Daidzein and genistein glucuronides: synthesis, estrogen receptor binding and effect on human natural killer cell activity

by

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ABSTRACT

To examine the potential biological activities of isoflavone glucuronides, daidzein and genistein glucuronides were synthesized by using 3-methylcholanthrene-induced rat liver microsomes in the presence of 15 mM UDP-glucuronic acid at pH 7.4. The Km and Vmax for the formation of daidzein and genistein glucuronides were 9.64 and 7.95 μM, and 0.68 and 1.65 μmol/mg protein/min, respectively. The absence of UV absorbance maxima shift in the presence of sodium acetate confirmed that our synthesized products were 7-O glucuronides. Daidzein and genistein glucuronides were further purified by Sephadex LH-20 column for use in the estrogen receptor binding and natural killer (NK) cell activity studies. Daidzein and genistein glucuronides were evaluated for their abilities to compete with the binding of 17β-(^{3}H) estradiol to estrogen receptors of B6D2F1 mouse uterine cytosol. The concentrations required for 50% displacement of 17β-(^{3}H) estradiol (CB_{50}) were: 17β-estradiol: 1.34 nM; diethylstilbestrol: 1.46 nM; daidzein: 1.6 μM; genistein: 0.154 μM; daidzein glucuronide: 14.7 μM; genistein glucuronide: 7.27 μM. In human peripheral blood NK cells, genistein at concentrations < 0.5 μM and daidzein and genistein glucuronides within the range of 0.1-10 μM enhanced NK cell mediated target K562 cancer cell killing. At concentrations > 0.5 μM, genistein inhibited NK cytotoxicity. The glucuronides only inhibited NK cytotoxicity at a concentration of 50 μM. Isoflavones, and especially the isoflavone glucuronides, enhanced activation of NK cells by interleukin-2 (IL-2), additively. Our study indicated that at physiological concentrations, daidzein and genistein glucuronides are weakly estrogenic and can activate human NK cells in vitro, probably by a mechanism independent of IL-2.

INTRODUCTION

People are becoming increasing aware that certain foods or constituents within foods may promote health. It is not a novel concept that food is intimately linked to optimal health. "Let food be your medicine and medicine be your food" was a tenet espoused by Hippocrates in approximately 400 B. C. (Jones,1932). From the point of view of traditional Chinese medicine, food is not only a source of nutrients, it is also tonic and medicine. The term *medicinal food* was used as early as 1000 B. C. in the West Zhou Dynasty and was frequently used in the literature of the East Han Dynasty (about 100 B. C.) (Weng & Chen, 1996). In the western world, since the 1980s, scientists , especially nutritionists, are becoming more and more interested in components of foods besides their nutrients, the ability of non-nutrient food components to modulate physiological systems of the human body, including immune, endocrine, nervous, circulatory, digestive and other cellular systems. Several bodies such as the National Research Council in the United States and the World Health Organization have directed their attention to foods that have a positive role in maintaining health and preventive diseases.

The expanding scientific knowledge has supported the idea that foods play a vital role in overall health and well-being. Six of the ten leading causes of death in the United States are believed to be related to the food we consumed: cancer, coronary heart disease, stroke, diabetes, atherosclerosis, and liver disease. Although cardiovascular disease is currently the leading cause of mortality in the United States and caused the death of one American every 34 seconds in 1995, cancer will surpass cardiovascular disease as the

leading killer by the turn of the century. A comprehensive review of the cause of cancer conducted by Doll and Peto in 1981 estimated that up to 70% of certain cancers are attributable to diet. In 1982, the National Academy of Sciences published public health guidelines regarding dietary practices and cancer risk reduction. The foods recommended by the guideline included fruits, vegetables, and whole-grain cereal products in the daily diet, especially citrus fruits and carotene-rich and cabbage-family vegetables. It was thought that such foods could reduce cancer risk because they contained certain phytochemicals.

Phytochemicals are nonnutritive secondary plant metabolites present in relatively small amounts. To date, most research related to phytochemicals has focused on cancer prevention. A dozen distinct classes of nonnutrient phytochemicals in fruits and vegetables with demonstrated anticancer activity have been identified (Table 1). This may explain in large part the overwhelming epidemiological evidence linking the increased consumption of fruits and vegetables with reduced cancer risk (Steinmetz and Potter, 1991; Block et al., 1992). Cancer fighting phytochemicals are thought to function by one of several mechanisms: induction of detoxification enzymes, inhibition of nitrosamine formation, provision of substrate for formation of antineoplastic agents, dilution and binding of carcinogens in the digestive tract, alteration of hormone metabolism, and antioxidant activity.

Soybeans contain several classes of phytochemicals, such as saponins, protease inhibitors, isoflavones and inositol hexaphosphate (phytates) (Table 1). Although several

Table 1. Classes of potentially anticarcinogenic phytochemicals in fruits and vegetables (modified from Steinmetz and Potter, 1991)

Phytochemical class	Food source
Carotenoids	Fruits and yellow/orange or dark-green leafy vegetables
Dithiolthiones	Cruciferous vegetables
Glucosinolates/indoles	Cruciferous vegetables
Isothiocyanates/thiocyanates	Cruciferous vegetables
Coumarins	Vegetables and citrus fruits
Flavonoids	Most fruits and vegetables
Phenols	Most fruits and vegetables
Protease inhibitors	Seeds and legumes, particular soy
Plant sterols	Vegetables
Isoflavones	Soybeans
Saponins	Plants, particularly soybeans
Inositol hexaphophate	Plants, particularly soybean and cereals
Allium compounds	Onions, garlic, leeks, chives
Limonene	Citrus fruits
Epigallocatechin	Tea
Melanoidins	Coffee
Phytic acid	Cereals
Curcumin, sesaminols	spices

studies attributed the anticarcinogenic effect of soybeans to protease inhibitors, a study conducted by Barnes et al. (1990) showed that after inactivation of protease inhibitors by autoclaving, soy diet still strongly inhibited carcinogen-induced mammary tumors in rats. Many epidemiological and animal studies, including Barnes's observation have led to the hypothesis that soybean isoflavones may be responsible for the anticarcinogenic effect of soy.

Epidemiological studies showed that soybean consumption may contribute to the lower rates of breast, colon and prostate sex-hormone dependent cancers in Asian countries (Adlercreutz et al., 1991 and 1995). Urinary excretion of isoflavones and their metabolites was 20-30 times higher among Japanese women and men consuming a traditional diet than in people living in Boston and Helsinki (Adlercreutz et al., 1991). Compared with vegetarian and omnivorous healthy controls, breast cancer patients excreted about 21% less of the isoflavone metabolite equol in urine (Adlercreutz et al., 1982). When rats consume a soybean-based diet, they developed less mammary tumors following the administration of the carcinogen initiator of N-methylnitrosourea and 7,12-dimethylbenz[α]anthracene (DMBA) than rats on isonitrogenous and isocaloric diet without soybeans (Barnes et al., 1990). Genistein and daidzein inhibited the growth of both estrogen receptor-positive (MCF-7) and -negative (MDA-468) human breast cell lines with IC_{50} of 24-44 μmol/L and 79-134 μmol/L, respectively (Peterson and Barnes, 1991). By measuring hepatic glutathione peroxidase activity and quantifying liver glutamyltransferase-positive (GGT^+) and placental glutathione transferase-positive ($PGST^+$) altered hepatic foci (AHF), Lee et al. (1995) found that isoflavones suppressed early early stage of hepatocarcinogenesis promoted by phenobarbital in diethylnitrosamine (DEN) initiated rats. These epidemiological studies and experimental results strongly suggested that a Western diet may lack certain cancer-protective factors which are present in soybeans.

The main forms of isoflavones found in soybean are daidzin, genistin, glycitin, malonyl- and acetyl-glucosides and their corresponding aglycones daidzein, genistein and

glycitein (Wang and Murphy, 1994). It was found that the content of the total isoflavones and different forms of isoflavone are different; the bioavailability and biological activities of different forms of isoflavone also differ to some extent. For example, daidzein is more bioavailable (Xu et al., 1995) and more effective in potentiation of splenocyte proliferation than genistein (Wang et al., 1997). Before consumers get the green light to increase consumption of foods high in isoflavones or isoflavone supplements, it is important for them to know what kinds of food sources, in what forms (soy protein isolate, soy fiber, miso etc.), what amounts or in what kind of dietary pattern they should consume these compounds.

After absorption, except for a small amount of aglycone isoflavones and equol, over 95% of the isoflavones appear as conjugated forms (mainly as glucuronides and less as sulfates) (Lundh, 1995) in the circulation. Classically, conjugation has been considered as a detoxification step. But in some cases, after conjugation, the biological activities of the conjugates has been enhanced. The formation of aromatic amine glucuronides can cause bladder cancer (Thorgeirsson et al., 1983; retinoyl β-glucuronide (RAG) and retinyl β-glucuronide (ROG) are more active than their parent compounds in inhibiting prolactin-induced DNA synthesis in organ cultures of mouse mammary glands (Olson et al., 1992). To our knowledge, the research about isoflavones has been focused on the biological activities of daidzein, genistein and equol. The activities of isoflavones conjugates have not been previously reported. In order to fully understand anticarcinogenic and other functions exhibited by soybean isoflavones, it is important to study all the active

metabolites and their potential biological activities. So, in this paper, we investigated the estrogen receptor binding ability and natural killer cell activation activity of daidzein and genistein glucuronides.

LITERATURE REVIEW

Isoflavones are phytochemicals. The amount that is present in plants depends upon many factors, including growth (Hanson et al.,1965) and genetic background (Morley and Foaucis, 1968). For soybeans, the location also influences the content of isoflavone present (Wang and Murphy, 1994a). The amount of isoflavones present in soybeans is about 1-3 mg/g (Wang and Murphy, 1994b). The major isoflavones found in soybeans are the polar aglycosylated form: daidzin, genistin, glycitin, malonyl- and acetyl-glucosides of these forms and their respective aglycone forms: daidzein, genistein and glycitein. The distribution of the isoflavone isomers in commercial soybean foods are quite different. For example, texturized vegetable protein (TVP) and tofu contain relatively high levels of isoflavone glycosides, whereas in fermented soybean products, such as miso, approximately 80% of the isoflavones are presented as aglycones.

