase inhibitors (CDKIs) in different phases of the cell cycle. Cancer cells treated with different concentrations of genistein showed a dose-dependent decrease in the expression of cyclin B, which plays important roles in the positive regulation of CDK activity and is necessary for forming cyclin B/CDK complex during the G2/M phase procession. These observations are in concordance with the G2/M cell cycle arrest, suggesting that genistein-induced cell cycle arrest in cancer cells is partially due to the down-regulation of cyclin B [33;34]. The activities of cyclins/CDKs complexes are negatively regulated by several CDK inhibitors (CDKIs) including p21\textsuperscript{WAF1}, p27\textsuperscript{KIP1} and p16\textsuperscript{INK4a}. We have found a significant dose-dependent up-regulation of p21\textsuperscript{WAF1} expression in genistein treated cancer cells including MDA-MB-231, MDA-MB-435 and MCF-7 breast cancer cells; PC3 and LNCaP prostate cancer cells; H460 and H322 non-small cell lung cancer cells; and HN4 head and neck squamous carcinoma cells [33;34;37–39]. Touny and Banerjee [40] reported the involvement of upstream kinases Myt-1 and Wee-1 in the transcriptional repression of cyclin B1 and the activation of p21\textsuperscript{WAF1} in prostate cancer cells. They found genistein treatment increased Myt-1 levels and decreased Wee-1 phosphorylation, providing better insight into the possible mechanism of genistein-induced G2/M arrest. These findings closely parallel with results on the inhibition of cancer cell growth and cell cycle arrest, suggesting that genistein can inhibit the growth of cancer cells by modulating the expression of genes that are involved in the regulation of cell growth and the cell cycle.

2.2. Effects on the induction of apoptosis

In addition to cell cycle arrest, another specialized event of genistein action involves the induction of programmed cell death known as ‘apoptosis’. This is mediated by a diverse group of protein moieties in cells, namely the Bcl-2 family, along with a concerted cascade of proteolytic activity of a family of asparate-specific cysteinyl proteases, or caspases activation, leading to the digestion of structural proteins, DNA degradation, and ultimately phagocytosis. The Bcl-2 family is the best characterized group of apoptosis mediating factors and can be divided into two main groups according to their functional properties: anti-apoptotic proteins, for example Bcl-x\textsubscript{L} and Bcl-2; and pro-apoptotic proteins, such as Bax, Bak, and Bad. The data from our laboratory showed that genistein could induce apoptosis in MDA-MB-231, MDA-MB-435 and MCF-7 breast cancer cells; PC3 and LNCaP prostate cancer cells; H460 and H322 non-small cell lung cancer cells; HN4 head and neck squamous carcinoma cells, and pancreatic cancer cells [33;34;37–39;41;42]. Using multiple assay techniques as hallmark to detect apoptosis, we found genistein induced apoptosis in all cancer cells tested. Flow cytometry revealed that the number of apoptotic cells increased 43–57% with longer genistein treatment. These results are consistent with studies reported by other investigators [43;44], clearly attesting to the fact that genistein induces apoptosis in cancer cells. This was further corroborated by recent findings of Moiseeva et al., who reported that physiological concentrations of a dietary phytochemical including genistein results in reduced growth and induction of apoptosis of in cancer cells [45].

To explore the molecular mechanism by which genistein induces apoptosis, we studied the effect of genistein on Bcl-2, Bax and caspases in multiple cell lines and found down-regulation of Bcl-2 protein expression, up-regulation of Bax expression, and activation of caspasas after treatment with genistein. Other investigators have also reported that soy isoflavone genistein could induce apoptosis in a variety of human cancer cells through caspase-3 activation and down-regulation of Bcl-2, Bcl-x\textsubscript{L}, and HER-2/neu [46;47]. Furthermore, the p53 and p21\textsuperscript{WAF1} tumor suppressor genes are also known to be involved in apoptotic processes, and we have detected the expression of p53 gene in MDA-MB-231 breast cancer cells, which are ER-negative and harbor mutant p53. Although the treatment of these cells with genistein down-regulated the expression of the dysfunctional p53, the expression of p21\textsuperscript{WAF1} was induced.
within 24 h [37]. These results suggest that the induction of \( p21^{\text{WAF1}} \) and apoptosis by genistein is functionally operated through a p53-independent pathway. A study reported by Kazi et al. [48] showed that genistein induced apoptosis by inhibiting proteosome and induction of \( p27^{\text{KIP1}} \), \( \text{IxBa} \), and \( \text{Bax} \). A recent study showed that in hepatocellular carcinoma, genistein induced apoptosis by the activation of several endoplasmic reticulum (ER) stress-relevant regulators, which include the transcription factor-GADD153, \( m\)-calpain, GRP78 and caspase-12 [49]. Taken together, these findings suggest that ER stress, caspase activation, inhibition of proteosome, down-regulation of \( \text{Bcl-2} \), \( \text{Bcl-xL} \), and \( \text{HER-2/neu} \) may partly represent the molecular mechanism by which genistein induces apoptosis, and the existing evidence suggests that many of these cascades may also be regulated either directly or indirectly by nuclear factor-\( \kappa \)B (NF-\( \kappa \)B).

