Biochemical Targets of the Isoflavone Genistein in Tumor Cell Lines (43840)

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Abstract. **Dietary intake of soy is associated with a decreased risk of both hormone-dependent and hormone-independent cancers. It has been proposed that genistein, the predominant isoflavone in soy foods, is responsible for this effect. In this review, the potential mechanisms of action of genistein at the cellular level are critically examined to determine which are physiologically relevant. We concluded that (i) only those mechanisms requiring genistein concentrations below 5 μg/ml (18 μ*M*) should be considered, and (ii) more emphasis should be placed on the effects of genistein on events in normal cells or those from the early stages of the cancer process.**

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Dietary intake of soy is associated with low incidence rates of hormonally dependent and independent cancers (1). Soy contains large amounts (1–3 mg/g) of the isoflavones, genistein (4′,5,7-trihydroxyisoflavone) and daidzein (4′,7-dihydroxyisoflavone) (Fig. 1) (2). These isoflavones have been implicated in the prevention of cancer (3–5). Animal models of cancer and cell culture studies with human tumor-derived cell lines support this hypothesis (1).

The soy isoflavones have estrogenic properties in some animals (6–9) and belong to the broad class of plant estrogens (phytoestrogens). Nonetheless, even though genistein apparently binds to the estrogen receptor (ER) *in vitro* (10), its inhibitory effects on tumor cell growth are not ER-dependent (11). The principal target of genistein in tumor cells has not yet been identified, although the discovery in 1987 (12) that genistein is a specific inhibitor of protein tyrosine kinases (PTKs) has led to its extensive use as a chemical probe to explore the pathways of signal transduction in both normal and transformed cell types. In addition to its effects on PTKs that are part of signal transduction pathways, genistein modulates events controlling entry into and out of specific phases of the cell cycle (13, 14), inhibits DNA topoisomerase II activity (15), modifies cell differentiation (16), and inhibits the production of reactive oxygen species (17).

In this review, the relative importance of the individual biochemical mechanisms is assessed with particular regard to the identification of those mechanisms which may operate at the physiologically relevant concentrations of genistein.

Isoflavones As Estrogens

The interest in the mechanism of action of isoflavonoids originated in the discovery that isoflavones in subterranean clover were the cause of antifertility effects in sheep in Western Australia (6, 7). Estrogenic effects of isoflavonoids have been observed in several, but not all, species. In 1986, Setchell et al. (18) reported that soymeal (a rich source of the isoflavones) in the diet of captive cheetahs was the component responsible for both infertility and a veno-occlusive disease of the liver observed in these animals. However, other species such as cattle are not susceptible to estrogenic-like effects when consuming isoflavones. In rodents, isoflavones stimulate uterine growth in immature animals (8, 9). However, not all mouse strains are susceptible to isoflavone estrogenicity (19), suggesting that either nonsusceptible strains convert the isoflavones rapidly to inactive metabolites or they lack the metabolic pathway for formation of an active, estrogenic metabolite.

The active metabolite of the isoflavones in subterranean clover consumed by sheep has been presumed

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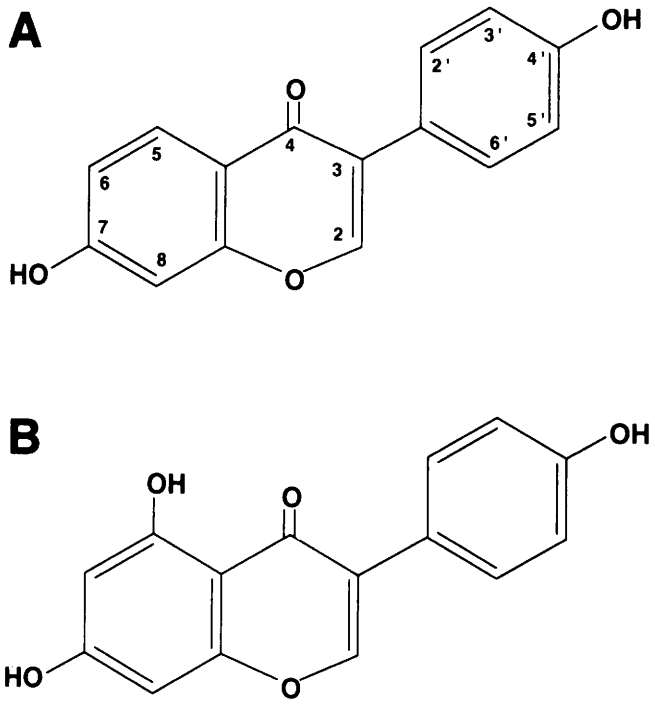

