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ISOFLAVONES IN BREAST CANCER CHEMOPREVENTION: WHERE DO WE GO FROM HERE?

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1. ABSTRACT

Based on the evidence from epidemiological, animal, in vitro data and human clinical trials, it is evident that isoflavones are promising agents for breast cancer chemoprevention. It is also evident that the form of isoflavone used (purified vs soy products), dose of isoflavone used (low vs high), timing and duration of exposure of isoflavones appears to play a major role in determining agonistic or antagonistic effects. Collectively, these isoflavones have enough evidence to warrant use in a number of clinical trials to examine its efficacy as a potential chemopreventive agent for breast cancer. In this comprehensive review, we attempt to summarize the evidence demonstrating the potential use of isoflavones in breast cancer chemoprevention and the rationale to examine a combination of biochemical, morphological and molecular intermediate endpoint biomarkers of breast cancer risk to examine the efficacy of this promising agent.

2. INTRODUCTION

It is estimated that 211,300 invasive breast cancers were diagnosed in the United States during 2003 and 40, 200 women died from the disease (1). Despite advances in the early detection and treatment of breast carcinoma, the mortality and morbidity burden from this disease remains high. It is thus well recognized that the most promising approach to breast cancer control is a national commitment to prevention. The feasibility of chemoprevention in humans using antiestrogens such as Tamoxifen (TAM) and Raloxifine has been well documented in the literature (3-4). However, both drugs increased risk for venous thromboembolic disease, menstrual abnormalities, bone loss in young premenopausal women, sexual dysfunction, cataracts and hot flashes; TAM increased risk for endometrial cancer and stroke (4). Concerns about the risk: benefit ratio has decreased the use of TAM for prevention in women over 50 (4). Clearly, there is a need to identify other agents for breast cancer prevention.

Research has identified plant-based nutrients and non-nutrients that inhibit mutagenesis and proliferation. These compounds are relatively non-toxic, low cost, and can be taken orally or as a part of the daily diet.

3. PROMISING NUTRIENTS FOR BREAST CANCER PREVENTION

Some of the most promising nutrients identified as chemopreventive agents in breast cancer include plant isoflavonoids, also referred to as isoflavones or phytoestrogens (4).

These plant derived phytochemicals have been shown to modulate tumor cell proliferation (5), angiogenesis (6), tumor cell invasion (7), and tumor metastasis (8), and can function as anti-oxidants (9-10) It is only logical to further examine the efficacy of phytochemicals in cancer chemoprevention, as an alternative to or in combination with, pharmacological agents, especially in high-risk populations,

4. ISOFLAVONES & BREAST CANCER

Isoflavones are nonsteroidal plant compounds

primarily found in soy foods as the β glycoside conjugates, genistin, diadzin, and glycetin. These conjugated isoflavones are inactive compounds, but after ingestion and hydrolysis by β glucouronidases result in the biologically active aglycones: genistein, diadzein and glycetein. Diadzein is further metabolized to equol. Bioavailability of dietary flavonoid components depends on relative uptake rates of free forms, hydrolysis of glycosides by gut bacteria or gut wall enzymes, further metabolism, for example to glucuronides within the liver, and excretion rate. The bioavailability of isoflavones depends largely on intestinal flora for bacterial degradation. It has been demonstrated that human microbial gut flora also naturally transform daidzein into equol, which is absorbed in the intestine and is 10- to 100-fold more estrogenic than daidzein and genistein. Differences in absorption and conversion by intestinal flora might result in variations in serum levels and thus effectiveness of these compounds. It has even been suggested that the ability to produce equol after ingesting soy isoflavones may be a significant factor in the effectiveness of soy supplementation (11, 12).