Metabolism of isoflavones

Most of the isoflavones occur in soybean as glycosides and aglycones, as stated above. In ruminants, the glycosides are hydrolyzed by β-glucosidases in the rumen and further demethylated and reduced by the microorganisms. A very minor part of these hydrolyzed isoflavones is absorbed very quickly from the rumen, and reaches the blood circulation unconjugated. The bulk are conjugated in gastrointestinal epithelium by phase II biotransformation enzymes, namely UDP-glucuronyltransferases and sulfotransferases (Fig 1). The remaining isoflavones unconjugated when entering the blood circulation are

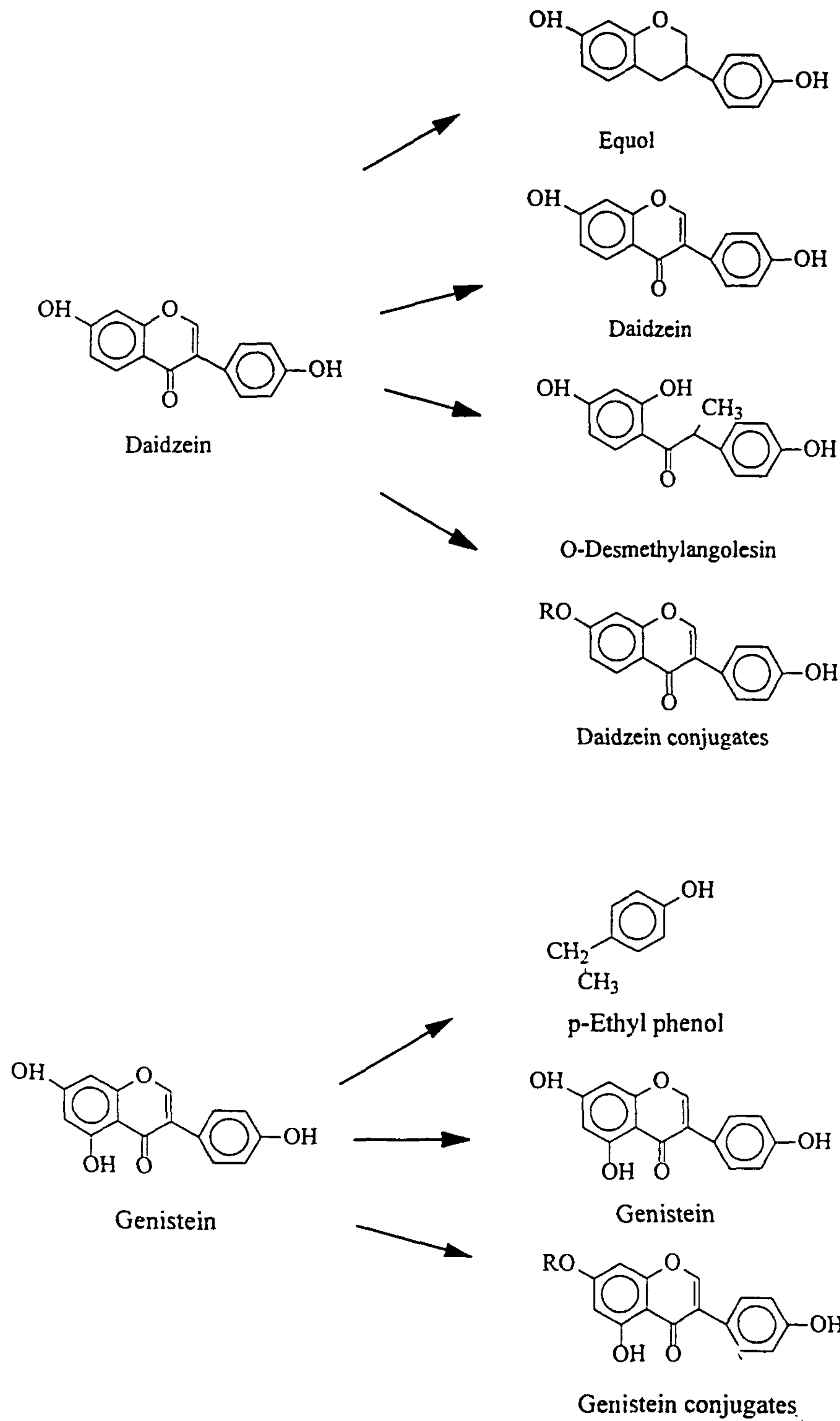

Figure 1. Metabolic pathways of dietary isoflavones Adapted from Setchell and Adlercreutz, 1988)

mainly conjugated by the liver and perhaps also by other extrahepatic tissues such as kidney. Lundh (1995) reported that in cattle and sheep, the conjugation of isoflavones mainly happened in gastrointestinal epithelium, which indicated that the livers are not the predominant detoxifying organ for isoflavones in ruminants. Rat intestinal microflora culture study have confirmed the metabolism of isoflavones. P-ethylphenol, a metabolite of genistein was identified by paper chromatograph and thin layer chromatograph (TLC) and confirmed by gas chromatograph (GC). Equol was also detected (Griffith and Smith, 1972).

The mode of formation of isoflavone metabolites in humans seems to be similar to that in rats. Administration of soy protein resulted in a marked increase in the excretion of equol (a metabolite of daidzein formed by intestinal bacterial action) (Axelson et al., 1984). Some subjects may not be able to form equol (Axelson et al., 1984) and germ-free rats did not excrete equol when given commercial pelletted food (Axelson and Setchell, 1981). O-desmethylangolensin (ODMA) seems to be a minor metabolite in man (Adlercreutz et al., 1986).

Different isoflavone component in food may result in different metabolites. When Yasuda et al. (1994) treated male Sprague-Dawley rats (orally) (120-160 g) with daidzein (60 mg/kg), the urine of rats contained daidzein 7-O-β-D glucuronide, daidzein 4'-O-sulfate and daidzein. The urine of rats treated with daidzin (100 mg/kg) contained daidzein 7, 4'-di-sulfate and the other three compounds mentioned above.

Epidemiology of soy's anticancer effects

Diet has been considered to play an important role in cancer prevention. Although high intake of fruits and vegetables has been associated with lower cancer risk (Willett, 1994), evidence also suggests that higher intake of isoflavones from soy rich diets can decrease the incidence of hormone-dependent cancers, such as breast, colon and prostate cancers. British intake of isoflavones has been estimated to be < 1 mg/day (Jones et al., 1989), while the average consumption of these compounds in Asian countries is 50-100 mg/day (Barnes et al., 1990). Equol excreted in Japanese was up to twenty times more in urine than in Western populations, and the circulation levels of isoflavones in plasma was also much higher. In 1978, Nomura et al. found an inverse association between intake of miso soup or tofu and subsequent risk of breast cancer. Hirayama (1986) also showed that a high intake of soybean paste soup was associated with a reduced risk in Japanese women. Lee et al. (1991) in their case-control study found a significant ($p<0.01$) inverse association in premenopausal women between breast cancer risk and soybean protein intake. These epidemiological observations have been verified by certain experiment studies.

Compared with a casein diet, Barnes et al. (1990) showed that N-methylnitrosourea (NMU) induced female rat mammary tumor load was reduced by 20% powdered soybean chips from 8 to 3. However, when the isoflavones were chemically extracted from the soybean product, no reduction in mammary carcinogenesis was observed in rats (Barnes et al., 1990). Hawrylewiez et al. (1991) also noticed that soybean protein isolate decreased

tumors by 50% in a NMU (40 mg/kg body weight) model of Sprague-Dawley female rats (7 weeks) breast cancer compared to casein (20%) feeding group. When rats consumed a powdered soybean diet (15%), there was a 50% reduction in X-ray irradiated mammary tumors (Troll et al., 1980). Lamartiniere et al. (1995) also showed that administration of genistein (5 mg subcutaneous injection) to female Sprague-Dawley CD rats (50 days) suppressed mammary cancer induced by dimethylbenz[α]anthracene (DMBA) (80 μg/g body weight), compared to vehicle (dimethylsulfoxide) treated group.

In explaining the anticarcinogenic functions of isoflavones, several mechanisms have been proposed. Initially, isoflavones, especially genistein were considered to have estrogen agonist/antiagonist activity because of their structural similarity to the physiological estrogens such as 17β-estradiol and because of its estrogenic effects (mouse uterine weight incresing) when administered 1 or 2 mg genistein, 2 mg genistin to immature mice (Cheng et al., 1956). However, other mechanisms have been proposed, including protein tyrosine kinase (PTK) inhibition, topoisomerase II inhibition, induction of differentiation and antiproliferation, and inhibition of oxidation events.

Antioxidant functions of isoflavones

Hepatic neoplastic nodules from rats initiated with a single injection of diethylnitrosamine (DEN), 0.15 μmol/kg body wt, intraperitoneal) and promoted by 0.05% phenobarbital showed increased levels of reactive oxygen species (ROS), predominantly superoxide anion radicals ($O_2^{\bullet-}$) and hydroxyl peroxide (H_2O_2), which was measured by the emission of lucigenin-chemiluminescenic signals (Scholz et al., 1990).

Certain ROS, such as hydroxyl radicals (•OH), $O_2^{\bullet-}$ and H_2O_2, and their subsequent modification of macromolecules (such as protein, RNA and DNA) may be involved in development of multistage carcinogenesis (Fisher et al., 1988; Frenkel, 1992; Cerutti, 1985; Sun, 1990). Antioxidants may protect cells from the oxidative damage that occurs during carcinogenesis (Sun,1990). Antioxidant effects of isoflavones may inhibit tumor promoter-induced oxidant formation and inflammatory responses which could lead to anticarcinogenic activities. Naim et al. (1976) reported that isoflavones inhibited lipoxygenase action and prevented peroxidative hemolysis of sheep erythrocytes in vitro. Feeding a soybean isoflavone extract at 240 mg/kg diet for one week, rat hepatic cumene hydroperoxidase activity was increased significantly. In female CD-1 mice (6-7 weeks old) fed 250 ppm genistein for 30 days, the activities of antioxidant enzymes including catalase, SOD, GSH-PX and GSSG-R in skin and small intestine enhanced about 10-30%. When comparing the effect of isoflavones on the H_2O_2 production by 12-*O*-tetradecanoylphorbol-13-acetate (TPA) activated HL-60 cells and $O_2^{\bullet-}$ generation by xanthine/xanthine oxidase, Wei et al. (1995) showed that genistein and daidzein was among the most potent inhibitors, and biochanin A had little effect.

Antiproliferation and differentiation induction properties of isoflavone

Another main mechanism proposed for the anticarcinogenic function of isoflavones is antiproliferation and inhibition of cell growth. As the main transduction signal, increased activity of protein tyrosine kinases (PTKs) will give cells proliferation

advantages. It is possible that tyrosine phosphorylation plays an important role for cell proliferation and cell transformation.

Genistein is the most potent inhibitor of PTK, so the investigation of antiproliferation activities of isoflavones is mainly focused on genistein, even though there is some reports about daidzein. Genistein has been shown to inhibit the PTK activity of growth factor receptors, such as epidermal growth factor (EGF) receptor and $pp^{66v\text{-}src}$ PTK with a IC_{50} of 22-26 μmol/L, and inhibited intact A431 PTK with an IC_{50} of 148 μmol/L (Akiyama et al., 1987 and 1991). Aluminum tetrafluoride stimulated inositol phosphates in 3T3 cells was inhibited by genistein and daidzein (Higashi and Oganara, 1992). Genistein inhibited both estrogen and growth factor stimulated proliferation of human breast cancer cell lines, including MCF-7, T47D ER^+, T47D ER^-, BT-20 and ZR-75-1 (Peterson and Barnes, 1991 and 1996). However, the requirement for the presence of functional estrogen receptor or inhibition of epithelial growth factor PTK activity is not the only mechanism for the growth inhibition effect of genistein. Interfering with signal transduction events stimulated by estradiol or growth factors may also account for the mammary cell growth blockage function of genistein, like the blockage of human colon cancer cell proliferation stimulated by estradiol which stimulates cell proliferation by triggering a tyrosine-specific protein kinase, erk-2 (Domenico et al., 1996).

The other major cellular effect of genistein is to induce differentiation. Genistein induced erythroid differentiation of erythroleukemia cells (Watanabe et al., 1991). Myeloblastic ML-1 cells were induced to differentiate into promyelocytes, and

promyelocytic HL-60 cells were induced to differentiate into mature granulocytes (Makishma et al., 1991; Constantinou et al., 1990). Mouse C1 line cells which are megakaryoblastic cells established by coinfection of Abelson murine leukemia virus and recombinant simian virus were induced to differentiateand and the *v-abl* tyrosine kinase activity was inhibited by genistein (4.6 µmol/L) (Honma et al., 1991). The differentiation effects have also been attributed to PTK inhibition. In some studies, DNA damage have been noted associated with the differentiation, and it has been attributed to the inhibition of topoisomerase II (Markovits et al., 1989).

Estrogenic and antiestrogenic property of isoflavones

The anticarcinogenic effect of isoflavones may also be exerted via their antiestrogenic activity, especially in hormone-dependent breast cancer. Breast cancer is associated with hormone (estrogen, prolactin) activity (Welsch, 1976). Estrogens bind to cytoplasmic receptors, elicit their translocation to the nucleus estrogen receptor complex for a short time. This events initiate DNA transcription, RNA synthesis, which in turn result in protein synthesis and cell growth (Anderson, 1977). So, increasing the levels, intensity and duration of exposure of estrogen would increase the risk of breast cancer. Several studies have examined the biological effect of isoflavones in women. Consumption of 45 mg glycosylated isoflavones as 60 g textured vegetable protein over a 1-month period significantly ($p < 0.01$) prolonged the length of menstrual cycle of premenopausal women, and suppressed luteinizing hormone (LH) ($p < 0.05$), and follicle-stimulating hormone (FSH) ($p < 0.01$) (Cassidy et al., 1994, 1995). These findings suggested that soybean

isoflavones may exert weak estrogenic effect on the hypothalamic-pituitary-gonadal axis, and may be beneficial with respect to factors stimulating breast cancer.