### 2.3. Effects on inhibiting the activation of NF-\( \kappa \)B

NF-\( \kappa \)B plays important roles in the control of cell growth, differentiation, apoptosis and stress response. Under non-stimulating conditions, NF-\( \kappa \)B is sequestered in the cytoplasm through tight association with the impeding \( \text{IxB} \) proteins. Following stimulation, \( \text{IxB} \) protein is phosphorylated and degraded, allowing the NF-\( \kappa \)B to translocate to the nucleus, bind to the NF-\( \kappa \)B-specific DNA-binding sites or interact with other transcription factors, and thus regulate gene transcription. We have reported that genistein treatment could modulate NF-\( \kappa \)B DNA binding activity in prostate, breast, head and neck, and pancreatic cancer cells by electrophoresis mobility shift assay (EMSA) [37;50;51]. In concordance with our findings Natrajan et al also found that in human myeloid leukemia cells genistein blocked activation of NF-\( \kappa \)B concomitant with degradation of \( \text{IxBa} \) [52].

We have further investigated whether genistein could block NF-\( \kappa \)B induction by known inducers such as \( \text{H}_2\text{O}_2 \) and TNF-\( \alpha \), both of which has been previously shown to induce NF-\( \kappa \)B DNA binding activity. After treatment with \( \text{H}_2\text{O}_2 \) or TNF-\( \alpha \), we observed an increase in NF-\( \kappa \)B DNA binding activity in prostate cancer cells, which supports the findings reported by Natrajan et al. [52]. However, when the cells were treated with 50 \( \mu \text{M} \) genistein for 24 h prior to stimulation with the inducing agent, genistein abrogated the induction of NF-\( \kappa \)B DNA binding activity elicited by either \( \text{H}_2\text{O}_2 \) or TNF-\( \alpha \) [53]. Furthermore, we found that genistein inhibited the phosphorylation of \( \text{IxB} \). By immunohistochemistry and confocal microscopic analysis we also found that the treatment of cells with genistein significantly decreased the nuclear staining of the NF-\( \kappa \)B. These results indicate that genistein inhibits the translocation of NF-\( \kappa \)B to the nucleus, preventing NF-\( \kappa \)B from binding to its target DNA and thereby inhibiting the transcription of NF-\( \kappa \)B downstream genes. This process ultimately inhibits cell growth and also induces apoptotic cell death. Although controversies are sprouting whether NF-\( \kappa \)B could also function as tumor suppressor gene and thus inactivation of NF-\( \kappa \)B could be tumor promoting although the exact role of NF-\( \kappa \)B certainly merits further investigation.

It has been reported that in the NF-\( \kappa \)B signaling pathway, \( \text{IxBa} \) is phosphorylated by \( \text{IxB} \) kinase \( \alpha \) (\( \text{IKK}\alpha \)) and \( \text{IxB} \) kinase \( \beta \) (\( \text{IKK}\beta \)), while \( \text{IKK} \) is phosphorylated and activated by the upstream molecule, mitogen activated kinase 1 (MEKK1) [54;55]. We have found that genistein treatment did not alter the protein expression of MEKK1; however, genistein treatment inhibited MEKK1 kinase activity when tested by a kinase assay. These results demonstrate that genistein inhibits MEKK1 activity, which may be responsible for the decreased phosphorylation of IxB, thereby, resulting in the inactivation of NF-\( \kappa \)B (unpublished data).

Genistein has also been found to potentiate the antitumor activity of chemotherapeutic agents through regulation of NF-\( \kappa \)B. It has been reported that some chemotherapeutic agents such as cisplatin, gemcitabine and docetaxel induce the activation of NF-\( \kappa \)B in cancer cells and this may be responsible for drug resistance in cancer cells [42;56;57]. By \textit{in vitro} and \textit{in vivo} studies, we have found that pre-treatment with genistein followed by treatment with lower doses of...
docetaxel or cisplatin elicited significantly greater inhibition of cell growth and induction of apoptosis compared to either agent alone [41;42;56-58]. By EMSA, we found that NF-κB activity was significantly increased by docetaxel, gemcitabine or cisplatin treatment, and the NF-κB inducing activity of these agents was completely abrogated in cells pre-treated with genistein. These in vitro results were also recapitulated in our in vivo studies [41;42;59]. Our results clearly suggest that genistein pre-treatment, which inactivates NF-κB activity, together with other cellular effects of genistein, may contribute to increased cell growth inhibition and apoptosis with non-toxic doses of docetaxel, cisplatin, or gemcitabine.