5. EVIDENCE FROM EPIDEMIOLOGICAL STUDIES

Several epidemiological studies show an inverse relationship between dietary isoflavone intake or urinary excretion of phytoestrogens and the incidence of hormone-dependent cancers (14). Differences in menstrual cycle lengths between breast cancer cases and controls and between populations of women with significantly higher soy intake compared to the Western world with different breast cancer risk are consistent with the theory that menstrual cycle length may moderate breast cancer risk (14, 15). Most of the epidemiological evidence to date, have been based on retrospective study designs, measuring urinary metabolites at diagnosis, with significant differences in methodology used in measurement of biomarkers. However, most recently, in a large population-based (209,354 women), prospective cohort study in Japan, Yamamoto S *et al.* (2003), observed miso soup and isoflavones consumption was associated with a reduced risk of breast cancer (the adjusted RR for breast cancer for women in the highest quartiles was 0.46 [95% CI=0.25 to 0.84] compared to women in the lowest quartile) with this inverse relationship stronger in postmenopausal women (16).

6. EVIDENCE FROM ANIMAL STUDIES

Lamartiniere *et al.* (33) have demonstrated that rats treated with purified isoflavones at supraphysiological as well as physiological levels during the neonatal and prepubertal stages had a significant protection against DMBA-induced experimental breast cancer (17). In another study where purified genistein was given perinatally to postnatally,a significant protection was offered even at lower doses (18). However, in a review of experimental studies using soy isoflavones in rodents concluded that using soy products did not confer a significant preventive effect (19). In summary, the results of animal studies, while inconsistent reinforce the need for further investigation of the use of purified isoflavones, in the modulation of breast cancer risk.

7. EVIDENCE FROM *IN VITRO* STUDIES

Isoflavones have also demonstrated an inhibitory effect *in vitro* on human tumor cell lines. (5) Most of the *in vitro* studies have been conducted with purified forms of isoflavones, specifically genistein, which has been demonstrated to have a mixed agonistic and antagonistic effect on estrogen sensitive MCF-7 cell lines and numerous other cellular effects. The effect of isoflavones on breast tissue may be complex, however *in vitro* data has consistently demonstrated that exposing cultured breast cancer cells to *purified isoflavone*, genistein, inhibits cell proliferation, or induction of apoptotic cell death, indicating the potential for its use as a chemopreventive agent.(20-21) Genistein produced a dose and time dependent *in vitro* growth inhibition against high concentrations of several breast cancer cells lines and breast epithelial cell lines.(22) On the other hand, other *in vitro* studies demonstrate that the isoflavones contained in *soy foods* show mainly agonistic activity (23) suggesting that isoflavones increase rodent mammary gland proliferation and proliferation in breast tumor cell lines, which may be a dose dependent phenomena.(24)

In vitro, purified isoflavone-genistein has also been shown to exert both estrogenic and antiestrogenic effects in human cell lines. In a human breast cancer cell line, genistein behaved as a pure estrogen agonist at low physiologically relevant concentrations, but was growth inhibitory at higher concentrations, that were higher than physiological levels observed in Japanese subjects (13). Isoflavones are considered selective estrogen receptor modulators (SERMs) and have different binding affinities for ERβ vs. Erα (11, 23). Although isoflavones contain at least one aromatic ring with a hydroxyl group, their relative affinities for the estrogen receptors are lower than for estradiol, probably because they form unstable complexes. Still, these compounds can induce conformational changes that result in transcription activation. The estrogenic potency of a compound is the result of several factors, such as the transactivation functionality of the receptor, the specific coactivators recruited, and the particular cell context.

Furthermore, we observed that genistein inhibits the proteasomal chymotrypsin-like activity *in vitro* and *in vivo* (10). Our computational docking studies confirm the interaction of genistein to the proteasomal α5 subunit that is responsible for the chymotrypsin-like activity of the proteasome. Inhibition of the proteasome by genistein in prostate cancer LNCaP and breast cancer MCF-7 cells is associated with accumulation of ubiquitinated proteins and three known proteasome target proteins, the cyclin-dependent kinase inhibitor p27Kip1, inhibitor of NF-κB (IκB-α), and pro-apoptotic protein Bax. Genistein-mediated proteasome inhibition was accompanied by induction of apoptosis in these solid tumor cells. Furthermore, prior to induction of apoptosis, genistein also

induced p53 protein accumulation, associated with increased levels of p53 downstream target proteins such as p21Waf1 (10).