The phenolic ring of isoflavones, which is a prerequisite for binding to the estrogen receptor, is similar in structure to mammalian estrogen17β-estradiol. However, the estrogenicity of daidzein and genistein were about 10^3-10^5 times less than estradiol or the synthetic estrogen diethylstilbestrol (DES) (Farmakalidis et al., 1985; Kitte et al., 1980; Newsome and Kitte, 1980). The sheep uterine estrogen receptor binding of daidzein and genistein were only 0.1% and 0.9% that of 17β-estradiol, respectively (Shutt and Cox, 1972). Equol and O-desmethylangolensin (ODMA), the metabolites of daidzein, had also been found to bind to estrogen receptors and to have weak estrogen activity (Shutt and Cox, 1972; Adlercreutz et al., 1992). Certain soybean foods, such as tofu and soymilk, contain comparatively large amount of isoflavones. After consumption, the plasma concentration of isoflavones and their metabolites may exceed endogenous estrogens by several orders of magnitude, which could effectively compete with estrogen by competitive binding to estrogen receptor, suppressing estrogen stimulated cell growth (Adlercreutz et al., 1995).

In addition to the competitive estrogen receptor binding ability, isoflavones may also exert their antiestrogenic effects by directly or indirectly increasing the synthesis of sex-hormone binding globulin (SHBG), a protein that binds dihydrotestosterone, testesterone and estradiol. Mousavi and Adlercreutz (1993) found that at concentration of 5-40 μM, genistein can not only significantly increase hepatocarcinoma (Hep-G2) cell

SHBG production, but also suppress the proliferation of these cancer cells at a stage when SHBG production continues to be high. The urinary excretion of the phytoestrogen equol had a positive relationship with plasma SHBG and negative association with plasma free estradiol, found by Adlercreutz (1987). The high SHBG levels in plasma will decrease hormone exposure by reducing the metabolic clearance rate or lowering the uptake of sex hormone in many tissues (Adlercreutz et al., 1987; Monsavi and Adlercreutz, 1993), in turn will decrease the risk of sex-hormone dependent cancer. Decreasing estrogen levels by isoflavones can also be achieved by inhibition of aromatase activity, a cytochrome p-450 enzyme that catalyzes the conversion of androgens to estrogens (Kellis et al., 1984; Adlercreutz et al., 1993). The competitive binding of lignans to aromatase with a binding affinity of 1/75-1/300 to natural substrates testosterone and androstenedione will result in a decreased substrate binding affinity (Adlercreutz et al, 1993).

Modulation of immune system

In malignancy, natural killer (NK) cells appear to represent a first line of defense against the metastatic spread of blood-borne tumor cells, and normal NK activity may be important in immune surveillance against cancer. The development and progression of cancer has been found to be associated with decreased natural immunity, as measured in vitro by NK activity and/or absolute numbers of circulating NK cells (Whiteside and Herberman, 1989). Many cells of the body including macrophages and neutrophils can produce prostaglandins (PG) (Kunkle et al., 1984), which suppress the activation of all of the immune system: B cells (Thompson et al.,1984), T cells (Parhar et al., 1988), NK cells

(Herman and Rabson, 1984), lymphokine-activated killer (LAK) cells (Parhar and Lala, 1988), as well as macrophages (Parhar and Lala, 1988). Tumors which produce PGE_2 are able to down regulate the immune system and escape lysis (Earnest et al., 1992). For the T cells, the PGE_2 inactivation is mediated by blockage of interleukin-2 (IL-2) production (Lala et al,. 1988; Chouaib et al., 1985), down-regulation of IL-2 receptors (production (Lala et al., 1988) and transferrin receptors (Chouaib et al., 1985). Due to the dependence of NK cells on interferons as well as IL-2 for their activation, PGE_2 action on both of these pathways remains a strong possibility. Isoflavones as antioxidants can increase glutathione peroxidase activity (Hendrich et al., 1994) which inhibits prostaglandin production by removing the hydroperoxides required for prostaglandin synthetic activity (Marshall et al., 1988). So, it is possible that isoflavones may also enhance IL-2 production and NK activity.

The IL-2 activation of NK cells is via the induction of tyrosine phosphorylation of multiple proteins (Einspahr et al., 1990 and 1991). The IL-2 receptor itself lacks intrinsic tyrosine kinase activity (Waldman, 1991). Other non-receptor, membrane-associated tyrosine kinases, such as *src*-family tyrosine kinases, may associate with the IL-2 receptor and thereby relay information from the receptor to the cell interior. Einspahr et al.(1991) found that protein tyrosine phosphorylation was an early and obligatory signal in the activation of NK cell cytotoxic function. As a PTK inhibitor, genistein also demonstrated concentration-dependent suppression of direct and FcR-mediated NK cellular cytotoxicity.

Therefore, the effect of isoflavones on NK cell activity may depend on the balance between their inhibition of tyrosine kinase activity and suppression of prostaglandin production.

An experiment performed by Drs. Cunnick and Hendrich (unpublished data) showed that after feeding 240 mg isoflavones /kg diet for 10 days, rat liver associated NK cell activity was two fold greater than that in the control group. However, rats receiving a dose of 480 mg isoflavone/kg diet did not differ in NK cell activity compared with the control group. This study indicated that soybean isoflavone extracts can affect NK cell activity in a dose dependent manner.

In addition to the possible effect on NK cell, isoflavones, especially genistein, also have some influences on B-cell and T-cell. In human B-cell lineage lymphoid cells, genistein inhibited multiple effects of CD-40 (in B cells function as signal transduction), namely tyrosine phosphorylation of several proteins, phosphoinositide turnover, and activation of serine/threonine protein kinase (Uckun et al., 1991). In 1995, Uckun et al. reported that genistein (1.8 x 10^5 cpm/nmol of B43-^{125}I-genistein), conjugated to the B-cell specific CD-19 receptor with monoclonal antibody B43 by one molecule of genistein conjugated to one molecule of mAb B43 through SANPAH cross-linker, was highly effective in treating leukemia in a nude mouse in a model of pre-B cell human leukemia. At a concentration of 30 μg/mL. genistein completely blocked mitogen induced T-cell receptor (TCR) ξ phosphorylation. In the presence of the same concentration of genistein (30 μg/mL), T-cells stimulated with phytohemagglutinin (PHA) or with OKT3, a

monoclonal antibody to CD3, failed to undergo the blast transformation (Mustelin et al., 1990).

When Swiss mice were fed 20 or 40 mg daidzein/kg (Zhang et al., 1997), their peritoneal macrophages and thymus weights were increased. Both of the humoral and cell-mediated immunity were enhanced. In an in vitro study, at concentration of 0.01-10.0 μM, daidzein significantly inhibited proliferation of mixed mice splenocyte cultures activated with concanavalin A or lipopolysaccharide in a dose dependent manner, whereas genistein had no influence on the response (Wang et al., 1997).

There are few reports on effects of isoflavones to the immune system. Clearly, more work needs to be done on isoflavones' immunoregulatory function.

It is hard to know that the relevance of in vitro studies in understanding the potential chemopreventive effects of phytoestrogenic isoflavones. Many of the studies were carried out by using high concentration of isoflavones, and few of the previously described mechanisms in tumor cells are sensitive to physiological serum concentrations of genistein. Petrokis et al. (1996) described that daily ingestion of a commercial soy protein isolate containing genistein and daidzein stimulated the premenopausal female breast, as shown by an increase in nipple aspirate fluid volume and by the presence of hyperplastic epithelia cells. In a recent study by Zava and Duwe (1997), it was clearly shown that, in several human breast cancer cell lines, genistein acts as a growth stimulator at low concentrations and as a growth inhibitor at high concentrations. In azoxymethane (AOM) induced rats rats fed 250 ppm genistein, Rao et al. (1997) found that genistein enhance the

formation of noninversive adenocarcinomas in colon. These results indicated that the effect of genistein and other soybean isoflavones may be organ specific.

When DEN initiated, PB promoted female F344/N rats were fed soybean isoflavone extract at 920 μmol/kg or 1840 μmol total isoflavone/kg diet for 3 months, hepatocarcinogenesis were suppressed. But when they were fed 1840 μmol/kg ispflavone extracts for 11 months, there was a greater development of GGT+ and PGST+ AHF than in the group fed the basal diet alone (Lee et al., 1995). This experiment indicated that the safety margin for these soybean isoflavones is relatively narrow.

As we know, after consumption, glycosidic isoflavones are cleaved to aglycone forms by gut β-glucosidase. These aglycones may be conjugated in gut mucosal cells or in the liver by the phase II enzymes, mainly UDP-glucuronyltransferases and sulfotransferases However, the sulfate conjugates are probably in general only a minor part of the total isoflavone conjugates. The biological activity of the isoflavone glucuronides is still unknown. It is well accepted that not all glucuronide forms are inactive. For example, retinyl β-glucuronide (ROG) is about 40-fold more active than retinol and fourfold more active than 13-*cis* retinoic acid (RA) in increasing NK cell number (Prabhala, 1989). It is possible that isoflavone glucuronides may have similar biological functions as ROG has. In order to completely understand the function of mechanisms of isoflavones as anticarcinogens or as activators of certain cancers, and to establish their possible utility and safety for the use in humans, it is important to understand the potential biological activities of isoflavone metabolites under common

physiologically achievable concentrations, e.g. concentrations resulting from dietary exposure.

MATERIAL AND METHODS

Chemicals

Daidzein and genistein were chemically synthesized by T. T. Song in Dr. Murphy's lab (ISU). Uridine 5'-diphosphoglucuronic acid (trisodium salt), uridine 5'-diphospho-N-acetylglucosamine (sodium salt), D-saccharic acid-1,4-lactone, diethylstilbestrol (DES), Tween 80, 17ß-(2,4-^{3}H) estradiol (23 Ci/ mmol), 17ß-estradiol, dextran coated charcoal (DCC), bovine serum albumin, interleukin-2 (IL-2) and other chemicals were all obtained from Sigma Chemical Co., St. Louis, MO. Scintiverse® (BD, scintanalyzed) fluid was purchased from Fisher Scientific Co. (Pittsburgh, PA). Hank's balanced saline solution (HBSS), RPMI-1640, HEPES, L-glutamine, gentamicin and fetal bovine serum (FBS)-heatinactivated were all from Life Technologies, Gaithersburg, MD.

Rats microsome preparation and isoflavone glucuronide synthesis

All the animals used in this experiment were purchased through laboratory animal resources, Iowa State University (ISU), Ames, Iowa. All animal uses were approved by the ISU Animal Care Committee. Rats (190-200 gram Sprague-Dawley female) were gavaged each day for four consecutive days with 3-methylcholanthrene in corn oil (40 mg/kg body weight) before sacrifice. Microsome preparation and glucuronide synthesis were modified from Lundh et al (1988). Rat livers were washed with ice cold 0.25 M sucrose and 50 mM tris-HCl buffer (pH 7.4), and homogenized in a Kinematika-Gmbh homogenizer (Luzern, Switzerland). The homogenate was adjusted to 10% (w/v) with buffer and centrifuged at 10,000g for 10 min. The supernatant fraction was centrifuged at

105,000 g for 60 min and the resulting microsomal pellet was washed with sucrose-Tris buffer and rehomogenized slightly. Protein was measured by the Lowry procedure (Lowry, 1956) with bovine serum albumin as the reference standard.

