Rationale for the Use of Genistein-Containing Soy Matrices in Chemoprevention Trials for Breast and Prostate Cancer

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Abstract  Pharmacologists have realized that tyrosine kinase inhibitors (TKI) have potential as anticancer agents, both in prevention and therapy protocols. Nonetheless, concern about the risk of toxicity caused by synthetic TKIs restricted their development as chemoprevention agents. However, a naturally occurring TKI (the isoflavone genistein) in soy was discovered in 1987. The concentration of genistein in most soy food materials ranges from 1-2 mg/g. Oriental populations, who have low rates of breast and prostate cancer, consume 20-80 mg of genistein/day, almost entirely derived from soy, whereas the dietary intake of genistein in the US is only 1-3 mg/day. Chronic use of genistein as a chemopreventive agent has an advantage over synthetic TKIs because it is naturally found in soy foods. It could be delivered either in a purified state as a pill (to high-risk, motivated patient groups), or in the form of soy foods or soy-containing foods. Delivery of genistein in soy foods is more economically viable ($1.50 for a daily dose of 50 mg) than purified material ($5/day) and would require no prior approval by the FDA. Accordingly, investigators at several different sites have begun or are planning chemoprevention trials using a soy beverage product based on SUPRO™, an isolated soy protein manufactured by Protein Technologies International of St. Louis, MO. These investigators are examining the effect of the soy beverage on surrogate intermediate endpoint biomarkers (SIEBs) in patients at risk for breast and colon cancer, defining potential SIEBs in patients at risk for prostate cancer, and determining whether the soy beverage reduces the incidence of cancer recurrence. These studies will provide the basis for formal Phase I, Phase II and Phase III clinical trials of genistein and soy food products such as SUPRO™ for cancer chemoprevention. © 1995 Wiley-Liss, Inc.

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In 1994, a total of 400,000 new cases of breast and prostate cancer will be diagnosed in the US [1]. In addition, there will be 45,000 breast cancer deaths and 33,000 prostate cancer deaths. The death rate from these cancers when expressed per 100,000 of the American population has not changed over the past 60 years [2], despite improvements in diagnosis, treatment and therapy. The lifetime risk for breast cancer in women is one in nine; for men, prostate cancer risk is one in eleven [1].

Chemoprevention, as opposed to chemotherapy, has been underexplored as an approach to reduce the risk of and death from cancer. In order to systematically develop rational strategies in chemoprevention, investigators have attempted to determine the reasons for marked country-to-country variations in specific cancer incidences and mortality.

The peoples of Southeast Asia (e.g., China, Indonesia, Japan, Korea, Singapore) have a four-to ten-fold lower incidence of and death from breast and prostate cancer [1]. However, following emigration to the US, the risk of these can-
cers in Asian peoples rises rapidly in one generation to equal that of Americans [3]. Differences in the diet are thought to account for a large part of this variation [4,5].

The American diet's much higher fat content compared to the Asian diet was initially considered the most important factor leading to increased cancer risk in Americans [6], i.e., suggesting that fat is cancer-causing. However, clinical trials performed in this country relating breast cancer risk to dietary fat intake have not supported this hypothesis [7,8]. A new hypothesis is being examined to determine whether one or more components of the Southeast Asian diet are cancer-preventing.

Asian diets contain a higher percentage of vegetable products. Soy is used to prepare soy milk, miso (used in soups and gravies), tofu (used in place of meat) and a variety of fermented products including soy paste and tempeh. The average intake of soy protein in Southeast Asia varies from 10 g/day in China to 30-50 g/day in Japan and Taiwan [9]. In contrast, Americans eat no more than 1-3 g/day. In a recent review of the epidemiological literature, Messina et al. [10] found that nearly two-thirds (16/27) of the reported studies associated soy intake with a reduction of cancer risk.

Whole soybeans and various soy foods have chemopreventive properties in animal models of cancer. Troll et al. [11] first reported that adding whole soybeans to the diet reduced the incidence of mammary tumors in female rats irradiated with X-rays. Barnes et al. [12] confirmed this observation in an N-methyl-N-nitrosourea (MNU)-induced model of breast cancer after first carefully controlling the diets so that they were isocaloric and isonitrogenous.