2.4. Effects on regulation of Akt signaling pathway

Akt signaling is another important transduction pathway that plays a critical role in controlling the balance between cell survival and apoptosis [92]. Evidence suggests that Akt also regulates the NF-κB pathway via phosphorylation and activation of molecules in the NF-κB pathway [60;61]. Thus, strategies to block the activity of Akt would ideally lead to the inhibition of proliferation and the induction of apoptosis. By immunoprecipitation, Western blot and kinase assays we found that genistein treatment reduced the level of the phosphorylated Akt protein at Ser473 compared to control cells, resulting in a dose dependent induction of apoptosis after genistein treatment of cells that display constitutively active Akt [41]. Additional studies were carried out to examine the status of Akt in the PC-3 prostate cancer cells treated with genistein followed by EGF stimulation. We found that EGF treatment alone activated Akt kinase as expected, while genistein pre-treatment abrogated the activation of Akt by EGF [25]. This data demonstrates that genistein inhibits the activation of Akt, which may result in the inhibition of survival signals ultimately leading to induction of apoptotic signals.

We have explored the molecular cross talk between Akt and NF-κB signaling pathways by conducting transfection experiments and found that genistein exerts its inhibitory effects on NF-κB pathway through the Akt signaling pathway [25]. Several reports from other investigators also showed similar regulation between Akt and NF-κB pathways [60–62] and these results strongly suggest molecular cross-talk between NF-κB and Akt pathway and that ‘dual’ disruption of these pathways by genistein could be an effective strategy for the inhibition of cancer cells. Stoica et al., demonstrated that genistein exerted inhibitory effect on Akt activation induced by estradiol in MCF-7 cells [63;64]. El Touny and Banerjee [65] recently documented that the chemopreventive action of genistein in vivo is mediated through the Akt-GSK-3β signaling downstream effectors retarding cancer progression. Collectively, these results demonstrate that genistein exerts its inhibitory effect on NF-κB signaling through Akt pathway. Thus, abrogation of NF-κB and Akt signaling pathway by genistein may be one of the molecular mechanisms by which genistein inhibits cancer cell growth and induces apoptosis.

2.5. Effects on the regulation of androgen-mediated carcinogenesis

Research on androgens showed that androgens are involved in the development and progression of prostate cancer via activating the androgen receptor (AR) [66]. Prostate-specific antigen (PSA) is a clinically important AR-responsive gene which is used to monitor treatment response, prognosis, and progression in patients with prostate cancer [67]. It has been demonstrated that the transcriptional regulation of PSA occurs via AR binding to the AR-responsive element (ARE) in the promoter region of PSA [68]. The expression of PSA is initially regulated by androgen through the regulation of AR, and undergoes a sharp decline after medical castration [69]. The tumor then becomes androgen-independent and PSA expression is constitutively up-regulated through an unknown mechanism, suggesting the importance of PSA in prostate carcinogenesis.
We have previously demonstrated that genistein has different effects on ARE binding and the expression of AR and PSA in androgen-sensitive (LNCaP) and androgen-insensitive (VeCaP) prostate cancer cells. Genistein transcriptionally down-regulated AR, decreased nuclear protein binding to ARE, thereby, inhibiting the transcription and protein expression of PSA in androgen-sensitive LNCaP cells [70]. Genistein treatment also resulted in a dose and time dependent decrease in the secreted PSA in the media collected from LNCaP cells treated with low concentration of genistein (0.1–5 μM). In contrast, genistein did not alter AR expression and binding of nuclear AR to the ARE at low concentration in VeCaP cells. However, higher concentrations (10–50 μM) of genistein were able to significantly inhibit PSA secretion in VeCaP cells. Further studies using transient transfection of a PSA promoter construct revealed that genistein can inhibit PSA synthesis in prostate cancer cells through an androgen-dependent or androgen-independent pathway highlighting the fact that genistein inhibits cell proliferation independent of androgen and PSA signaling pathways. These studies strongly support the role of genistein as a chemopreventive/therapeutic agent for prostate cancer, irrespective of androgen responsiveness. Genistein has also been shown to bind directly to the estrogen receptor and modulate its function [71], suggesting the inhibitory effects of genistein on both androgen and estrogen-mediated carcinogenesis. However, further studies are required to fully understand the complex regulation of ER and AR pathways during genistein induced cell growth inhibition and apoptosis.

2.6. Effects on the regulation of MAPK Pathway

MAPK pathway consists of a three tiered kinase core where MAP3K activates a MAP2K that activates a MAPK (ERK, JNK, and p38), resulting in the activation of NF-κB and cell survival [72;73]. It has been reported that activation of the MAPK pathways may cause the induction of phase II detoxifying enzymes, and inhibition of MAPK pathways may inhibit AP-1-mediated gene expression [74].