The final standard incubation mixture contained 100 μM daidzein or genistein, usually added in 50% ethanol. The final concentration of ethanol was 5%. Other reagents were 15 mM uridine 5'-diphosphoglucuronic acid (UDPGA), 50 mM Tris-HCl buffer, 0.1 mM MgCl, 0.25 mM uridine 5'-diphosphoacetylglucosamine (UDPAG) and 10 mM saccharic acid 1,4-lactone. The reaction mixture was prewarmed for 20 sec. before adding 5 mg microsomal protein/mL to initiate the reaction. The mixture was then incubated for 60 min at 37°C. Reaction was stopped by adding of methanol and 0.4 M trichloroacetic acid-0.6 M glycine buffer (pH 2.0). The mixture was centrifuged and the supernatant was concentrated by rotovapor (R-114, BÜCHI, Flawil, Switzerland). Ten mL concentrated reaction mixture was subjected to a Sephadex LH-20 column with 40% ethanol as the eluant, the separated isoflavone aglycone and its glucuronide were detected by a single path monitor UV detector (Pharmacia, Ulppsala, Sweden) and collected by a Ultrorac fraction collector (LKB-7000 UltroRAC, LKB-producter, Bromma, Sweden). The glucuronide fraction was lyophilized and stored at 20°C for further using.

The purity and identification of daidzein glucuronide and genistein glucuronide were done by HPLC, UV spectral analysis and mass spectral analysis. UV spectral analysis was performed on a Hewlett Packard 8452A diode array spectrophotometry. The effects of $AlCl_3$-HCl, sodium methoxide, sodium acetate and boric acid in methanol on the UV

absorption maxima were determined according to Mabry et al. (1970). Mass spectral analysis was done on a Finnigan model TSQ-700 (Finnigan MAT, San Jose, CA).

HPLC assay

Twenty µl of sample was injected onto a reversed phase 5 µm C18 AM 303 column (250 x 4.6 mm) (YMC Co. Ltd. Wilmington, NC). A linear HPLC elution gradient was composed of (A) 0.1% glacial acetic acid in water and (B) 0.1% glacial acetic acid in acetonitrile. Following sample injection, solvent B was kept at 20 % for 5 min, then increased to 30% in 25 min, after that increased to 50% in 15 min and decreased to 20% at the end of 45 min. The solvent flow rate was 0.8 mL/min at the first 5 min, then increased to 1 mL/min and kept there for 40 min. The HPLC system was a Hewlett Packard 1050 series. Wavelengths used to detect isoflavone compounds and their conjugate products were 254 nm. Ultraviolet absorbence spectra were recorded and area responses were integrated by Chem station^{3D} software (Hewlett Packard Company, Scientific Instruments Division, Palo Alto, CA) to identify and quantify isoflavones and their conjugates. Purified aglycones and glucuronides were used as external standard to calibrate the results.

Cytosol preparation and estrogen receptor assay

Female B6D2F1 mice (4 weeks) were obtained from Harlan Sprague-Dawley (Madison, WI). Their uteri were homogenized in Tris-EDTA (10 mM Tris and 1 mM EDTA, pH 7.4) buffer (1:10 wt/vol). The diluted cytosol fraction was obtained by centrifugation of the homogenate at 100,000 g for 1 hour. The binding assay was modified

from Verdeal et al. (1980). Total binding was determined by adding 0.2 mL Tris-EDTA buffer, 0.2 mL 1.5 ng (^{3}H) estradiol/mL and 0.2 mL cytosol fraction. Non-specific binding was determined by replacing the Tris-EDTA buffer with 0.2 mL of 17ß-estradiol (1500 ng/mL) in the pH 7.4 buffer. Blanks contained 0.4 mL buffer and 0.2 mL (^{3}H) estradiol. Competitive binding was determined by replacing the 0.2 mL buffer with 0.2 ml of solution containing 150, 750, 1500, 7500 and 15000 ng/mL daidzein or genistein, or 150, 750, 1500, 7500 and 15000, 30000 and 75000ng/mL daidzein glucuronide or genistein glucuronide at pH 7.4. Duplicates were performed for each estrogen at different concentrations. Mixtures were incubated at room temperature (23°C) for two hours. Half mL dextran coated charcoal (2.5% in pH 7.4 buffer) was added to each mixture and incubated for another 15 min. After 10 min centrifugation at 1,000g, the supernatants were decanted into scintillation vials containing 10 ml scintillation fluid. The samples were counted for 10 min on a Packard liquid scintillation analyzer model 1900TR (Packard Instrument Co., Downers Grove, IL).

Natural killer activity assay

Six human subjects (three female and three male, between 18 and 29 years old) , with informed consent, participated in this experiment. The use of human subjects was approved by ISU subjects Committtee. Twenty mL peripheral blood samples were collected from each subject in heparinized vacutainers by a licensed medical technologist under stringent aseptic conditions. Mononuclear cells were isolated from diluted blood (1:1 with HBSS) using density gradient media Ficol-hypaque (Pharmacia). Cells collected

from the interface was washed and diluted with complete medium (RPMI-1640 with 25 mM HEPES, 2 mM L-glutamine, 50 μg/mL gentamicin and 10% FBS-heat inactivated) to 2.5 x 10^6 cells/mL before plating. The target K562 cancer cells (ATCC) were maintained in complete medium. Cancer cells in log growth phase (7 x10^6) were labeled with 200 μCi of ^{51}Cr for 70 min. The targets were washed three times prior to dilution and reactions containing 2.0 x 10^5 cells/mL were prepared. Five concentrations, 0.1, 0.5, 5, 10, 50 μM of daidzein glucuronide, genistein glucuronide and genistein (dissolved in DMSO; 0.0825% of DMSO in total incubation volume) with and without interleukin-2 (1.25 ng/mL) were incubated with 2.5 x 10^5 isolated peripheral blood lymphocyte (PBL) in the 96-well plate (Model 3595, Costar, Cambridge, MA) in triplicate for 30 min. After enriched chromium targets (1 x 10^4 cells/well) were added to each well, the plates were incubated for 4.5 h in a humidified CO_2 incubator (5% CO_2) (Fisher scientific, Pittsburgh, PA), and centrifuged at the end of incubation for 5 min. 100 μL of supernatant was collected to determine the amount of ^{51}Cr released by dying cells using a Gamma Trac 1191 counter (TM Analytic, Inc., Elk Grove Village, IL). Data was expressed as percentage of NK killing obtained from cells incubated with isoflavones and with or without IL-2.

Statistical analysis

For the natural killer cell study, means of treatments were used to estimate treatment effects by using SAS program. An ANOVA to compare three isoflavones across five concentrations with and without interleukin-2 was used to assess the significance of the

variances. The differences between enzymatic kinetics were also analyzed by ANOVA program. A p value of < 0.05 was considered to be statistically significant.

RESULTS

Isoflavone glucuronides synthesis and identification

HPLC analysis. The acetonitrile and water mobile phase system in combination with a YMC C_{18} reverse phase column was used for the separation of isoflavone aglycones and their glucuronides. A typical chromatogram of a reaction mixture is shown in Fig 2. Isoflavone conjugates were more hydrophilic and would appeared earlier than their aglycone counterparts. The retention times were: daidzein glucuronide at 8.5 min, genistein glucuronide at 14.6 min, daidzein at 26 min and genistein at 37.3 min. After LH-20 separation, the purity of the isoflavone conjugates was evaluated by peak spectral analysis, enzyme hydrolysis and mass spectrophotometer. The peak spectral analysis and wavelength scan after separation and deconjugation all matched their respective aglycone compounds. The mass spectral results also showed that these conjugated matched their speculated molecular weight and structure. The purity was about 95%.

Maximal UV absorption of glucuronides. When dissolved in different solvents, the maximal UV absorption of the glucuronides differed. As shown in Table 2, in 30 % acetonitrile, which was the same solvent used in our HPLC system, the maximal absorption was at 250 nm for daidzein glucuronide (DG) and 262 nm for genistein glucuronide (GG). In 100 % methanol or ethanol, the maximal absorption was 262 nm for both DG and GG. After acidifying these two solvents with glacial acetic acid to pH 6.0, the maximal absorption of DG shifted to 250 nm and there was no change for GG.

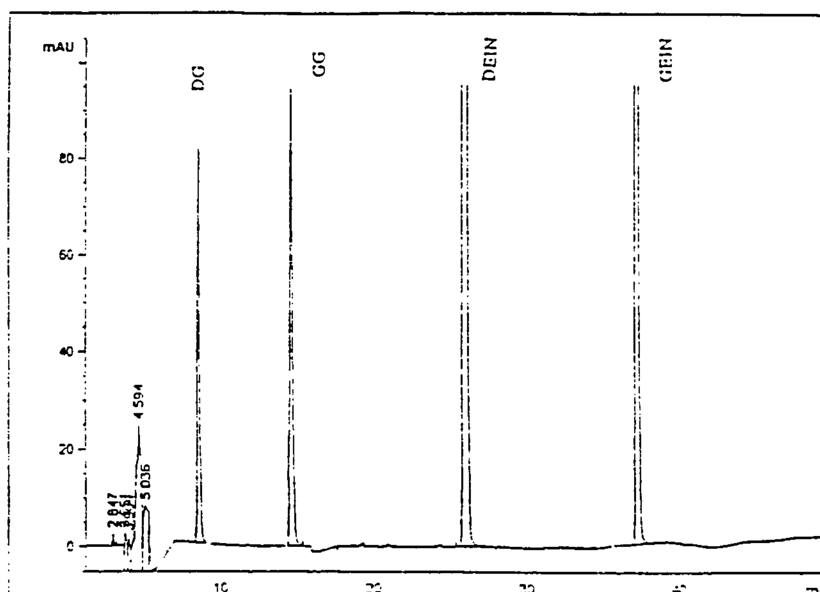

Figure 2. HPLC chromatograms of enzymatic synthesis of daidzein and genistein glucuronides (Dein = daidzein; Gein = genistein; DG = daidzein glucuronide; GG= genistein glucuronide). A YMC C18 reverse phase column was used to separate the aglycones and their glucuronide products. The elution and gradient conditions were as described in the method.

Table 2: UV absorption maxima (nm) of daidzein and genistein glucuronides

Reagent	Daidzein glucuronide	Genistein glucuronide
30% acetonitrile	250	262
ethanol	262	262
Methanol	262	262
Methanol/NaOMe	282	272
Methanol/$AlCl_3$	264	272
Methanol/$AlCl_3$:HCl	264	274
Methanol/NaOAc	262	264
Methanol/NaOAc:H_3BO_3	262	264

When adding $AlCl_3$/HCl, sodium methoxide, and sodium acetate to the glucuronide methanol solution, the change of the absorption maxima differed between these two glucuronides (Table 2), which indicated the hydroxyl group differences in the structure.

Mass spectra analysis of glucuronides. The purified glucuronides were analysis by solid probe electron impact or chemical ionization of mass spectra. The ion peak 255 and 270 indicated the parent compounds of daidzein and genistein; the ion peak 488 and 465 indicated the daidzein and genistein glucuronides, respectively, as shown in Fig 3.

The stability of glucuronides. The conjugates were dissolved in 30 % acetonitrile, methanol or ethanol, and stored at 4°C or -20°C refrigerator. At different times (0,15, 30 and 100 days), the concentration change of the conjugates were measured by HPLC. The percentage changes were calculated by the formula $Y(\%) = (Xt - Xo)/Xo * 100\%$. As shown in Table 3, the concentration changes were within ± 5 %. At day 30, daidzein glucuronides gave a 7% changes.

Table 3. The percentage changes of glucuronide concentration (μM) in methanol*

		Days			
		0	15	30	100
Daidzein glucuronide	4°C	0±0.07	1.18±0.12	6.99±0.31	1.17±0.11
	-20°C	0±0.04	-3.34±0.52	6.97±0.19	0.78±1.07
Genistein glucuronide	4°C	0±0.08	-0.81±1.21	0.56±0.76	4.38±0.52
	-20°C	0±0.03	-0.11±1.33	1.55±0.94	3.47±0.79

* The results were the average of four observation numbers (n=4).