In summary, the *in vitro* studies indicate that isoflavones are promising chemopreventive agents, with several cellular effects which are both genomic and non-genomic. The responsiveness observed with isoflavones in breast cancer cell lines varied with the concentration of purified isoflavones, with growth stimulation of these cells at low concentrations and inhibition at high concentrations,(higher than the physiological levels observed in Japanese subjects). In addition, our work indicates that proteasomes are breast cancer-related molecular targets of isoflavones.

8. EVIDENCE FROM HUMAN CLINICAL TRIALS

There is a general agreement that steroid hormones are involved in the development of breast cancer. In addition to genetic factors, number of pregnancies, use of oral contraceptives and lifestyle factors influencing age at menarche and menopause, levels of steroid hormones and menstrual cycle length are recognized risk factors for breast cancer. More recently, clinical studies, such as ours, have examined the effects of soy isoflavones on serum estrogen levels and have demonstrated moderate reductions in ovarian steroid levels.(15, 16) In our study, free estradiol decreased in 53.85% of experimental subjects compared to 37.5% in the placebo group (15). Other clinical studies have shown that increased intake of isoflavones converts the endogenous estrogens to the protective 2-hydroxylated estrogens in women and may play a critical role in lowering 17-alpha hydroxyestrone (13), a known stimulant of breast proliferation, thereby decreasing the long-term risk of breast cancer.

Our data also demonstrated that the subjects in the experimental group consuming soy had their mean menstrual cycle length increase by 3.52 days compared to a mean decrease of 0.06 days for the placebo group (P=0.04) from baseline to the 3rd menstrual cycle. In addition, subjects on soy had their mean follicular phase increase by 1.46 days compared to a mean increase of 0.14 days for subjects on placebo (P=0.08) (15).

In contrast, there have been some controversial findings with regard to the effect of soy isoflavones on breast tissue. Investigators have recently demonstrated that supplementation with isoflavones for 14 days affected histologically normal breasts in premenopausal women, as indicated by an increase in the number of cells in the S-phase of the cell cycle and a small increase in progesterone receptor expression.(25, 26) However, in a more recent report of one of the same studies with double the number of subjects, Hargreaves *et al.* failed to see any difference in breast proliferation, apoptosis or progesterone receptor expression as observed in the preliminary report of 1998 (27). These clinical trials, which report higher proliferation with short-term supplementation, are at most pilot trials with small sample sizes and short duration of interventions, not exceeding one month (25-27). In addition, currently there are no clinical trials demonstrating the agonist effect of isoflavones in post menopausal women. Earlier, it was suggested that isoflavones may exert an estrogenic effect in postmenopausal women. However, this was merely speculation based on 4 rodent studies that suggested that in a low estrogenic environment (post menopausal) isoflavones is estrogenic and has a proliferative effect on breast tissue, but in a high estrogenic environment, such as in premenopausal women, it has antiproliferative properties (28). Although postmenopausal serum estradiol levels are 10% of the level of premenopausal women (29), some data indicate that the concentration of estradiol in breast cancer tissue of postmenopausal women is similar to that of premenopausal women (57) and that breast tissue estradiol concentrations are higher than paired serum concentrations (30). Consequently, identifying a high *vs* a low estrogen environment may not be consistent in pre and postmenopausal breast cancer patients (28).

In human studies, elevated thyroid stimulating hormones (TSH) were observed in healthy, iodine-sufficient adults without known thyroid disease when fed 30grams of soy for a month (59), although changes were not observed in T3 and T4 levels (30, 31). Thus serial measurement to monitor for these changes during the study period in future clinical trials is warranted (31).

In summary, the human clinical trials have continued to demonstrate that isoflavones have interesting antimutagenic properties and holds promise to be tested in clinical trials, in high-risk populations. The most important question that emerges from these human clinical trials is whether the agent can be effectively and safely administered as a chemopreventive agent for a long duration of time, in a population of women at high risk for breast cancer.