Figure 1. Chemical structures of the isoflavones, daidzein (A) and genistein (B).

to be equol (7,4′-dihydroxyisoflavan) (Fig. 2) (20), formed by intestinal bacteria from the isoflavone precursor formononetin (7-hydroxy-4′-methoxyisoflavone). Welshons et al. (21) reported that serum-stimulated growth of the estrogen receptor-positive, human breast cancer MCF-7 cell line is stimulated by low concentrations of equol. Although formation of equol has been demonstrated in humans eating soy (22), some subjects do not produce it at all but instead metabolize its precursor, daidzein, to the ring-opened O-desmethylangolensin (Fig. 2).

In order to better understand the action of the isoflavonoids, it is important to consider their structure. Genistein has an additional 5-hydroxy group compared with daidzein. Although one might think that genistein as a trihydroxy isoflavone would be more hydrophilic than the dihydroxy isoflavone daidzein, this is not the case; as noted previously (23) and verified by molecular modeling,[2] hydrogen bonding of the 5-hydroxy group with the 4 ketonic oxygen makes genistein a more hydrophobic isoflavone than daidzein. This is observed when analyzing these isoflavones by reversed-phase HPLC where daidzein is eluted before genistein (2). The difference in hydrophobicity may also be a factor in explaining some of genistein's biochemical and biological effects.

[2] When the genistein molecule was examined using the Sybyl force field (Version 6), a hydrogen bond was formed between the 4-carbonyl and 5-hydroxyl oxygens (inter-oxygen distance 2.651 Å) (Larry Hendry, PhD, Medical College of Georgia, Augusta, GA, personal communication).

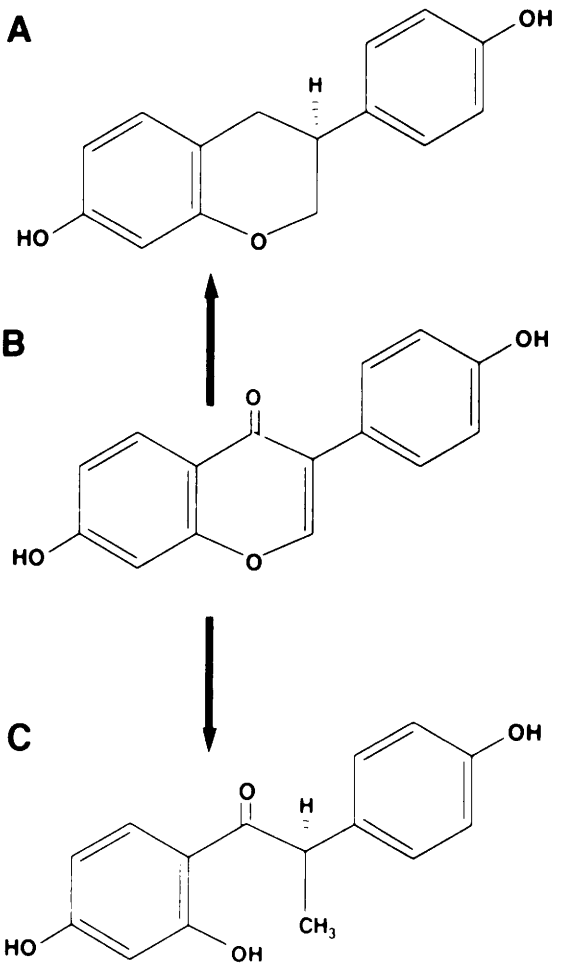

Figure 2. Metabolism of daidzein (B) to equol (A) and O-desmethylangolensin (C).