Several substances with chemopreventive properties have been identified in soy [13]. These include isoflavonoids, soybean protease inhibitors [see A. Kennedy and H. Manzone, this issue], phytosterols and saponins, phenolic acids and phytic acid.

This review summarizes the experimental evidence for soy isoflavonoids having a role in the prevention of cancer based on data obtained from animal models and studies in cell culture, and the mechanisms responsible. We conclude by considering the optimal way of delivering a chemopreventive agent that naturally occurs in an edible food.

ANIMAL MODELS OF CANCER

Soybeans contain large amounts (1–2 mg/g) of the isoflavones daidzein (4',7-dihydroxyisoflavone) and genistein (4',5,7-trihydroxyisoflavone) [14–16]. Most soy foods also contain high levels of isoflavones because of their binding to soy proteins. The exceptions are soy sauce and soy protein products which have been washed with aqueous alcohol during their processing [14]. Much of the data from animal models was obtained without knowledge of the isoflavone content of the soy material used [17]. Nonetheless, two-thirds of the reported studies found the soy food product either reduced the incidence of tumors, inhibited the rate at which tumors appeared, or reduced the total tumor number or tumor size. Interestingly, a soy protein concentrate which had been washed with aqueous alcohol (removing the isoflavones) had no effect on the appearance of mammary tumors in the 7,12-dimethylbenz[a]anthracene model of breast cancer. On the other hand, the aqueous alcohol extract, concentrated to remove the alcohol, did have a chemopreventive effect in this model [18].

Since genistein is expensive ($250/g) and available only in small amounts, only a limited number of studies in animal models have been carried out using the pure compound. In colon cancer models, genistein inhibited aberrant colonic crypt formation induced by two different carcinogens [19,20]. Genistein has also inhibited incidence, multiplicity and tumor size in the DMBA-initiated, phorbol ester-promoted skin cancer model in mice [21]. A fascinating recent study, found administration of genistein only during the neonatal period lowered mammary tumor incidence, multiplicity and size, and increased latency, in the DMBA-induced breast cancer model [22]. Future studies will need to address when genistein administration is most effective, doses required to obtain chemopreventive effects, and also whether there are positive synergistic effects on chemoprevention when genistein is administered in combination with daidzein or other non-isoflavone soy components.

MECHANISMS OF ACTION

At this time, many mechanisms have been put forward to account for the observed and pre-
dicted chemopreventive effects of isoflavones. Until 1987, genistein was considered a phytoestrogen, acting either as an estrogen agonist or antagonist. Since then, a variety of non-estrogenic mechanisms have been proposed. These include inhibition of protein tyrosine phosphorylation, induction of differentiation, inhibition of DNA topoisomerases, inhibition of specific cell cycle events, induction of apoptosis, inhibition of angiogenesis and antioxidant activity. The relevance of these events in vivo has not yet been firmly established.

Most important is whether genistein concentrations at the cancer cell level in the body reach concentrations used in cell culture experiments to demonstrate these biochemical and biological events. Daily dietary intake of genistein in heavy soy consumers is approximately 50 mg (18.5 µmol) [17]; in Asian diets, soy is the principal source of these isoflavones [23]. Assuming that this daily dose distributes through body water, the maximum serum genistein concentration is unlikely to exceed 3–4 μM [17]. This has been confirmed by studies in which human adults were fed 50 mg of genistein and daidzein orally [24]. The peak serum concentrations observed with this dose were 0.7 μM (0.2 µg/ml). Thus, in many experiments, genistein only shows its effects at supraphysiological, if not pharmacological, concentrations.

On a cautionary note, satisfactory analytical procedures to measure isoflavones in blood and tissues have not yet been established, and it is possible that cells are exposed to higher concentrations of genistein than currently thought. The possibility of genistein accumulation in enterohepatic circulation has not been considered. Current analytic methodologies based on gas chromatography-mass spectrometry (GC-MS) require extensive steps in the work-up procedure to separate individual conjugated forms of the isoflavones, hydrolyze the conjugates and prepare suitable volatile derivatives for GC. The variable and extensive losses in these procedures demand isotopically labeled standards suitable for reverse isotope dilution analysis. However, the deuterated isoflavones used for this purpose are themselves unstable [25], calling into question the validity of existing methods.

