CHAPTER 1

INTRODUCTION

Menopause is the permanent cessation of menstruation and ovarian function characterized by lack of estrogen production (1). Estrogen deficiency may cause climacteric symptoms that include a variety of symptoms, such as hot flushes, headache, nervousness, loss of libido, insomnia, depression, irritability, palpitations as well as joint and muscle pain (2). These changes are caused by a fall in estrogen production by the ovaries (3). Postmenopausal hormonal therapy is used to manage the climacteric symptoms that impair the quality of life of a substantial number of women. Nonetheless, it is quite difficult to achieve the desired effects without side effects or other adverse health risks (4).

Estrogen therapy is highly effective for relieving menopausal symptoms and for preventing osteoporosis as well as colon and gastric cancer, thus improving quality of life and increasing life span. Nonetheless, the use of estrogen-only hormone-replacement therapy (HRT) increases the risk of endometrial cancer (5), whereas several lines of evidence reported in the 1970s have shown that the increased incidence of endometrial cancer in women taking estrogen alone could be avoided by using HRT with estrogen plus progestin (6, 7). However, recent studies indicate that the progestin component in HRT might increase the risk of breast cancer and stroke and counteracts the beneficial effects of estrogen on the heart and lipoprotein lipids (6, 8, 9).

The concern over potential adverse effects of HRT leads many women to look for other alternatives. One modality that has been widely investigated is soy supplement. Soy foods contain isoflavones that structurally similar to estrogen but have weaker hormonal effects: binding weakly to the estrogen receptor α (ERα) of the uterus, ovaries and breast, but more strongly to the estrogen receptor β (ERβ) that found in the brain, arteries and bone (10). Isoflavones are an integral part of many Asian diets and have received considerable attention in recent years for their potential role in reducing risk of CHD (11), osteoporosis (12, 13), and certain cancers (14, 15). In addition, soy and isoflavones have been studied for the ability to alleviate hot flushes in postmenopausal women (16).

Osteoporosis is a problem experienced by about one third of postmenopausal women. Estrogen deficiency is a major cause of postmenopausal osteoporosis (17). There is both a reduction in the efficiency of absorption of calcium from gastrointestinal tract (18, 19) and an increased rate of bone resorption (20). In prevention and treatment of postmenopausal osteoporosis, active form of vitamin D, $1,25_2D_3$ or calcitriol, is able to stimulate normal bone growth, and it is of fundamental importance in the mineralization processes. It mainly increases the intestinal absorption of calcium and phosphates, allowing these components for the deposition of crystals onto the collagen fibers of the osteoid proteic matrix (21). Thus, vitamin D and calcium intake is the cornerstone of prevention and treatment of osteoporosis. There is compelling scientific evidence that vitamin D doses of 700 to 800 IU/day reduce the risk of both hip and non-vertebral fractures in postmenopausal women (22).

Thus, it's possible that both peri- and postmenopausal women might consider using the combination of vitamin D, calcium and soy isoflavones for prevention and treatment of postmenopausal osteoporosis in stead of HRT. However, it is hypothesized that co-administration of vitamin D (calciferol) and isoflavones, in which their structures are similar to classic steroid hormones, might interfere the absorption (either rate or extent, or both) of each other. In addition, although vitamin D is predominantly metabolized by phase I, whereas isoflavones are largely converted to their conjugated metabolites via phase II, it has been found that drugs predominantly metabolized via phase I, such as rifampicin, phenytoin and pentobarbital, possibly induce UDP-glucuronosyltransferase 1A (UGT1A) involved in phase II biotransformation (23-26). Conversely, drugs predominantly metabolized via phase II, such as chloramphenicol and isoniazid, could also inhibit cytochrome P450 (CYP) such as CYP2C19 and CYP3A4 involved in phase I biotransformation (27, 28). The evidence proposed above supports the probable existence of pharmacokinetic drug interactions between vitamin D and isoflavones, or even with calcium supplement. Thus, the purpose of this study is to determine the effect of vitamin D plus calcium supplement on isoflavone pharmacokinetics in Thai postmenopausal women.