9. INTERMEDIATE ENDPOINT BIOMARKERS

In the following sections, we provide the rationale to examine a combination of validated biochemical, morphological and molecular intermediate endpoint biomarkers (IEBs) as response indicators to purified isoflavones, including a.) biochemical IEBs (urinary and serum markers of estrogen metabolism), b.) morphological IEBs (mammographic density, ductal cytology, Ki-67 from ductal lavage), and the c.) potential molecular mechanisms that produce these effects.

10. BIOCHEMICAL INTERMEDIATE ENDPOINT BIOMARKERS

Isoflavonoid phytoestrogens have been shown to increase serum sex-hormone binding globulin (SHBG) which decreases the bioavailability of estrogen by lowering of free estradiol. (32) This may also be due to the weak estrogenic effect of phytoestrogens which, like TAM, stimulate the synthesis of sex-hormone-binding –globulin (SHBG) in the liver. Several studies also indicate that

phytoestrogens reduce the bioavailability of estrogens by occupying estrogen binding sites exerting a weak estrogenic effect, by decreasing the availability of estrogen receptors to endogenous, biologically active estrogen. (29)

Other candidate risk biomarkers include the serum hormone insulin-like growth factor-1 (IGF-1), which has been shown to have potent mitogenic and anti-apoptotic properties (33, 34). Further, IGFs are also involved in angiogenesis. In general, the data to date suggest that plasma IGF-1 is positively related to risk, and could be useful both for diagnosis and surveillance.

Thus, steroid hormonal changes, both in serum (free and total estradiol, SHBG and IGF-1) and urinary metabolites of estrogen metabolism(2-hydroxylated estrogens and 16-alpha hydroxyestrone), are valid IEBs for breast cancer and have been demonstrated to be potentially reversible intermediate biomarkers in prevention trials with isoflavones and must be incorporated as IEBs to be monitored as outcome variables in chemoprevention trials.

11. MORPHOLOGICAL INTERMEDIATE ENDPOINT BIOMARKERS

In addition to altering production, metabolism and excretion of estrogens and their impact on target tissues, there is increasing evidence that isoflavones have been shown to inhibit breast proliferation *in vitro*. Based on these observations, researchers in clinical trials have previously used change in breast cytology as main study endpoint to evaluate the efficacy of chemopreventive agents. All ductal and lobular breast cancers originate in epithelial cells that line the preterminal ducts of the mammary lobules. Recently a large multi-center clinical trial, and other groups, including our collaborators, have demonstrated the efficiency and ease of obtaining adequate ductal lavage samples for evaluation, thus demonstrating feasibility and efficiency of obtaining cytological data from women at high risk for breast cancer, using a relatively non-invasive technique (35). Several studies, including two prospective studies with long-term follow-up have shown that women with cellular atypia detected by the cytological examination of breast specimens are at significantly higher risk of developing subsequent breast cancer compared to women without cellular atypia. Epithelial cells in nipple aspirate fluid (NAF) and ductal lavage have been cytologically analyzed successfully in both premenopausal and postmenopausal women (35) Markers of proliferation such as Ki-67 are reliable and quantitative IEBs of risk of developing carcinoma that can be detected from breast cells obtained from ductal lavage. Recent studies have demonstrated that Ki-67 proliferation index is an established, valid, reliable marker of proliferation and changes in Ki-67 in ductal lavage is a valid IEB of chemoprevention efficacy (35, 36). As reported in these studies, Ki-67 proliferation index changes in relation to hormonal receptor status and plasma hormonal levels Menard and his group used histopathological techniques and found evidence of fluctuation in breast cancer tissue with respect to number of mitoses during menstrual cycle phases (36). They observed that the frequency of primary breast carcinomas with a high number of mitoses is at a minimum during the follicular phase and maximum in the luteal phase ($p=0.05$) decreasing in premenses and menses phases, concluding that tumors, like normal breast epithelial tissue, respond to physiological fluctuations of hormone concentrations. Findings by Soderqvist *et al.* using breast epithelial cells procured through needle aspirates from healthy premenopausal women, observed a greater preponderance of Ki-67 immunoreactivity during the luteal (2%) than the follicular phase (1.6%) of the menstrual cycle ($P=0.04$) (37). It is logical to infer that these intermediate endpoint biomarkers (proliferation), which are the outcome variables that may prove efficacy of chemoprevention, may be affected by the timing of menstrual cycle. Thus timing of menstrual cycle must be accounted for when examining these biomarkers.