The phenyl group attached to C_3 of the chroman ring system for genistein undergoes rotation about the C_3-C_1, bond. It has many minimum energy conformers clustered at a ±40° angle to the plane of the chroman ring system (24). Accordingly, the actual conformer utilized in interactions with a protein or DNA binding site will be mostly determined by the shape of the binding site.

Both equol and O-desmethylangolensin, unlike the isoflavones daidzein and genistein, have an asymmetric center at C_3. One of equol's isomers is strikingly nonplanar (Fig. 3). If this isomer is the one found biologically, it is very hard to understand how it can mimic the action of the steroidal estrogens and bind directly to the estrogen receptor.

Genistein and Tumor Cell Growth

At low concentrations (100–200 n*M*) and in the absence of estrogenic steroids, genistein has been reported to increase the serum-stimulated growth of cultured human breast cancer, estrogen receptor-positive, MCF-7 cells (10, 25). At higher concentrations (greater than 2 μ*M*), genistein inhibited the

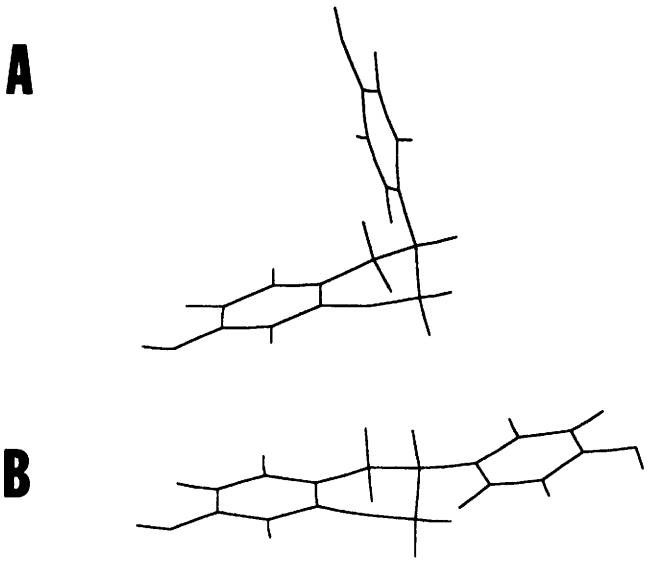

Figure 3. The R- and S-isomers of equol (A and B, respectively). Their structure was deduced using the algorithms of Alchemy III.

growth of these cells (11, 25) with IC_{50} values of 16–27 μM. In addition, genistein inhibited estradiol-stimulated growth with an IC_{50} value of 10 μM^2. Genistein also inhibited the serum-stimulated growth of the MDA-468 human breast cancer cell line, which does not contain estrogen receptors (11), but had no stimulatory effect at low concentrations. To minimize phenotypic differences between an ER^+ and an ER^- cell line, we have studied the effect of genistein on the growth of T47D cells with and without expressed ER (courtesy of Dr. Craig Jordan). Again, genistein had the same inhibitory effect on both cell lines (T. G. Peterson and S. Barnes, unpublished data).

These data suggest that genistein has a biphasic mechanism of action in these cell lines. It is clear that the inhibitory action of genistein in both these cell lines does not rely on its effects on the estrogen receptor. Indeed, Migliaccio et al. (26) have shown that estradiol stimulates a very rapid onset (within 10 sec) of protein tyrosine phosphorylation in MCF-7 cells, suggesting that estrogens activate the signal transduction machinery. Genistein may therefore have its inhibitory effects on signal transduction rather than activation of the estrogen receptor.

Genistein and Protein Tyrosine Kinases

An alternative mechanism for the growth inhibitory effects of genistein arose following the discovery that about half of the known oncogenes (cancer causing genes) encode mutated forms of normal cellular proteins involved in growth factor-stimulated signal transduction (27). This group of proteins in the signal transduction pathways have the property of catalyzing tyrosine phosphorylation of themselves (autophosphorylation) and other proteins, or are substrates of other cellular PTKs. Accordingly, the hunt began for inhibitors of this biochemical mechanism, and soon reports appeared of the discovery of a substance derived from a microorganism which inhibited the activity of PTKs (28). In another study, a differentiating agent (initially called differenol A) for mouse erythroleukemia cells was described (29). In both cases, the active agent turned out to be genistein. These groups were fortunate to make these discoveries, since genistein was not formed by the microorganisms as such, but rather was derived from the hydrolysis of the glycosidic conjugates of genistein in the soymeal used as a protein source for the microorganisms.