1.1 Isoflavones

Isoflavones are phytoestrogen, compounds of plant origin that have effects similar to estrogen. They have a common diphenolic structure that resembles the structure of the potent synthetic estrogen diethylstilbesterol (Figure 1).

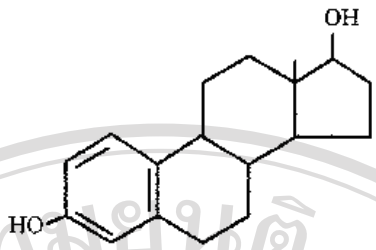


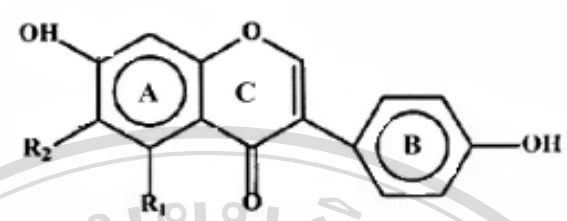
Figure 1 Structure of 17β-estradiol

1.1.1 Structures of isoflavones

Isoflavones are a subclass of the more ubiquitous flavonoids. The basic structure feature of flavonoid compounds is the flavone nucleus, which is composed of 2 benzene rings (A and B) linked through a heterocyclic pyrane C ring. The position of the benzenoid B ring is the basis for separate the flavonoid class into flavonoids (2-position) and isoflavonoids (3-position).

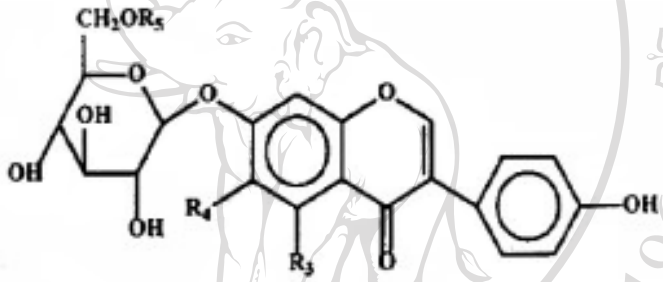
The principal isoflavones found in soy proteins and soy foods are daidzein, genistein, and glycitein. Each of them is found in four chemical forms: the unconjugated form, or aglycone; the conjugated form, or glycoside (daidzin, genistin, and glycitin); acetylglycoside; and malonylglycoside. The soy isoflavones daidzein and genistein primarily appear in the form of their glycosides, daidzin and genistin, respectively (Figure 2) (29, 30).

Aglycone



R1	R2	Compound
H	H	Daidzein
OH	H	Genitein
H	OCH_3	Glycitein

Glycoside



R3	R4	R5	Compound
H	H	H	Daidzin
OH	H	H	Genitin
H	OCH_3	H	Glycitin
H	H	$COCH_3$	Acetyldaidzin
OH	H	$COCH_3$	Acetylgenitin
H	OCH_3	$COCH_3$	Acetylglycitin
H	$HCOCH_2$	COOH	Malonyldaidzin
OH	$HCOCH_2$	COOH	Malonylgenitin
H	OCH_3	$COCH_2COOH$	Malonylglycitin

Figure 2 Classes and chemical structures of isoflavones in soybean

The structures of isoflavones have two characteristics that are similar to the

structure of 17-estradiol, one of the most potent estrogens: (i) both 17-estradiol and isoflavones have an aromatic ring with a hydroxyl group; (ii) a nearly identical distance exists between two hydroxyl groups in both 17-estradiol and isoflavones (31). It is not surprising, then, that isoflavones can bind to the estrogen receptor because of their structural similarity to estradiol (Figure 3) (32-34).