Genistein has been found to regulate the molecules in the MAPK pathway. Huang et al., reported that genistein inhibited TGF-β–mediated p38 MAP kinase activation, matrix metalloproteinase type 2, and cell invasion in human prostate epithelial cells [75]. In other studies, genistein has been found effective in preventing cytokine- induced ERK-1/2 activation and promoted apoptotic cell death [76;77]. Since genistein is a well known inhibitor of tyrosine kinase, it is possible that genistein may inhibit tyrosine kinase upstream of p38 MAPK and subsequently inhibit the phosphorylation of tyrosine on p38 MAPK, leading to the inactivation of MAPK pathway.

2.7. Anti-oxidation effects

Isoflavones, including genistein, are known antioxidants. Genistein has been shown to protect cells against reactive oxygen species (ROS) by scavenging free radicals and reducing the expression of stress-response related genes [21;22]. It has been demonstrated that genistein inhibits tumor-promoter, 12-O-tetradecanoylphorbol-13-acetate- induced hydrogen peroxide production as well as its function in human polymorphonuclear leukocytes, and HL-60 cells [78;79]. Furthermore, as a follow up to genistein action showing antioxidant capacity, its effect on activation of the transcription factors- Nrf1 and Nrf2, which have been implicated in the regulation of genes involved in response to oxidative stress, was investigated [80]. These transcription factors are involved in the regulation of γ-GCS and other detoxification proteins. Genistein was found to induce the cytosolic accumulation and nuclear translocation of Nrf1 and Nrf2 which closely paralleled changes in glutathione peroxidase (GPx) mRNA levels and also the activity of GPx [80]. Genistein has also been shown to stimulate antioxidant protein gene expression in Caco-2 cells [81]. It has been reported that oxidative stress activates NF-κB [82] and our data showed that genistein is an antioxidant by virtue of its inhibition of the activation of NF-κB stimulated by oxidant stress [53]. Thus, the ability of genistein in inhibiting...
the generation of ROS, resulting in the inhibition of NF-κB activation, make it a strong candidate as an antioxidant and a powerful chemopreventive agent.

2.8. Regulation of other pathways

Cyclooxygenase-2 (COX-2) is a critical enzyme catalyzing synthesis of bioactive prostaglandin E2 (PGE2) from the substrate arachidonic acid (AA) and is found to be overexpressed in many human tumor tissues. COX-2 is known to increase cell proliferation and VEGF production, induce angiogenesis, and possess anti-apoptotic effects. Genistein and other soy isoflavones have been found to be effective not only in reducing COX-2 expression but also for antagonizing AA for controlling PGE2 production and invasiveness of the breast cancer MDA-MB231 cells through downregulation of EGFR and HER-2/neu activity and by modulating the level of NF-κB expression. Further transcriptional control studies by Lau and Leung [83] identified activator protein-1 (AP-1)/cyclic AMP response element binding protein (CREB) binding site in the COX-2 promoter which is critical for COX-2 expression. Genistein suppressed AP-1/CREB binding, resulting in reduced COX-2 expression, which could be important in the post-initiation events of breast carcinogenesis. In addition, genistein has shown to be beneficial in combination with 5-Flurouracil (5-FU) in the treatment of colon cancer through the COX pathway [84].

Genistein also inhibits insulin-like growth factor-1 receptor (IGF-1R) signaling, resulting in the inhibition of cell proliferation and induction of apoptosis. Moreover, Raffoul et al. reported that genistein also enhanced prostate cancer radiotherapy through the downregulation of apurinic-apyrimidine endonuclease 1/redox factor-1 expression [85]. Among the STAT family of transcription proteins, constitutive activation of STAT-3 and STAT-5 has been identified as responsible for cell survival and growth by preventing apoptosis through increased expression of antiapoptotic proteins such as Bcl-2 and Bcl-xL. Genistein has been shown to inhibit phosphorylation of these transcription proteins which, in turn, may inhibit the constitutive and abnormal signaling cascade, promoting survival and growth of tumor cells [76]. Expression profiling of rat mammary epithelial cells by Su et al., [86] confirmed the differential expression of Wnt (Wnt5a, Sfrp2) and Notch (Notch2, Hes1) signaling components by soy protein isolate and/or genistein using quantitative real-time PCR. Wnt pathway inhibition by genistein was supported by reduced cyclin D1 immunoreactivity in mammary ductal epithelium in the genistein treated group, despite comparable levels of membrane-localized E-cadherin and beta-catenin.

3. Inhibition of carcinogenesis in vivo by genistein

There is growing in vivo evidence demonstrating the inhibitory effects of genistein on carcinogenesis. Although Hawrylewicz et al [87] and Ravindranath et al [88] have published data pertaining to animal studies and anticancer potential of soy isoflavone genistein, here we summarize the in vivo studies in a comprehensive fashion.