Observing the changes in breast cell cytology, combined with other quantitative markers of breast proliferation such as Ki-67 in ductal lavage and mammographic breast density may be useful, valid outcome markers for surveillance in chemoprevention trials in healthy women.

12. BREAST DENSITY AND BREAST CANCER RISK

Dense breast tissue as observed from a breast radiograph is a composite of stromal and epithelial tissue. Both constituents have similar attenuation properties at the typical energies in planar X-ray breast imaging and are therefore indistinguishable. The other component in the image is the fat tissue, which appears relatively dark in the developed image (less ability to block x-rays). Early on John Wolfe observed a relationship with the presence of prominent ductal patterns, as observed in breast radiographs, and breast cancer [Wolfe 1967a,b 1969, 1976,a,b,c 1977]. Roughly thirty years of research was spawned by this assertion, most of which indicates its validity to varying degrees, that is, dense breast tissue is a risk factor. Subsequent work , which reexamined some of the earlier pattern-risk research by imposing strict methodological standards, found an increased risk associated with dense breasts (38). Similarly, other retrospective considerations indicate that carefully conducted studies support Wolfe's original assertions (38). Reviews of tissue related risk are also given by Heine and Malhotra (39).

Briefly, the density-related risk research indicates that women with increasing proportions of dense breast tissue are at a significantly increased breast cancer risk, which appears to be greater than any of the other accepted factors. This work also indicates that density and reproductive (hormonal) factors are related. Older women (post menopause) in general experience a decline in breast density with increasing age, which is termed involution. Although, the rate and manner of decrease is not understood, it can be assumed to occur gradually over many years. Likewise, non-parous women tend to have greater densities compared with parous women, women on hormone replacement therapy (HRT) tend to show increased densities relative to non-users or past users, and late childbirth tends to postpone involution. The important

conclusion here is that in some fashion density is tracking risk as observed from these relations, but the interactions and degree are not well understood. Although, the density-age and risk relation is confounding when observed at face value; we speculate that it may be yesterday's density that puts a women at risk today, but the degree of time lag is not understood.

13. TAMOXIFEN-SERIAL DENSITY RELATED RESEARCH

For future clinical trials, it may be important to draw on past clinical intervention trials as guide, where serial breast density changes were assessed following some risk-reduction measure. More specifically, a close review of the Tamoxifen-density related trials is provided due to the close relation with Tamoxifen and the isoflavones.

The work by Atkinson *et al.* showed that downward shifts in Wolfe patterns (decreasing risk) were recognizable within 0-25 months of therapy (38). Similarly, the work by Brisson *et al.* show that the change in both Wolfe-patterns and visually assessed density proportions occurs within a 12-40 month period with the change more pronounced in pre-menopausal women (40). Earlier research performed by Ursin *et al.* indicates measurable density changes over a 12-month window using visually derived tissue measures (41). It is important to note that the work discussed above was performed with essentially qualitative tissue measures and all show trends in decreasing tissue-related risk due to the intervention. Likewise, a case study of one pre-menopausal woman with unilateral invasive ER positive disease taking a5-year TAM regimen showed a progressive loss of tissue from the baseline heterogeneously dense contra-lateral breast to mostly fatty tissue after theree years. When the therapy was discontinued after 5-years the breast tended to baseline after one-year (42).

Research investigating the biological reasons that support the density-risk relation has demonstrated a significant positive correlation with growth factors (IGF-I) and increasing proportions of dense breast tissue (43). Other evidence indicates that it is possible to detect density changes due to monthly menstrual timing with film-mammography based on the radiologist's assessment (88) or magnetic resonance imaging techniques (43-45). The evidence here indicates that it is feasible to use serial density change as a non-invasive novel marker for evaluating the impact of chemoprevention, in the short term with quantitative methods (one year duration or greater).