Enzymatic conjugation of isoflavone aglycones

The reaction time course. The reaction was performed from 2 minutes until 60 minutes with 15 mM UDPGA and 5 mg/mL protein in the reaction. As shown in Fig 4, the formation of both daidzein and genistein glucuronides was increased with increasing time. Since the reaction was almost linear during the one hour assay, 20 minutes was chosen for the reactions performed later.

Protein concentration. Protein concentration was determined according to the method of Lowry et al. (1951) using bovine serum albumin as standard. The activity of both daidzein and genistein UDP-glucuronyltransferase was shown to be constant when the assay mixture contained between 3 to 15 mg of microsomal protein per milliliter (Fig.5). Five mg microsomal protein/mL was used in all the reactions.

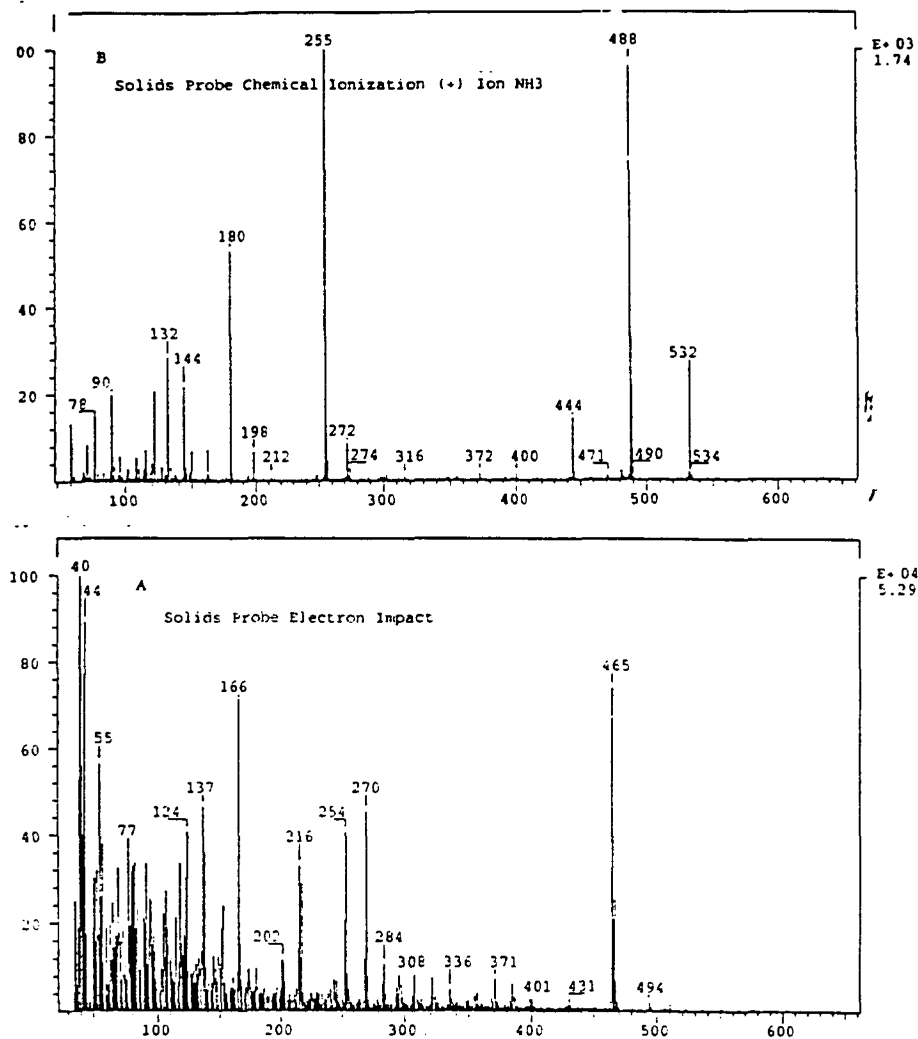

Figure 3. Mass spectrum of (A) daidzein glucuronide and (B) genistein glucuronide.

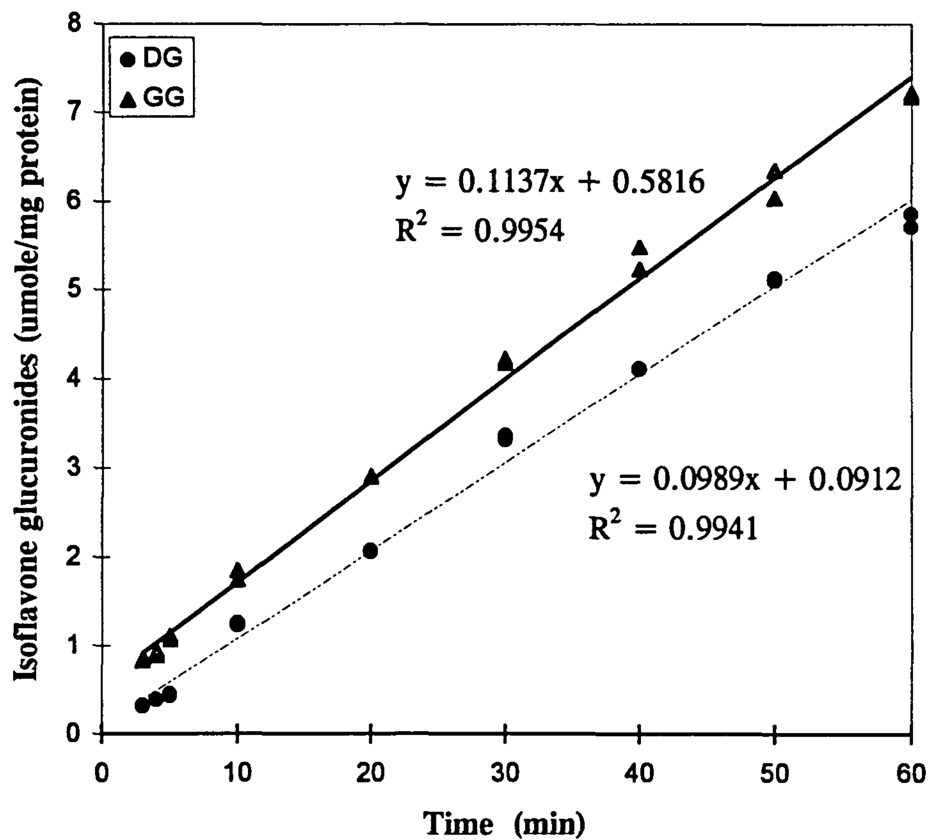

Figure 4. Glucuronide conjugation of daidzein and genistein measured as a function of time. The formation velocity was expressed as µmol/mg protein. The reaction conditions were as described in the method section.

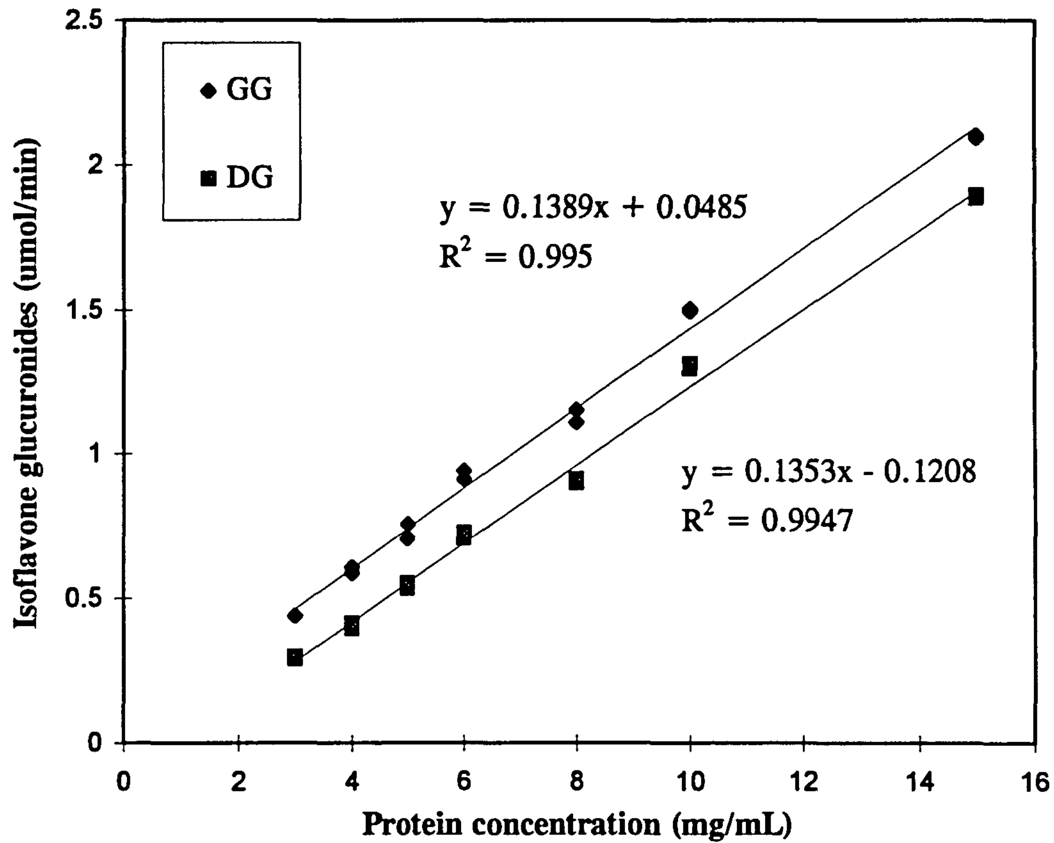

Figure 5. Daidzein and genistein glucuronides formation measured at different concentration of microsomal protein with 15 mM UDPGA and 3 mM genistein in the reaction medium for 20 minutes.

UDPGA concentration. Five concentrations (1.5, 3, 10, 15 and 30 mM) of UDPGA were tested. As shown in Fig 6, daidzein and genistein glucuronides formation were increased progressively with increasing UDPGA concentration. At 15 mM, the formation of the glucuronide was almost reached saturation and it was used across all the reactions in our assay.

PH. The optimal pH for daidzein and genistein glucuronides formation was 7.4, as shown in Fig 7.