3.1. Inhibition of cancers in animal

It has been reported that genistein has a protective role against carcinogenesis in animals. Prepubertal exposure to soy or genistein reduced mammary carcinogenesis in rats treated with carcinogens, possibly by modulating the development of the mammary end buds [27;89]. One of the early studies revealed that soy-containing diets reduced the severity of prostatitis in rats [90]. Soy isoflavone supplemented diets also prevented the development of adenocarcinomas in the prostate and seminal vesicles in a rat carcinogenesis model [91]. It has also been reported to be effective in chemical carcinogen-induced rat ovarian carcinogenesis [92]. The soy diet reduced growth of transplantable prostate adenocarcinomas and inhibited tumor cell proliferation and angiogenesis of transplantable prostate cancer in immunodeficient mice.
A diet rich in soy also inhibited pulmonary metastasis of melanoma cells in C57BL/6 mice [95]. Genistein inhibited the growth of carcinogen-induced cancers in rats and human leukemia cells transplanted into mice [87;88;96;97]. Singh et al [98] evaluated the natural form of genistein, and the isoflavone-rich soy phytochemical concentrate (SPC) on the growth and metastasis of human bladder cancer cells 253J BV-induced tumors in an orthotopic site. Both treatment regimes were effective in reducing tumor weight by more than 50%, accompanied by induction of tumor cell apoptosis and inhibition of tumor angiogenesis in vivo. However, SPC treatment was significantly better, which inhibited lung metastases by 95% and reduced circulating insulin-like growth factor-I levels [98]. Furthermore, genistein protects the skin from the effect of long-term psoralen plus ultraviolet A radiation (PUVA) therapy which is associated with an increased risk of squamous cell carcinoma and malignant melanoma [99;100]. Additionally genistein is also reported to substantially inhibit skin carcinogenesis and cutaneous aging induced by ultraviolet (UV) light in mice [99]. In a murine PTEN (mPTEN) heterozygous (+/−) mutant mouse model for endometrial carcinoma, as well as in estrogen-related endometrial carcinogenesis, genistein exerted an inhibitory effect on PTEN-related tumorigenesis [101]. Genistein also attenuated gastric carcinogenesis promoted by sodium chloride in a rat model of gastric cancer [102]. Thus, a growing body of literature provide strong evidence to support the role of the various soy products containing genistein in the protection against carcinogenesis in animal models.

3.2. Inhibition of NF-κB activation supporting antioxidant effect of isoflavone in humans

We have investigated the effects of isoflavone supplementation on NF-κB activation in vivo in human volunteers [53]. Genistein is an antioxidant as indicated earlier, thus, soy isoflavone supplementation is expected to inhibit NF-κB activation and, in turn, may reduce the oxidative damage in human lymphocytes. The lymphocytes from healthy male subjects were harvested from peripheral blood and cultured for 24 h in the absence and presence of genistein. EMSA revealed that genistein treatment inhibited basal levels of NF-κB DNA binding activity by 56% and abrogated TNF-α induced NF-κB activity by 50%. Furthermore, when human volunteers received 50 mg of soy isoflavone supplements (Novasoy™) twice daily for three weeks, TNF-α failed to activate NF-κB activity in lymphocytes harvested from these volunteers, however lymphocytes from these volunteers collected prior to soy isoflavone intervention showed activation of NF-κB DNA-binding activity upon TNF-α treatment ex vivo [53]. These results demonstrate that soy isoflavone supplementation has a protective effect against TNF-α induced NF-κB activation in humans in vivo. We have also measured the levels of oxidative DNA damage in the blood of the six subjects before and after supplementation with Novasoy™. The results demonstrate that isoflavone supplementation reduced the levels of the 5-OhmdU and decreased oxidative damage in human subjects, which provided strong evidence that soy isoflavone functions as antioxidant. and these effects of genistein could be responsible for its chemopreventive activity [53].

4. Effects on the inhibition of angiogenesis and metastasis

Matrix metalloproteinases (MMPs) are proteolytic enzymes believed to provide cancer cells with their invasive potential by degrading the extracellular matrix. Genistein has been shown to reduce the angiogenic and metastatic potential of cancers [38;103;104]. Additionally, genistein also significantly decreased the incidence of cancer cell invasion into the lymphatic vessels attenuating cancer metastasis. Our laboratory has examined the inhibitory effect of genistein on tumor cell invasion and metastasis of MDA-MB-435 breast cancer cells transfected with c-erbB-2, which has been shown to promote secretion of MMPs and subsequent metastasis in experimental models [38]. We found that the expression of c-erbB-2, MMP-2, and MMP-9 in MDA-MB-435 cells stably transfected with c-erbB-2, was much higher than that in parental MDA-MB-435 cells. However, the high expression of c-erbB-2,
MMP-2, and MMP-9 in MDA-MB-435 transfectants was significantly down-regulated by genistein treatment. These results suggest that increased c-erbB-2 expression in MDA-MB-435 transfectants may result in increased secretion of MMPs, and that genistein may inhibit the expression of c-erbB-2 and subsequently decrease the secretion of MMPs in breast cancer cells. An interesting finding reported by Owen et al., showed that genistein was effective in decreasing the constitutively high levels of MMP-9 within T-lymphocytes harvested from mammary tumor bearing mice [105].