In in vitro experiments with the epidermal growth factor (EGF) receptor from plasma membrane of A-431 cells, genistein inhibited receptor tyrosine autophosphorylation and tyrosine phosphorylation of both natural and artificial substrates with IC_{50} values from 0.7 to 10 μg/ml (12). Thus began an explosion in the published work on genistein from a sleepy one to six papers a year prior to 1985 to over 200 a year in 1993. Interestingly, although genistein has been widely used, most investigators are unaware that genistein is a naturally occurring substance and therefore may have diet-based, chemopreventive benefits.

Structure-function experiments on the inhibition of tyrosine phosphorylation of proteins by isoflavones have revealed that the 5- and 4'hydroxy groups are essential (30). Substitution with a carboxylic acid group at the Position 2 maintains most of genistein's inhibition of TPK activity in vitro. However, ionized substituents in the Position 2 have no inhibitory activity on the growth of Rous-transformed cells in culture. On the other hand, hydrophobic alkyl esters in the Position 2 were inhibitory, strongly suggesting that genistein has to enter cells to have its effect (30). It will be, therefore, important in the future to determine the genistein content of cells, rather than just its serum concentration.

Targets of Genistein in Signal Transduction

Cells respond to circulating or locally produced factors that regulate their growth or function. These factors interact with receptors (e.g., the EGF receptor) in the cell membrane which alter structure of the cytoplasmic domain of the receptor. This triggers a change in the enzyme activity of the receptor (i.e., tyrosine kinase activity) or the way in which it binds to protein complexes. The activity of critical targets downstream of membrane receptors (p21 Ras, mitogen-activated protein [MAP] kinase) are altered in such a way that rates of DNA transcription and protein synthesis are changed so that the cell may either enter a cellular differentiation program (e.g., the production of hemoglobin by leukemia cells [29, 31]) or gather itself for replication (proliferative growth). The way in

which these critical targets are regulated is not necessarily the same from cell to cell (32). For instance, convergence of α-interferon (its receptor does not have an intrinsic PTK activity) and EGF stimuli have been suggested to occur via p91, a part of the SIE transcription complex (33). Clearly, although the EGF receptor is important for stimulation of cell growth, there are several steps in the other pathways that could be regulated by genistein.

Genistein and biochanin A, but not daidzein, inhibit the serum-stimulated growth of the human prostatic cancer cell lines, LNCaP and DU-145, although inhibition was weaker (IC_{50} values of 20–30 μg/ml) than in the breast cancer cell lines (34). Since the growth of tumors in the prostate is highly dependent on local production of transforming growth factor alpha (TGF-α) which binds to the EGF receptor, we also examined the effect of genistein on EGF-stimulated growth. Genistein's growth inhibitory effect was much greater in this experiment (IC_{50} values of 4–6 μg/ml) and paralleled that observed with tyrphostin, a synthetic PTK inhibitor (34). These data were consistent with genistein being a PTK inhibitor.

However, in experiments designed to determine whether genistein altered the tyrosine phosphorylation of the EGF receptor in the prostate cancer cells following stimulation with EGF, although EGF-stimulated tyrosine phosphorylation of the EGF receptor, genistein (in the presence of EGF) had no inhibitory effect, even up to 50 μg/ml (34). In contrast (and in the same experiment), tyrphostin at its IC_{50} for cell growth (6 μg/ml) reduced EGF receptor tyrosine phosphorylation to control levels. We have repeated this experiment with several other human prostate and breast cancer cell lines and obtained the same result (T. G. Peterson and S. Barnes, unpublished data). Therefore, we are forced to conclude that genistein does not inhibit EGF receptor tyrosine phosphorylation in intact cells. Most reports of inhibitory effects of genistein on tyrosine phosphorylation of membrane receptors and of intracellular proteins in experiments with intact cells have necessitated genistein concentrations in excess of 30 μg/ml (12, 35–37).