Influence of substrate concentration on the synthesis of daidzein and genistein glucuronides. The effects of daidzein and genistein substrate concentrations on the kinetics of UDP-glucuronyltransferase-mediated formation of daidzein and genistein glucuronides were analyzed in rat microsomal preparations. Incubations were performed using a wide range of daidzein and genistein concentrations (1.56-400 μM) and a saturating concentration of the cosubstrate UDP-glucuronic acid (15 mM). With increasing concentration of daidzein and genistein, the rates of formation of both daidzein and genistein glucuronides increased progressively and approached saturation. The Lineweaver-burk plot (Fig. 8) exhibited Michaelis-Menten kinetics. However, the calculated apparent affinity constants (Km) and maximal velocities (Vmax) for the two substrates were significantly different (Table 4). Compared to genistein glucuronide, daidzein glucuronide synthesis was a higher capacity, lower affinity reaction with rat microsomal UDP glucuronyltransferase. The product ratio (GG/DG) (Fig 9) showed that at substrate saturation concentrations, four and half times more GG than DG was present,

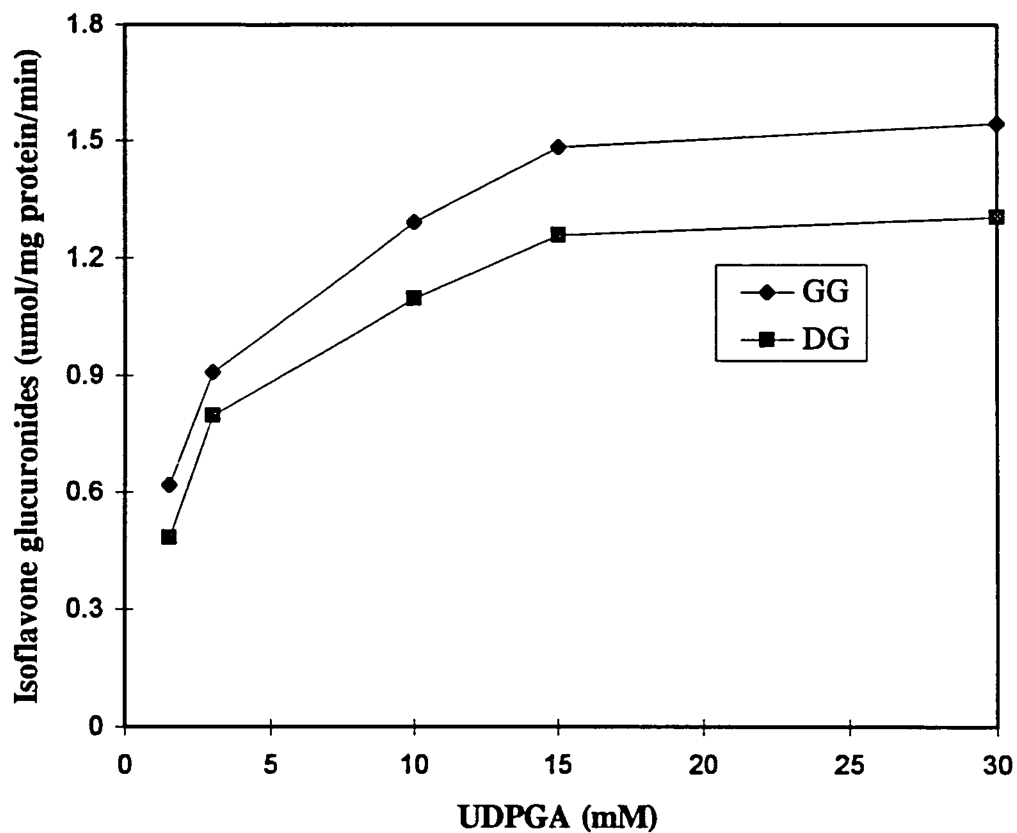

Figure 6. The influence of variable concentration of UDP-glucuronic acid (UDPGA) on the formation of daidzein and genistein glucuronides. The measurement was performed at pH 7.4 with 5 mg/mL hepatic protein and 3 mM genistein in the reaction mixture for 20 min.

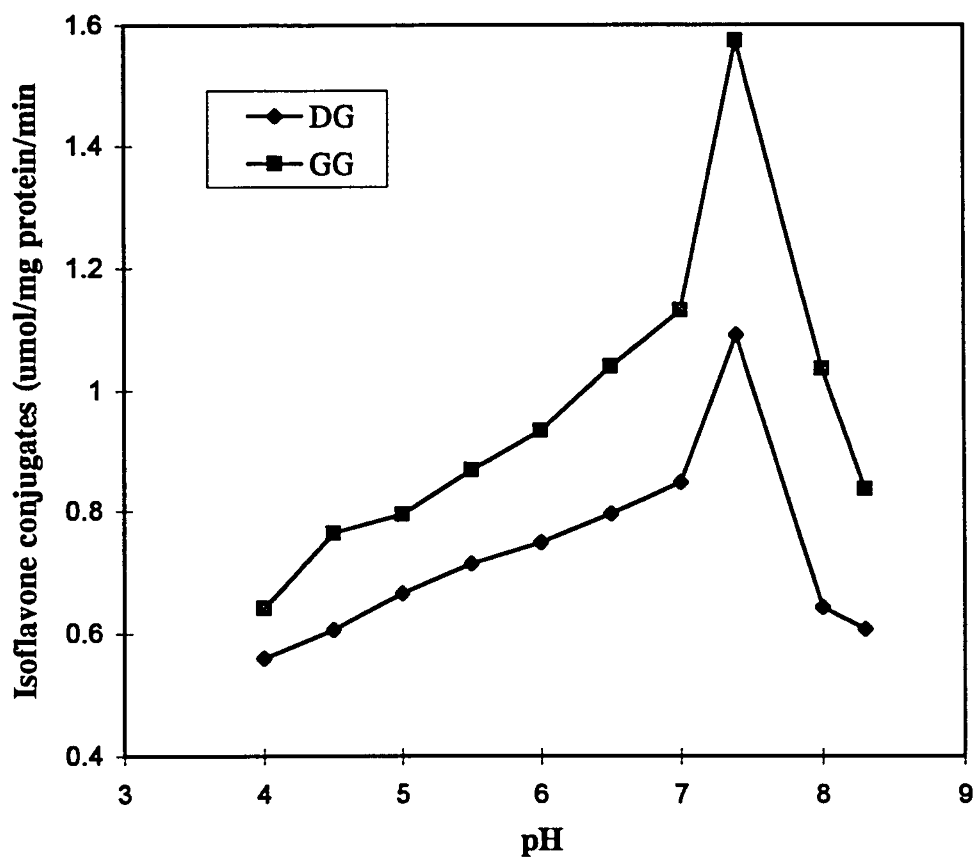

Figure 7. The influence of pH on the formation of daidzein and geinistein glucuronides. Other conditions used in this synthesis were 5 mg/mL hepatic microsomal protein, 15 mM UDPGA, 3 mM daidzein or genistein, pH7.4 and reacted for 20 min.

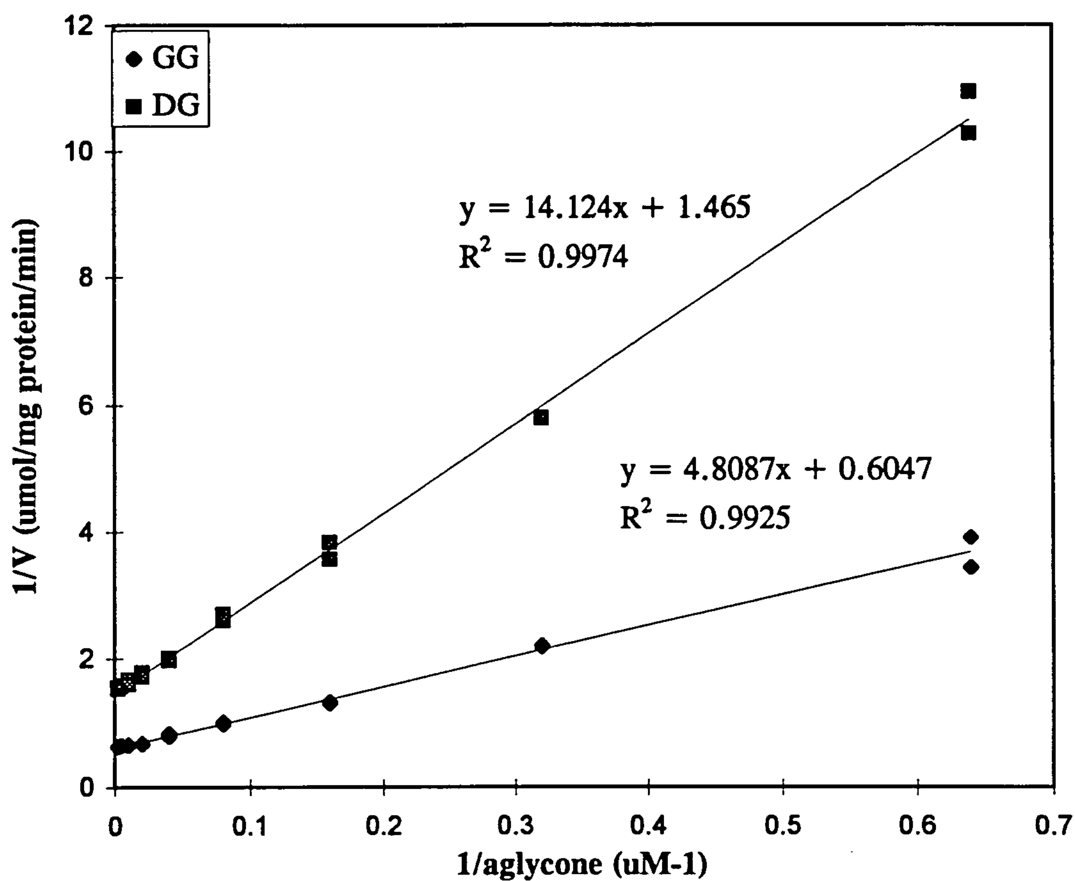

Figure 8. Lineweaver-Burk plot of UDP-glucuronyltransferase catalyzed formation of daidzein and genistein glucuronides versus daidzein and genistein substrate concentration. The reciprocal plot was linear and obeyed Michaelis-Menten kinetics.

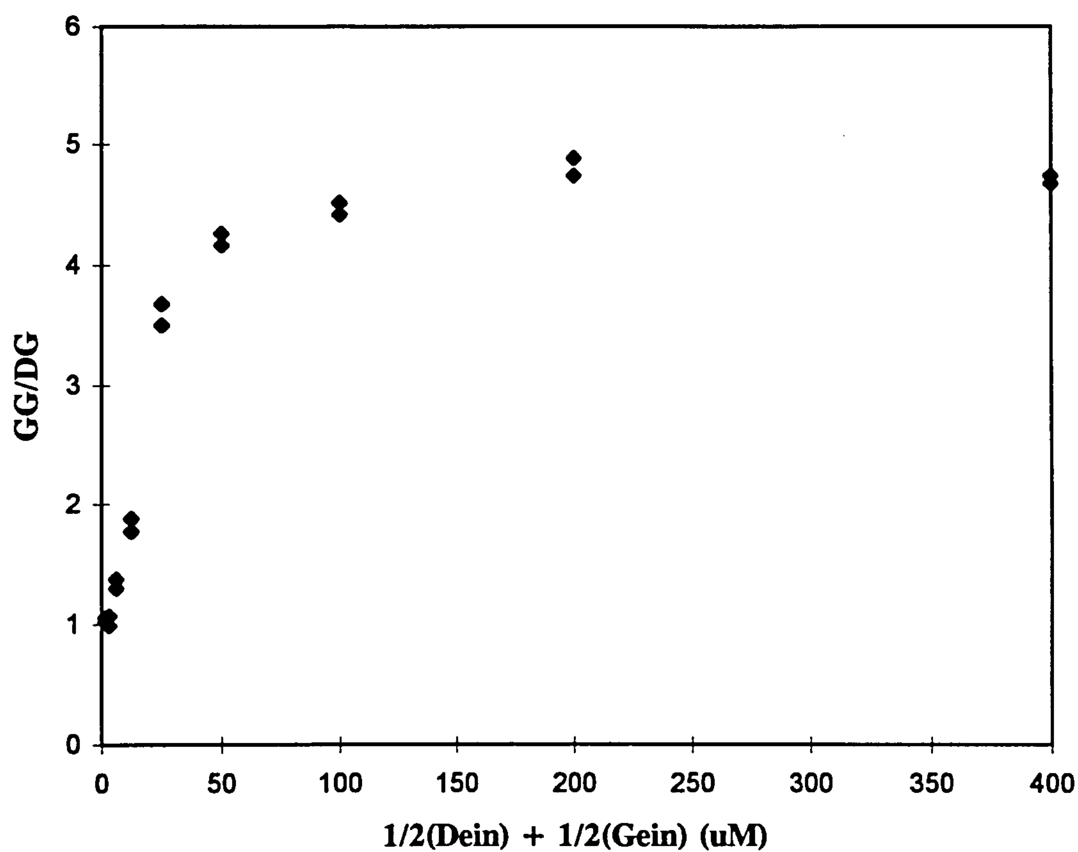

Figure 9. The daidzein and genistein glucuronide formation ratio when equal daidzein and genistein concentration were used in the reaction mixture. The actual concentration used were 1.563, 3.125, 6.25, 12.5, 25, 50, 100, 200 and 400 μM.