By gene expression profiling of genistein treated PC-3 prostate cancer cells and PC-3 bone tumor, we also found that genistein down-regulated the expression of MMP-9, MMP-2, protease M, uPAR, VEGF, neuropilin, TSP, BPGF, LPA, TGF-β, TSP-1, and PAR-2, and up-regulated the expression of connective tissue growth factor and connective tissue activation peptide [106]. All of these genes are related to angiogenesis and metastasis. These findings were further supported by studies reporting inhibition of MMP-2 activation and reduction of prostate cancer cell invasion by genistein [75;107]. Another oligonucleotide microarrays study has been reported by Lee wherein the gene expression profile by genistein treatment in breast cancer cells was investigated [108]. Accordingly, this author has shown that TFPI-2, ATF3, DNMT1, and MTCBP-1, which inhibit invasion and metastasis, were upregulated, and MMP-2, MMP-7, and CXCL12, which promote invasion and metastasis, were downregulated.

However, as a corollary to our in vitro study, we have further investigated the effect of dietary genistein on the growth of metastatic prostate cancer cells in a SCID-human experimental model of prostate cancer bone metastasis. Our results demonstrate that genistein inhibited prostate cancer cell growth in the bone environment and down-regulated the transcription and translation of genes critically involved in the control of tumor cell invasion and metastasis in vitro and in vivo, suggesting the possible therapeutic role of genistein for metastatic prostate cancer [109]. Other investigators have also demonstrated similar results showing that isoflavones inhibited bone metastasis of human breast cancer cells in a nude mouse model and metastasis of androgen-sensitive human prostate tumors in mice [110;111]. Furthermore, we documented that genistein also intervenes in the regulation of the osteoprotegerin/receptor activator of NF-κB (RANK)/RANK ligand/MMP-9 signaling in prostate cancer, suggesting that isoflavone genistein could be a promising non-toxic agent augmenting the therapeutic outcome of metastatic prostate cancer with chemotherapeutic drugs [59]. Since cancer metastases follows a multi-step pathway wherein invasion and cell motility is an early step, genistein at physiological relevant concentrations has been shown to be effective in exerting an inhibitory effect on the migration of prostate cancer cells [112]. Complementing these report, Craft et al., recently demonstrated that genistein also has the potential to therapeutically compensate endoglin deficiency- a key regulator of cell motility in prostate cancer [113]. Another recent report showed that genistein induced metastatic suppressor kangai-1 (KA11), suggesting that genistein could be used for anti-metastatic therapies [114].

Angiogenesis is the formation of new blood vessels and it is essential for normal reproductive function, development and wound repair processes. However, angiogenesis in solid tumors are important and necessary for promoting the proliferation, invasion and metastasis of cancer cells. It has been found that genistein inhibits vessel endothelial cell proliferation and in vitro angiogenesis at half maximal concentration of 5 and 150 μM, respectively, suggesting that genistein is a potent inhibitor of vascularization and cancer cell growth [104]. TGF-β is a known major factor that regulates cell proliferation [115], and TGF-β signaling is an important feature in the up-regulation of angiogenesis [116]. Genistein has been known to inhibit TGF-β signaling, and therefore inhibit angiogenesis [116]. Further evidence in support of soy-based foods as natural dietary inhibitors of tumor angiogenesis was reported in a study by Su et al. [117]. The efficacy of soy isoflavones on angiogenesis inhibition in vivo was examined by nude mice xenograft and chick chorioallantoic membrane bioassay. Factors analyzed included...
angiogenic factors, matrix-degrading enzymes, and angiogenesis inhibitors. Genistein was the most potent inhibitor of angiogenesis \textit{in vitro} and \textit{in vivo} among the isoflavone compounds tested. It may also account for most of the reduced microvessel density observed in xenografts and the suppressed endothelial migration by soy isoflavones. Genistein exhibited a dose-dependent inhibition of expression/excretion of vascular endothelial growth factor\textsubscript{165}, platelet-derived growth factor, tissue factor, urokinase plasminogen activator, and matrix metalloprotease-2 and 9, respectively. On the other hand, there was an up-regulation of angiogenesis inhibitors- plasminogen activator inhibitor-1, endostatin, angiostatin, and thrombospondin-1. In addition, a differential inhibitory effect between immortalized uroepithelial cells and most cancer cell lines was also observed. All these reports suggest that tissue factor, endostatin, and angiostatin are novel molecular targets of genistein.

Recently, Guo et al. [118] documented that genistein significantly reduced nuclear accumulation of hypoxia-inducible factor-1\textalpha in PC-3 cells, which is the principle transcription factor that regulates VEGF expression in response to hypoxia. These observations support the hypothesis that genistein may inhibit prostate tumor angiogenesis through the suppression of VEGF-mediated autocrine and paracrine signaling pathways between tumor cells and vascular endothelial cells. Hence, we believe that there is ample evidence to suggest that genistein is a potent anti-angiogenic agent and its application in human awaits further investigation.