In addition to the EGF receptor, we have examined the tyrosine phosphorylation of several other members of the known signal transduction pathways and of cell cycle regulation (MAP kinase, phosphatidylinositol-3-kinase, GAP, phospholipase Cγ, Ras, cdc2 kinase) in MCF-7 cells. Tyrosine phosphorylation of these proteins was determined by immunoprecipitation of cell lysates with an antiphosphotyrosine antibody, SDS-PAGE separation of immunoprecipitates, blotting onto nitrocellulose, and detection with antibodies to the individual proteins. Although the tyrosine phosphorylated form of each protein examined was readily detected, EGF-stimulation did not lead to any increases in tyrosine phosphorylation. This suggests that in transformed cells, these key targets are constitutively activated, even in quiesced cells—which is perhaps that is why they are cancer cells in the first place.

Many other investigators have shown that genistein inhibits tyrosine phosphorylation in certain cell systems. However, the concentration of genistein needed for these effects is always very high, far above the IC_{50} for growth inhibition. It is possible that in these cases genistein is not inhibiting a tyrosine kinase directly, but rather is interacting with an effector protein for the kinase. In the case of the thromboxane stimulation of platelets, McNichol (38) suggested that genistein inhibited protein tyrosine phosphorylation stimulated by the agonist U46619 by competitive inhibition of binding of U46619 to the thromboxane receptor and not by a direct action on intracellular tyrosine kinases (38).

DNA Topoisomerases

The structure of genistein suggested that it may bind to DNA as well as to proteins and therefore have DNA intercalation properties. However, experiments with supercoiled plasmid DNA and DNA topoisomerase revealed that genistein inhibited the DNA topoisomerase (L. Coward, W. Zacharias, and S. Barnes, unpublished data). Other investigators showed that genistein stabilized the transient DNA:DNA topoisomerase complex (15). Consequently, genistein induces DNA strand breaks during replication (16). The resulting fragmentation of DNA may be the cause of the genistein-induced apoptosis (programed cell death) (39).

Genistein As an AntiOxidant

Genistein may prevent cancer as a result of its biological antioxidant properties. It has been suggested that reactive oxygen species play an important role in mutagenesis and carcinogenesis, particularly tumor promotion (40). Genistein inhibits the production of hydrogen peroxide in response to the phorbol ester TPA in HL-60 cells, human polymorphonuclear cells and mouse skin (17). The effect could only partially be accounted for in terms of a chemical reaction between hydrogen peroxide and genistein, suggesting that the effect of genistein is at a biochemical level. Indeed, in the mouse skin model genistein inhibits the expression of the immediate early gene c-*fos* (41).

Genistein and Chemoprevention

In understanding the role of genistein in preventing cancer it is important to consider when in the overall process the compound is involved. Lamartiniere et al. (42) have recently shown that administering genistein in the immediate neonatal period alone is suf-

ficient to reduce cancer risk, suggesting that imprinting or cellular differentiation are important targets of its action. Genistein may also have a role during the period when the carcinogen is administered to the animal by increasing the activity of enzymes which metabolically inactivate the carcinogen or decreasing those that activate it (3). Genistein may interfere with the promotion and progression of cells which have undergone DNA damage but are not fully transformed, since Hawrylewicz et al. (43) have found that isolated soy protein (containing genistein conjugates) inhibits the appearance of mammary tumor growth even when first administered 3 weeks after the carcinogen. Finally, genistein may inhibit the proliferative growth of tumor cells and thereby prevent the appearance of the clinical form of cancer. The relative contributions of these activities remain to be elucidated for individual cancers.

Summary

Our current knowledge of the biochemical and biological actions of genistein in the prevention of cancer is incomplete. In the future, it will be important to distinguish between processes that occur in vivo and those that can be generated in vitro. Although the precise cellular target(s) of genistein is not known, it is clear that the inhibitory effects of genistein on the growth of tumor cells and those of the hemopoietic system do not depend on the expression of the estrogen receptor. In addition, data from our laboratory do not support the hypothesis that the antiproliferative action of genistein depends on its inhibition of the EGF receptor tyrosine kinase activity. The relatively high concentrations of genistein needed to inhibit the growth of many tumor cell lines suggest that the real targets of genistein are precancerous cell populations. In these cells, the signal transduction pathways may not yet be autonomously activated.

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