In conclusion, it is plausible that automated density measurements are possible, and warranted, for chemoprevention trials similar in design to current TAM studies.

14. MOLECULAR TARGETS FOR ISOFLAVONES IN BREAST CANCER PREVENTION

To date, the molecular mechanism for cancer-preventive effects of isoflavones is poorly understood. Recently, we reported (10) that ester bond-containing tea polyphenols, such as (-)-EGCG, inhibited the chymotrypsin-like activity of the proteasome. Our computational docking and *in vitro* and *in vivo* proteasome activity studies confirmed that genistein is a proteasome inhibitor. We also found that genistein at 1 μM could inhibit ~30% of the chymotrypsin-like activity of purified 20S proteasome. It has been reported that plasma levels of genistein are in a range of 0.5-2.5 μM and the concentrations of genistein also vary in different tissues and organs. It is therefore possible that a partial inhibition of the proteasome activity by genistein at a physiological concentration might contribute to its reported cancer-preventative effects. Among different soy compounds genistein was the most potent inhibitor of the proteasomal chymotrypsin-like activity, which was consistent with the previous reports where it has also been shown that genistein is the most potent soy isoflavone.

Inhibition of proteasome activity by genistein in prostate (LNCaP) and breast cancer (MCF-7) cells was associated with increased levels of p27Kip1, IκB-α, Bax, and ubiqutinylated proteins, accompanied by induction of apoptotic cell death. We also found that genistein was the most potent one among all the tested isoflavones to induce Bax accumulation and PARP cleavage. However, daidzein, daidzein, genistein and glycerin, in addition to genistein, were able to accumulate $p27^{Kip1}$ protein. These results suggest that accumulation of Bax and IκB-α is associated with apoptosis induction while $p27^{Kip1}$ accumulation is probably associated with G_1 arrest . Our studies are consistent with the previous studies about p53-dependent induction of apoptosis by genistein (46). One of the important criteria for potential anticancer drugs is the ability to selectively kill tumor cells, but not normal cells. In our study we have shown that genistein was able to selectively increased ubiquitinated proteins and induced apoptosis in SV40-transformed, but not in the parental normal, human fibroblasts. Our results suggest that the proteasome is a potential target of genistein in human tumor cells and that inhibition of the proteasome activity by genistein might contribute to its cancer-preventive properties. The mechanism for failure of normal human cells to respond to genistein remains unknown, which is clearly important for understanding the cancer-preventative effects of genistein.

15. SUMMARY

Based on the evidence from epidemiological, animal, *in vitro* data and human clinical trials, it is evident that isoflavones are promising agents for breast cancer chemoprevention. It is also evident that the form of isoflavone used (purified vs soy products), dose of isoflavone used (low vs high), timing and duration of exposure of isoflavones appears to play a major role in determining agonistic or antagonistic effects. (46, 47, 48, 49) Collectively, these isoflavones have enough evidence to warrant use in a number of clinical trials to examine its efficacy as a potential chemopreventive agent for breast cancer, using a purified agent at a dose sufficiently higher

than those used in previous clinical trials, yet safe to be used for a long duration- one year.

In addition, the utility of IEBs in the development of cancer chemoprevention studies is solidly established (50). With rigorous attention to methodology, identification of high-risk cohorts, validated intermediate endpoint biomarkers, the evaluation of efficacy, safety and the mechanism of action of potential agents for cancer chemoprevention clinical trials has become an efficient and an increasingly exciting and critical areas of research. In addition, chemopreventive agents have multiple chemoprevention-associated molecular activities. Some of these activities may be interrelated. Also, a single activity, even if it is the agent's predominant pharmacological activity, may not be the most important or the only one effecting chemoprevention. Thus, although observing chemopreventive effects at the cellular and tissue levels is a key approach to identifying potential chemopreventive agents, future clinical trials must complement these studies by examining the molecular targets of these agents.

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