5. The sensitizing effect of genistein in cancer treatment

In recent years, novel combination treatments with conventional cancer therapies and chemopreventive agents have received much attention in cancer research. More importantly, the published studies have shown that isoflavone genistein could potentiate the antitumor effects of chemotherapeutic agents in various cancers \textit{in vitro} and \textit{in vivo} in preclinical studies. We have reported \textit{in vitro} that genistein poteniated growth inhibition and apoptotic cell death caused by cisplatin, erlotinib, docetaxel, doxorubicin, gemcitabine, and CHOP (cyclophosphamide, doxorubicin, vincristine, prednisone) in cancers of prostate, breast, pancreas, and lung and lymphoma [56–58;119–121]. We have also found that dietary genistein \textit{in vivo} could enhance the antitumor activities of gemcitabine and docetaxel in a tumor model, resulting in apoptotic cell death and the inhibition of tumor growth [42;109]. Similar observations has been reported by other investigators showing that the antitumor effects of chemotherapeutics, including 5-fluorouracil (5-FU), adriamycin, cytosine arabinoside, tamoxifen and perifosine could be potentiated by genistein [84;122–126]. Genistein also enhanced the antitumor effect of bleomycin in HL-60 cells, but not in normal lymphocytes in an \textit{in vitro} study [127]. The synergistic action of genistein and cisplatin or carmustine (BCNU) on the growth inhibition of glioblastoma and medulloblastoma cells has also been observed [128]. In ovarian cancer, genistein potentiated the antiproliferative and proapoptotic effect of antibodies directed against the cell adhesion molecule L1-CAM [129]. Furthermore, despite limitations in the cytotoxic effect of the tumor necrosis factor-related apoptosis-inducing ligand (TRAIL/Apo2L) in gastric and pancreatic adenocarcinoma cell lines, subtoxic concentrations of genistein sensitized these TRAIL-resistant cells to TRAIL/Apo2L-mediated apoptosis [130;131]. In radiotherapy, experimental studies from Dr. Hillman’s laboratory have demonstrated that the combination of genistein and radiation exert enhanced inhibitory effects on tumor growth and progression of renal cell carcinoma and prostate tumor in orthotopic models [132;133]. Genistein also enhanced radiosensitivity in human esophageal, and cervical cancer cells, suggesting the beneficial effects of genistein in cancer radio-therapy [134;135]. These reports clearly demonstrate that genistein could be used in cancer treatment to further enhance the antitumor activities of conventional therapeutics.
6. Genistein analogues and related studies

To enhance the antitumor activity of isoflavone, several isoflavone derivatives have been synthesized and used in *in vitro* and *in vivo* experiments and in clinical trials. These compounds have shown a low IC$_{50}$ in the inhibition of cancer cell growth *in vitro*. Moreover, at low concentrations, these compounds were able to enhance the antitumor activity of clinically available chemotherapeutic agents, suggesting their potent effects as therapeutic agents for combination treatment. Phenoxodiol is one such analog of isoflavone genistein and has shown a broad-spectrum, anticancer effect. In an animal study, phenoxodiol inhibited dimethyl-benz (α)anthracene (DMBA)-induced mammary carcinogenesis in female Sprague-Dawley rats, suggesting that phenoxodiol is an effective chemopreventive agent against DMBA-induced carcinogenesis [136]. In experimental studies and clinical trials, phenoxodiol has been used both as a mono-therapy and in combination with standard chemotherapeutics. These studies have shown that in some cancers phenoxodiol appears to be strong enough to work on its own as a monotherapy. However, one of the major benefits of phenoxodiol is its ability to sensitize cancer cells to the antitumor effects of conventional chemotherapeutics [137]. It has been found that cancer cells that have become resistant to the effects of conventional chemo-therapeutics, phenoxodiol could restore chemosensitivity [138]. Therefore, by exposing chemoresistant cancer cells to phenoxodiol first, long-standing drug resistance is removed, making cancer cells susceptible once again to standard chemotherapeutics, such as cisplatin, carboplatin, taxanes, and gemcitabine. Phenoxodiol is currently undergoing clinical studies in the USA and Australia. So far, phase I/II clinical trials have shown some disease stabilization without severe toxicity [138]. We have also synthesized structurally-modified derivatives of isoflavone based on the structural requirements for optimal anti-cancer effect [139]. We found that these synthetic derivatives of isoflavone exerted higher anti-cancer activity with lower IC$_{50}$. These derivatives of isoflavone also induced more apoptosis compared to genistein. Other investigators also synthesized a series of genistein derivatives and evaluated either their cytotoxic potential and/or protective efficacy against hydrogen peroxide induced endothelial cell damage [140;141]. Some of these have effect comparable to 5-Flurauracil in potency of their cytotoxicity [141]. These results suggest that genistein and synthetic structurally-modified derivatives of isoflavone may be promising agents for cancer chemoprevention and therapy either alone or in combination with existing chemotherapeutic agents.