Table 4: Apparent kinetic parameters for hepatic rat microsomal UGT-mediated formation of daidzein and genistein glucuronides*

Substrate	product	Km (μM)**$	Vmax (μmol/mg protein/min)**#
Daidzein	Daidzein glucuronide	9.038±3.567[a]	0.6674±0.0015[a]
Genistein	Genistein glucuronide	7.656±1.366[b]	1.6405±0.0018[b]

* Parameters were obtained by hyperbolic fit to the data according the method of Wilkinson (1961).
** Values in a column bearing different letters were significant different.
$ The least significant difference (LSD) for Km was 0.737 and $p < 0.03$.
# The LSD for Vmax was 0.024 and $p < 0.001$.

which confirmed that genistein reacted more easily with UDP-glucuronic acid, compared to daidzein.

Estrogenicity study

Fig 10 showed that daidzein and genistein glucuronide had weak affinity fior the mouse uterine cytosol estrogen receptor compared with 17β-estradiol (ESTR) and diethylstlbestrol (DES). As unlabeled isoflavone glucuronides (daidzein and genistein glucuronide) increased in concentration, the displacement of ^{3}H estradiol increased. The CB_{50} (concentration required to displace 50% of the ^{3}H labeled estradiol in the competitive binding study) of these compounds were: ESTR =1.34 nM; DES = 1.46 nM; Dein = 1.6 μM; Gein = 0.154 μM; DG = 14.7 μM and GG = 7.27 μM. The relative molar estrogen receptor binding affinity of mice was calculated by dividing the CB_{50} of unlabeled

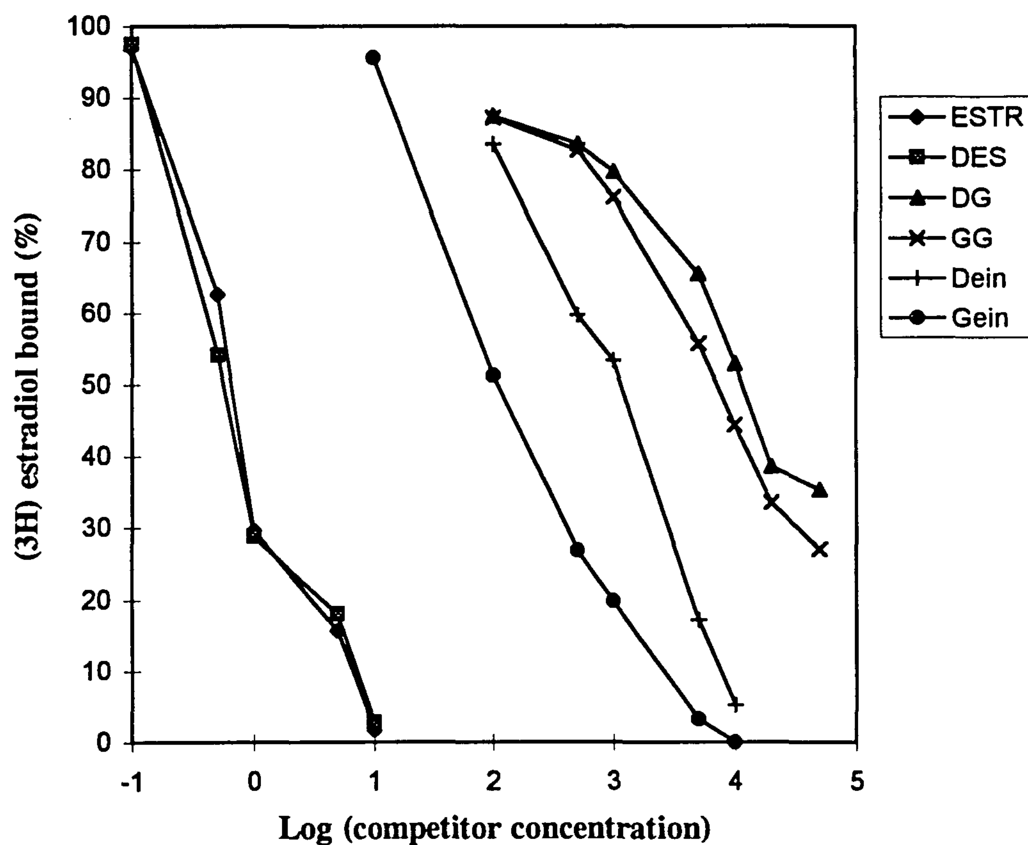

Figure 10. Competitive binding analysis of isoflavone glucuronides. Cytosol from B6D2F1 mouse uteri were incubated with 1.5 ng/ml 17β-(^{3}H)estradiol in the presence of increasing concentrations of unlabeled estrogenic agents. (17β-estradiol = ESTR; diethylstilbestrol = DES; Daidzein = Dein; Genistein = Gein; daidzein glucuronide = DG; genistein glucuronide = GG).

17β-estradiol by the CB_{50} of competitor and then multiplying by 100. The results were shown in Table 5. Compared to these two glucuronides, DES had a much higher binding affinity with 91.8% of 17β-estradiol binding affinity. Between these two glucuronides, genistein glucuronide had much higher binding affinity than daidzein glucuronide. These results were similar relative to the respective aglycone compounds in that genistein had greater estrogen receptor binding affinity then did daidzein.

Table 5. Relative affinities of estrogens for estrogen receptors*

Compounds	CB50(μM)	Relative affinity
17ß-estradiol(ESTR)	0.00134	100
Diethylstilbestrol(DES)	0.00146	91.8
Genistein(Gein)	0.154	0.87
Daidzein(Dein)	1.600	0. 082
Genistein glucuronide(GG)	7.27	0.018
Daidzein glucuronide(DG)	14.7	0.009

* Based on the molar concentration (CB_{50}) required to displace 50% of the (^3H) estradiol. n=2.

Natural killer cell assay

Peripheral blood leukocytes from healthy men and women were used as a source of NK cells to assay their activity against K562 target cancer cells in a ^{51}Cr-release assays of NK killing activity. The effect of various concentrations of genistein and the major two

metabolites (daidzein glucuronide and genistein glucuronide) of soybean isoflavones with or without interleukin-2 on target cell killing was investigated. The results of the assay were displayed in Fig 11. Individual variation of NK activity in the absence of IL-2 and isoflavone was set at 100% killing (data was transformed to percent baseline). IL-2 dose was chosen to give moderate not maximal activation. The activation of IL-2 was similar for all individuals and ranged from 107-117% of baseline NK activity. The mean activation with IL-2 alone was 113% which was shown in Fig. 11 as "baseline + IL-2".

Genistein, a known tyrosine kinase inhibitor, activated NK cell killing maximally at 0.1-0.5 μM. With increasing of genistein concentration above 5 μM, NK activity decreased linearly. At a concentration of 50 μM, genistein inhibited NK cytotoxicity. Genistein in the presence of IL-2 activated NK activity in a more narrow range (< 0.5 μM). The suppression of IL-2 activated NK cells by genistein linearly increased in a dose dependent manner from 5-50 μM.

In the absence of IL-2, daidzein and genistein glucuronide activated NK cell activities as much as IL-2 within the range of 0.1-10 μM. The enhancement with glucuronide alone was equivalent to IL-2. In the presence of IL-2, both glucuronides additively increased NK activity at concentrations of 0.1-10 μM. Across all concentrations, genistein gave significantly less activation than daidzein glucuronide, and genistein glucuronide gave significantly more activation than daidzein glucuronide ($p<0.01$). There were significant interactions between isoflavones and IL-2 ($p<0.01$). IL-2 in the presence of daidzein glucuronide and genistein glucuronide activated NK killing

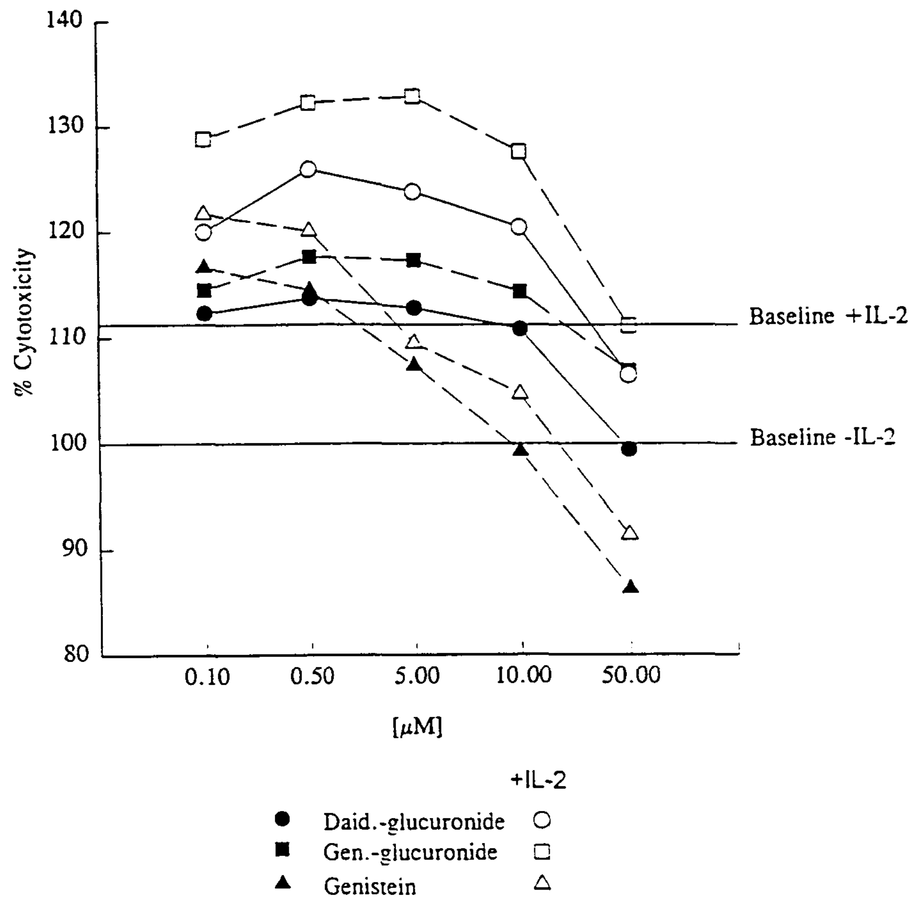

Figure 11. Cytotoxicity of human peripheral blood NK cells against K562 target cells, expressed as percentage of baseline cytotoxicity. Each point represent the mean cytotoxicity of six subjects. Results of incubation with three different isoflavones (0.1-50 μM) with (open symbol) and without interleukin-2 (1.25 ng/ml) (solid symbol) were shown in the graph.

more than did genistein with IL-2 ($p < 0.01$). Individual responses to the isoflavones were also significantly different ($p < 0.01$).

DISCUSSION

The most abundant isoflavones in soybean are the glucoside forms, which constitute over 97% of the isoflavones in soybean (Wang and Murphy, 1994). After consumption, glucoside isoflavones reach the colon and can be cleaved by bacterial glucosidases. The released free aglycones can be absorbed by the colonic mucosa (Brown, 1988) and transported to the liver via the portal vein where they are rapidly reacted with phase II enzymes, especially UDP-glucuronyltransferase and sulfotransferase to form glucuronide and sulfate conjugates. Glucuronidation has low affinity but high capacity whereas sulfation has a high affinity but low capacity. Therefore, following administration of relatively large amount doses of phenol compounds, glucuronidation would be the major pathway of biotransformation (Parkison, 1995). When rats were orally dosed with 50 mg/kg (0.3 mmol/kg) of ^{14}C-2-hydroxybiphenyl, almost 90% of the dose was recovered in urine within 24 hours and about 75% of the dose was excreted as the glucuronide or sulfate esters of the hydroxybiphenyl and its metabolites. As doses were increased from 5 mg/kg to 500 mg/kg, the proportion of glucuronides in urine was increased by 50% (Reltz et al., 1983).