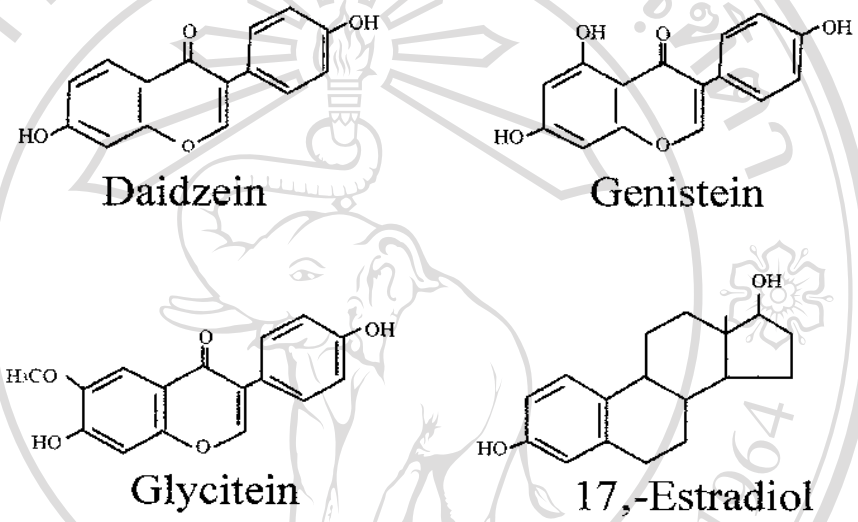


Figure 3 Structures of daidzein, genistein, and glycitein in comparison to estradiol

1.1.2 Major sources of isoflavones

Soybeans and soy products are a particularly abundant source of isoflavones. They contain approximately 0.2–1.6 mg of isoflavones/g dry weight (35). Chick peas and other legumes, as well as clover, toothed medic, and bluegrass are other isoflavone sources (36). Processing and fermentation of the soybean are known to influence the forms of isoflavones. In nonfermented soy foods, the isoflavones appear mostly as the conjugate, whereas in fermented soy products such as miso, the aglycones dominate. Many of the bacteria used in their preparation are capable of hydrolyzing the glycoside conjugates and modifying the composition. In second generation soy foods made by adding soy ingredients to a wide variety of

manufactured foods, the isoflavone content is diminished, for example tofu, yoghurt, and soy noodle (37).

Heat processing, enzymatic hydrolysis and fermentation can significantly alter the isomeric distribution of the 3 isoflavones. For example, the malonylglycosides and acetylglycosides are susceptible to heat and readily converted to the more stable β-glycosides (38); therefore, depending on the extent of processing of the soybean, the relative proportions of these conjugates can vary considerably among different soy foods (37).

1.1.3 Pharmacokinetic of isoflavones

Absorption of isoflavones

Dietary isoflavones exist in soy foods primarily as glycoside conjugates. Conjugated isoflavones require a series of steps prior to intestinal absorption (39, 40). The absorption of isoflavones by GI mucosa seems to partly depend on the relative hydrophobicity/hydrophilicity of these compounds. There is no evidence for facilitated or active transport of isoflavones. Isoflavone aglycones are of appropriate molecular weight (250 g/mol) to permit their diffusion. Isoflavone glycosides predominate in foods, but have not been detected in human blood plasma or urine (41, 42). Human intestinal or gut micro floral glucosidases seemingly cleave these moieties before the isoflavones can be absorbed. Therefore, most of absorbed isoflvones are the aglycones. In humans, the aglycone forms of daidzein and genistein also appear to be more bioavailability than the conjugate forms because of their lower hydrophilic and molecular weight (41, 43). In addition, daidzein has been shown to be more bioavailability than genistein in women (41, 42) suggesting that

small structural differences may have a significant effect on the bioavailability of isoflavones.

Isoflavones as aglycones enter the bloodstream within 15-30 min after ingestion. The time it takes to attain peak plasma concentrations after ingesting the isoflavones is 4-7 h, whereas when the glycoside conjugate are ingested, the T_{max} is shifted to 8-11 h, indicating that the rate-limiting step for absorption is initial hydrolysis of sugar moiety (44). Bioavailability of soybean isoflavones depends on relative uptake rates, hydrolysis of glycosides by gut bacteria or gut wall enzymes, further metabolism, for example to glucuronides within the liver, and excretion rate.