7. Clinical trials: effects on patients with prostate cancer

Several phase I and II clinical trials using isoflavone supplementation have been conducted in the patients with prostate cancer. In phase I clinical trials, the safety, pharmacokinetic parameters, and efficacy of orally administered isoflavones have been determined [10;142; 143]. No toxicity has been observed in the subjects. Oral administration of soy isoflavones gives plasma concentrations of genistein up to 16.3 μM that have been associated with anti-metastatic activity *in vitro* [10]. No genotoxicity has been found in subjects treated with a purified soy unconjugated isoflavone mixture [143].

Our *in vitro* data demonstrated the inhibition of prostate cancer cell growth and decreased PSA expression in LNCaP cells by genistein. Hence, we conducted a phase II clinical trial to investigate the modulation of serum PSA levels in patients with prostate cancer by soy isoflavone supplementation. Patients with prostate cancer were eligible to participate if they had rising PSA levels and were previously untreated (Group I), treated with local therapy (Group II), or treated with hormone therapy (Group III), and had either three successive rising PSA levels or a PSA of > 10 ng/ml at two successive evaluation. No other therapy or supplements were allowed during the study period. Patients received 100 mg Novasoy™ (Archer Daniels Midland Company, Decatur, IL, USA) orally twice daily for a minimum of three months in the absence of progression or toxicity. Novasoy™ contains genistein, daidzein,
and glycitin at a 1.3:1.0.3 ratios. Serum PSA, IGF-1 and IGFBP-3 levels were measured and toxicity was assessed. Serum PSA levels were monitored at baseline and monthly during the study. The results showed that soy isoflavone supplementation inhibited the linear rise in PSA in both androgen sensitive and androgen insensitive patient populations. There were no statistically significant changes in the plasma levels of IGF-1 and IGFBP-3. These data demonstrated that soy isoflavone supplementation decreases the rate of rise in serum PSA levels without any toxicity in prostate cancer patients. The lack of significant side effects of soy isoflavone makes it an ideal agent for patients with advanced disease for further studies. Nevertheless, the results of in vitro studies along with numerous in vivo studies in different animal models, and our pilot in vivo human studies collectively point towards a favorable application of genistein as chemopreventive and/or therapeutic agent for prostate and other cancers.

A phase II randomized, placebo-controlled clinical trial using purified isoflavones in modulating steroid hormones in patients with localized prostate cancer has been reported recently [144]. Although significant increases in plasma isoflavones (P < 0.001) was observed with no clinical toxicity, the corresponding modulation of serum SHBG, total estradiol, and testosterone in the isoflavone-treated group compared to men receiving placebo was nonsignificant. Increasing plasma isoflavones failed to produce a corresponding modulation of serum steroid hormone levels in men with localized prostate cancer, suggesting the need to explore other potential mechanisms by which prolonged and consistent purified isoflavone consumption may modulate prostate cancer risk [144].

Recently, the investigators from our institute reported the results from a phase II clinical trial designed to investigate the efficacy of lycopene alone or in combination with soy isoflavones on serum PSA levels in men with prostate cancer [145]. 35 of 37 (95%) evaluable patients in the lycopene group and 22 of 33 (67%) evaluable patients in the lycopene plus soy isoflavone group achieved stable disease, described as stabilization in serum PSA level. The data suggest that lycopene and soy isoflavones have activity in prostate cancer patients with PSA relapse disease and may delay progression of both hormone-refractory and hormone-sensitive prostate cancer [145]. Another human in vivo study also showed that a daily diet containing four slices of a bread rich in heat treated soy grits favorably influences the PSA level and the free/total PSA ratio in patients with prostate cancer, suggesting the inhibitory effects of phytoestrogen on PSA level [146]. Although these results are provocative, further clinical trials are needed to fully justify the use of isoflavones for cancer prevention and therapy.

8. Conclusions

In conclusion, genistein ingested through natural food sources exerts its anti-carcinogenic effects, mediated via its pleiotropic molecular mechanism(s) of action on cell cycle, cell apoptotic processes, angiogenesis, invasion, and metastasis. These effects may be primarily due to specific effects of genistein on Akt, NF-κB, MMPs and Bax/Bcl-2 signaling pathways. However, further basic and clinical research in this rapidly growing field of isoflavones should provide lessons for its ultimate application in the cancer field. Such advances will provide critical data that will be supported by definitive clinical trials to prove or disprove whether isoflavone, genistein could fulfill its promise as a chemopreventive and/or therapeutic agent against human cancers with utmost confidence.

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Table-1
Molecular targets of genistein

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