Numorous functional groups can undergo conjugations with glucuronic acid and form O-, N- and S-glucuronides, respectively. Certain nucleophilic carbon atoms have also been shown to form C-glucuronides. Isoflavones contain hydroxyl groups in their structure: daidzein at 7- and 4'- position, genistein at 5-, 7- and 4'- position. The hydroxyl in the 7- position is believed to be the most active group and will react with

glucuronic acid and form the glucuronides. Our UV absorption results has confirmed the 7- reaction position. As Table 2 showed, both DG and GG have a large bathochromic shift when NaOMe was added, which indicates that there are free 4'-OHs in their structure. When the weaker base of NaOAc was used, a modest bathochromic shift indicated that the 7- position was occupied by another group instead of the hydroxyl group. The bathochromic shift of GG in the presence of $AlCl_3$ and $AlCl_3/HCl$ was due to the presence of a 5-OH group, which was absent from DG. Both of the DG and GG bathochromic shift patterns were similar to daidzin (daidzein 7-O-glucoside) and genistin (genistein 7-O-glucoside) (Mabry et al., 1970), respectively. These results indicated that our synthesized compounds are 7-O-glucuronides which should be the same glucuronides as appear in the circulation after soy or soy product consumption.

The basic ion mass peak at 255 and 270 in Fig 2 confirmed that the structure containing the parent compounds daidzein and genistein. For the synthesized compounds, the expected molecular weights were 430 and 446 for daidzein and genistein glucuronides respectively. Unfortunately, the ion peak we obtained were not the ones we expected. In order to confirm the molecular weight of the glucuronides, other technology rather than chemical ionization (CI) or electron impact (EI)-Mass should be used.

There were slight changes of daidzein and genistein glucuronide concentrations when measured at different time (Table 3). These concentrations were calculated according to the same standard curve. The changes of the concentration (within 5%)

probably were due to the instrument and these glucuronide compounds will be considered stable for above 3 months.

Analysis of enzyme kinetics demonstrated that the rat microsomal UDP-glucuronyltransferase had a greater affinity for genistein. As the concentration of isoflavone substrate increased, the initial rates of formation of both glucuronides increased and approached saturation. However, as the concentration increased, less DG was formed relative to GG, as noted in the GG/DG ratio (Fig 9). The calculated apparent affinity constants and maximal velocities (Table 4) indicated that GG synthesis from genistein is a higher affinity, lower capacity reaction compared with DG synthesis, and the difference in the apparent affinity constants was significant different. The kinetic behavior of the enzyme seems not to agree with the pattern of the isoflavones excreted in urine. In Xu's study (1994), there was about 21% of ingested daidzein but only 9% of ingested genistein recovered from the urine. As we know, liver conjugation products can enter the general circulation and be excreted in the urine or with the bile into the gastrointestinal tract, where the conjugates are hydrolyzed by bacterial β-glucuronidase and the released aglycone may be reabsorbed via enterohepatic circulation. It is possible that as a better substrate for UDP-glucuronyltransferase, genistein is also a better substrate for β-glucuronidase. If there is more genistein glucuronide being deconjudated by β-glucuronidase, this will result in more free form genistein appeared in the blood than daidzein. If this is true, it can explain our human feeding study results that less genistein was recovered from urine but higher concentration of genistein was found in the plasma

than did daidzein. However, the relationship between the mammalian conjugation ability toward different isoflavones and the circulation or excretion patterns of the relative isoflavones needs further elucidation.

It is generally assumed that nonsteroidal estrogens exert their stimulatory effect on the estrogen receptor by binding to the same site occupied by steroidal estrogens such as 17β-estradiol. The distance between the two hydroxyl groups at the opposite poles of the molecules is similar to the distance between C-3 and C-17 hydroxyl groups of 17β-estradiol. Numerous studies (Jordan et al., 1985) have shown that isoflavone phytoestrogens had estrogen receptor binding ability and weak estrogenic activity. All of the main isoflavonoids (genistein, daidzein, equol, O-desmethylangolensin) and their precursors (biochanin A, formononetin) detected in human and animal urine bind to estrogen receptor. The estrogenicity of daidzein and genistein were reported to be roughly 10^3 to 10^5 times less than that of DES in mice (Bickoff et al., 1962). In sheep uterine estrogen receptor binding study, Shutt and Cox (1972) reported that the binding affinity of genistein and daidzein were 0.9% and 0.1% that of 17β-estradiol. Our in vitro mouse uteri binding study showed the same pattern as theirs. Our results agreed with most experiments results that genistein was a much more potent estrogen than daidzein was. Unlike the free form of isoflavones, for which the relative binding affinity of genistein was ten times higher than daidzein, genistein glucuronide was only two times greater than daidzein glucuronide in relative binding affinity. However, at higher concentration such as 10^2-10^3 times that of estradiol (close to the levels of isoflavones in human plasma after

soybean products consumption), these isoflavone glucuronides could effectively compete with the endogenous estrogens by competitive binding to estrogen receptors.

Only small amounts of daidzein, genistein and equol and a relatively large amount of daidzein and genistein glucuronides would appear in the body, depends on the intake of soy food. In addition to the estrogenic/antiestrogenic activities of daidzein, genistein, daidzein glucuronide and genistein glucuronide, equol, the metabolite of daidzein, has also been reported to have estrogenic/antiestrogenic effects (Shutt et al., 1980). When rats or other animals were fed with isoflavones, the estrogenic/antiestrogenic effects we observed may be resulted from the combined of all the isoflavone metabolites instead of glycosylated or aglycone isoflavone themselves.

Natural killer activity plays an essential role in immune surveillance against tumor development and progression as well as infectious agents. Tyrosine kinase activity is crucial for the activation of natural killer cell and genistein is a specific inhibitor of tyrosine kinase. It is reasonable to hypothesize that soybean isoflavones can either enhance or inhibit natural killer activity, and natural killer cell activity may vary with plasma isoflavone concentrations. Genistein inhibited purified epidermal growth factor (EGF) receptor and $pp60^{v\text{-}src}$ protein tyrosine kinase (PTK) with an IC_{50} of 22-26 μmol/L and inhibited intact human A431 PTK with an IC_{50} of 148 μmol/L (Akiyama et al. 1987 & 1991). The optimal genistein concentration that caused about 20-50% differentiation and therefore reduced number of K562 cells was 37 μmol/L (Constantinou and Huberman, 1995). It was also found that genistein could suppress both direct and FcR-mediated NK

cellular cytotoxicity in a dose dependent manner (Einspahr et al., 1991; Puente et al., 1996). In our experiment, we found that above the concentration of 0.5 μM, NK cell activity decreased. However, at low concentration, genistein increased NK cell activity. This was also true for the daidzein and genistein glucuronides. As we observed, the plasma concentration of isoflavones were around 5 μM after soy feeding (Xu e al., 1994 & 1995). So, under physiologically achievable concentrations in human, both free and glucuronide conjugated form of isoflavones would not seem to be toxic to NK cells.

IL-2 induces a rapid increase in the tyrosine phosphorylation of multiple proteins in NK cells. This function increases the cytotoxic activity of NK cells. In our study, after adding IL-2, the cytotoxic activity were consistently increased in every testing concentration. However, when IL-2 and isoflavone were both present, the NK cell activity was additively increased. This results indicated that the effect of physiological concentrations of isoflavone glucuronides and genistein would not be likely to inhibit PTK.

Olson (1992) proposed several modes of action to explain the possible biological mechanisms exerted by retinoid glucuronides. They were (1) immediate hydrolysis to the parent compounds, which then act on cells, (2) transport into cells followed by intracellular hydrolysis to the parent compounds at appropriate sites, (3) direct activation of appropriate receptors on membranes or in the nucleus. The first two possibilities cannot explain our results since the genistein glucuronide compounds was more active than genistein in NK activation. The third possibility was attractive but needs to be tested for isoflavones. However, these glucuronides functioned additively with IL-2, which stimulate NK cell

activity via membrane-associated tyrosine kinase, such as the *src*-family tyrosine kinase, it is possible that the glucuronides also stimulate NK activity via the *src*-family tyrosine kinase. Or on the other hand, it is possible that there are other mechanisms for this NK activation activity, as Kniss et al. (1996) reported that genistein could suppress EGF-induced prostaglandin biosynthesis by abolishing the response to calcium ionophores, a mechanism independent of EGF receptor tyrosine kinase inhibition. Tumor cells can produce high levels of prostaglandin and down regulate NK cell activity possibly through (1) inhibition of IL-2 and IL-2 specific receptor productions (Baxevanis et al., 1993), (2) blockage of the expression of transferrin (Baxevanis et al., 1993) and (3) reducing protein tyrosine kinase C activity (Ohnish et al., 1991), another transduction signal functioned in NK lytic activity (Hager et al., 1990). So, decreasing the level of prostaglandin would explain several possible mechanisms of the increasing of NK activity by isoflavones. If glucuronide and aglycone isoflavone could suppress prostaglandin levels at the same time increasing the protein tyrosine kinase activity, we would expect a large increase of NK cell activity. This possibility will be especially helpful for elderly people whose NK activity is decreased and prostaglandin production increased with age (Meydani et al., 1988).

El-Hag et al. (1986) reported that NK activity against K562 cells was susceptible to oxidative injury by H_2O_2. Genistein and daidzein have shown some antioxidant activities, as reported by Naim et al. (1976) and Wei et al. (1995). If the glucuronide of daidzein and genistein also had antioxidant activity, they may also enhance NK activity via reducing H_2O_2 formation. Another possibility needs further study.

In this experiment, we demonstrated that genistein, daidzein and genistein glucuronides are not toxic to NK cell under physiological concentrations. The glucuronide forms are more active than their parent compounds in activating NK cells. Overall, our study demonstrated that isoflavone glucuronides can not only compete with endogenous estrogen to inhibit the proliferation of cancer cell, but also can activate NK cell and increase the defensive ability of the body.

SUMMARY

The object of this study was to investigate the possible biological activities of daidzein and genistein glucuronides. Their biological activity was studied from two aspects: estrogen receptor binding ability and NK cell activation ability.

In order to perform this study, daidzein and genistein glucuronides were enzymatically synthesized. The kinetic study showed that genistein was easy to react with UDP-glucuronic acid than was daidzein. The Km and Vmax for daidzein and genistein were 9.038, 7.656 μM and 0.6674, 1.6405 μmol/mg protein/min, respectively.

The synthesized daidzein and genistein glucuronides were purified by Sephadex LH-20 column. The bathochromic shift of maxima UV absorption at different solution confirmed that glucuronic acid was joined at 7-O- position of daidzein and genistein. These purified daidzein 7-O glucuronide and genistein 7-O glucuronide were used for the biological study.

Daidzein and genistein glucuronides had weak estrogenic activity. This was supported by the B6D2F1 mice uteri cytosol estrogen receptor competitive binding study results with the CB_{50} value for 17β-estradiol, diethylstilbestrol, daidzein, genistein, daidzein glucuronide and genistein glucuronide of 1.34 nM, 1.46 nM, 1.6 μM, 0.154 μM, 14.7 μM and 7.27 μM respectively.

In a study of human peripheral blood NK cell activity, genistein, daidzein and genistein glucuronides enhanced NK cell mediated target K562 cancer cell destruction at the range of <0.5, 0.1-10 μM and 0.1-10 μM respectively. Above 5 μM, genistein

decreased NK cell cytotoxicity. Above 10 μM, daidzein and genistein glucuronides began to show decreased enhancement of NK cell activity.

Overall, our study showed that daidzein and genistein glucuronides were able to bind to estrogen receptor and may efficiently compete with endogenous estrogen and reduce estrogen stimulated proliferation of cancer cell. Compared to their parent compound, daidzein and genistein were less toxic to the NK cell and were relatively more efficient in the enhancement of NK cell activity. So, these results indicated that daidzein and genistein glucuronides not only are the detoxifying step, but also have some biological activity.

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