Reversed-phase HPLC greatly simplifies the analysis of isoflavones since the aglucones and their conjugates can be separated in one run without need for the derivatization required by GC-MS. However, sensitivity and specificity is a problem because of low blood concentrations of isoflavones [17,24]. The recent introduction of a combination of reversed-phase HPLC and electrospray ionization MS may overcome many of these difficulties [26]. However, much work needs to be done in the area of initial extraction of the isoflavones from the biological matrix.

ESTROGEN AGONISM AND ANTAGONISM

Genistein and daidzein are weak estrogens; however, several authors hypothesized chemopreventive properties similar to the estrogen antagonist tamoxifen [13,27,28], which has been used extensively in the treatment of estrogen receptor-positive breast cancer and is currently being tested in a breast cancer chemoprevention trial. The possibility of a weak estrogen agonist action of genistein should be considered seriously. Zava et al. [29] found that genistein added in low concentrations (1–100 nM) to estrogen-free, serum-containing media stimulates growth of the human breast cancer cell line MCF-7; however, at higher concentrations (1 μM) it inhibits growth. This may have relevance to postmenopausal women with low serum concentrations of physiologic estrogens. It may also account for the lack of association between soy intake and breast cancer incidence in postmenopausal women as opposed to premenopausal women [30].

PROTEIN TYROSINE PHOSPHORYLATION

During the 1980s, there was an intense search for compounds which inhibited protein tyrosine phosphorylation of enzymes in the signal transduction cascades stimulated by some growth factors. This was relevant to cancer chemoprevention since many of the expressed proteins of the oncogenes identified at that time were either activating ligands of tyrosine kinases (e.g., c-sis), tyrosine kinases (erb-B2, c-src), or substrates of tyrosine kinases [31]. A tyrosine kinase inhibitor therefore would have promise in chemoprevention. Genistein was "discovered" as a potent inhibitor of tyrosine phosphorylation using the epidermal growth factor receptor tyrosine kinase from A-431 cells as a screen of microbial metabolites [32]. The genistein did not come from the
microorganism but rather the soymeal used as the source of protein in the growth medium of the microorganisms that were studied. It was subsequently shown that genistein was a specific inhibitor of tyrosine, but not serine/threonine phosphorylation [33]. Now genistein is widely use as a probe to detect tyrosine kinase involvement in many cell signalling pathways, with the number of published papers doubling each year since 1989. Despite this wide use of genistein, it cannot be assumed that \textit{in vivo} genistein acts directly as a tyrosine kinase inhibitor [34,35]. McNichol [35] presented evidence that genistein inhibits thromboxane-induced pp50 tyrosine phosphorylation in platelets by competing with the thromboxane agonist U46619 for binding to the thromboxane receptor and not on the tyrosine kinase that phosphorylates pp50.

\textbf{INDUCTION OF CELL DIFFERENTIATION}

In addition to inhibiting growth of leukemia cell lines, genistein also causes them to differentiate and produce hemoglobin [36,37]. However, genistein inhibits differentiation in many hematopoietic cells, although it has no effect in others [38]. Since the various stimulators of differentiation use separate signal transduction pathways, it is not known which cellular target is affected by genistein.

\textbf{INHIBITION OF DNA TOPOISOMERASES}

Genistein has been reported to inhibit topoisomerase activity both \textit{in vivo} [39,40] and \textit{in vitro} at concentrations from 20–110 \text{\mu M} [39–41]. The relationship between topoisomerase II inhibition and inhibition of cell growth, however, is still a matter of debate [42]. From a mechanistic standpoint, genistein inhibits topoisomerase activity by inhibiting the interaction of ATP with its binding site on topoisomerase II [43,44], stabilizing the transient DNA-topoisomerase complex observed in several experiments.

\textbf{INHIBITION OF CELL CYCLE-SPECIFIC EVENTS}

Genistein arrests cell growth at various stages of the cell cycle. At 20–40 \text{\mu M} genistein, Jurkat T leukemia cells were arrested at the G2/M transition; whereas at 70–110 \text{\mu M}, they were arrested in the S-phase [45]. At the higher concentrations, tyrosine phosphorylation was also inhibited. In rat smooth muscle cells, genistein arrested in cells stimulated by PDGF at the G0/G1 transition; however, it had no effect on serum-induced stimulation [46]. Genistein also arrested K-562 leukemia cells [36], HL-60 cells [47] and HGC-27 gastric cancer cells [48] at the G2/M transition.

\textbf{INDUCTION OF APOPTOSIS}

Genistein causes apoptosis in many cell lines [49–53]. In each case the concentration of genistein (30–40 \text{\mu M}) was cytotoxic, and in excess of that obtained through a soy-based diet. In HL-60 cells, both genistein and the synthetic tyrosine kinase inhibitor tyrphostin AG82 caused apoptosis [49]. Since the action of genistein was only partially blocked by the tyrosine phosphatase inhibitor orthovanadate, whereas that of the tyrphostin was fully blocked, genistein must have additional or different targets from the tyrphostin. In thymocytes, genistein caused apoptosis, but unlike herbimycin, another tyrosine kinase inhibitor, did not alter tyrosine phosphorylation [53].

\textbf{INHIBITION OF ANGIOGENESIS}

Supply of blood and nutrients is a vital part of primary tumor growth and the process of metastasis. Genistein, by inhibiting angiogenesis, could have an important role in preventing the appearance of cancer. Although genistein inhibits angiogenesis [54], the IC\textsubscript{50} for this event is 150 \text{\mu M}, far in excess of observed serum concentrations in soy consumers.

\textbf{ANTIOXIDANT EFFECTS}

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 [55]. Genistein inhibits the production of hydrogen peroxide in response to the phorbol ester TPA in HL-60 cells, human polymorphonuclear cells, and mouse skin [56]. The effect could only be partially 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, genistein inhibits expression of the immediate early gene c-fos in the mouse skin model [21]. Genistein is particularly effective in inhibiting the production of superoxide anion stimulated by formyl-methionyl-leucyl-phenylalanine in granulocyte colony stimulating factor-primed human neutrophils with an IC₅₀ close to 1 μM [57]. More research is clearly indicated in this area.

**DELIVERY CHOICES FOR GENISTEIN**

Since genistein is found in a food source, there is the option in chemoprevention trials to use it either as the pure compound (extracted from soy or prepared synthetically) or as a genistein-containing, soy-based foodstuff. It should be noted that the chemical form of genistein in soy varies from the aglucone in fully fermented food (miso) to a variety of glucosides in non-fermented foods [15,16]. These differences have the potential to alter the initial uptake of genistein from the intestine and its pharmacokinetics—the aglucone should be absorbed rapidly in the upper part of the small intestine, whereas the glucoside conjugates would have to undergo hydrolysis to the aglucone before absorption. The latter may also lead to bacterial metabolism of genistein prior to uptake.

The principal advantage of using a soy food as the source of genistein is that the level of genistein (25–100 mg/day) in the food does not lead to any known toxicity, having been used by the peoples of Southeast Asia for centuries. By using purified or synthetic genistein, a much larger dose can be delivered (20–40 fold larger), but this opens up the possibility of toxic side effects. Toxicity experiments in animal models and subsequent Phase I clinical trials are needed to address this issue.

The safety of using soy food products as the delivery system for genistein has already led to the initiation of clinical trials in 1994 designed to examine the effect of isolated soy protein (Supro™) on surrogate intermediate endpoint biomarkers (SIEBs) of breast cancer [58] and prostate cancer [59]. It will be important to compare the effects of the soy protein with an equivalent amount of pure genistein to determine whether other components in soy have additive or synergistic effects on SIEBs.

The other main advantage of the use of soy protein over pure genistein is the relative cost. Synthetic genistein is currently sold for $250/g, *i.e.*, $12.50 per 50 mg (the daily intake). This figure may fall to $5/g if genistein is made on a larger scale. In contrast, 50 mg of genistein is delivered in 40 g (1.5 ounces) of Supro™ (a low-fat, high quality protein) at a daily cost of $1.50. The eight-fold lower cost of genistein in soy protein has been a large factor in the design of existing clinical trials.

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Genistein and Cancer Prevention

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