Biotransformation of isoflavones

After ingestion, the conjugated form of isoflavones is hydrolyzed by intestinal β-glucosidases and intestinal bacteria, which release the principal bioactive aglycones, daidzein and genistein. These compounds may be absorbed or further metabolized in the distal intestine with the formation of specific metabolites (30). Three native β-glucosidases are identified in humans (45). The first is glucocerebrosidase, being a lysosomal enzyme which hydrolyses glucoceramide from endogenous membrane glycolipids. Another is lactase phlorizin hydrolase, which is a membrane-bound enzyme found in the brush-border of the small intestine, and is primarily responsible for hydrolysis. The third β-glucosidase is a broad-specificity cytosolic enzyme found in abundance in the liver, kidney, and small intestine of mammals. Some intestinal bacteria produce β-glucuronidases, which can deconjugate these isoflavone metabolites when they pass through the intestine (30). Genistein is further metabolized by intestinal microflora largely to non -estrogenic metabolite product, *para*-ethylphenol, while daidzein is converted either to equol, a metabolite that has

estrogenic activity than daidzein (46) or *O*-desmethylangolensin (ODMA) by two different metabolic pathways (47-49). The metabolites can work in human body with various estrogenic activities, often higher or lower than their precursors (49-51). A variety of in *vitro* and in *vivo* assays have provided evidence for the difference in biological activities between the parent compounds and bacterial metabolites. It has been reported that genistein, daidzein and equol have relatively strong affinities for estrogen receptors, while *O*-DMA has a much weaker affinity and appears to be non-estrogenic (52, 53).

The unconjugated isoflavones are largely converted to their β-glucuronides by enzymes in the gut wall and liver. Isoflavones are predominantly conjugated with glucuronide conjugates and to a lesser extent sulfate conjugates (54). These conjugates are more readily transported in the blood and excreted in bile or urine than are the parent aglycones. After secretion into bile, the isoflavone β-glucuronides are hydrolyzed in the gut and undergo enterohepatic circulation. In general, genistein seems to be retained in the human body for longer time than daidzein (55). In clinical trial, $t_{1/2}$ values of daidzein and genistein are between 6 and 8 h. Some data indicated a significantly shorter $t_{1/2}$ for both daidzein and genistein, but this is likely to be due to insufficient sampling (56). Daidzein and genistein have large volume of distribution, indicating extensive tissue distribution. Daidzein exhibite a much higher volume of distribution than genistein, and this explains why genistein levels in plasma always exceed daidzein concentrations when equivalent amounts of the two isoflavones are ingested. Most of the daidzein and genistein are excreted in urine within the first 24 h after food intake (42, 57) and the rate of urinary excretion of daidzein is greater than that of genistein throughout the post meal period.

1.1.4 Factors influencing bioavailability of isoflavones

Microflora subgrouping and gut transit time

The increased bioavailability has been determined in subjects who have gut flora that degraded genistein more slowly in vitro than the gut flora of the remaining subjects (41). One logical explanation can be that the isoflavones are available for absorption for a longer period of time within these subjects due to the slow degradation rate, which leads to a higher bioavailability even though their gut transition time has increased. However, this hypothesis needs to be confirmed. Zheng et al have found that the group of Caucasian women has been shown homogenous and low urinary and fecal levels and a high gut transmission time independently of the rate by which their gut flora degraded genistein (58). Moreover, it is hypothesized by Setchell et al that equol production is influenced by several factors such as the intestinal microflora composition, gut transit time, and the redox potential of the colon(59). In addition, during the first months of life, plasma and urine levels of equol have been found to be significantly lower than in adults (60).

Frequency of ingestion

Lu and Anderson have shown that after 1 month of daily soymilk feeding, the urinary excretion of genistein and daidzein is decreased, whereas that of equol is increased (57). However, after 7 days of soy isoflavones, no significant differences are detected in the pharmacokinetics of daidzein or genistein (61). Furthermore, the frequency of soymilk powder feeding has no significant effect on the proportion of genistein and daidzein metabolites detected in plasma or urine when given as a single dose on two separate days 1 wk apart or after 6 days of consecutive feeding (58, 62).

Food matrix and diet

The effect of a fiber-rich food matrix is investigated; the recovery of urinary genistein from tofu o textures vegetable protein (TVP) is lower when ingested with 40 g wheat fiber than with 15 g dietary fiber but the urinary excretion of daidzein is not affected by the ingestion of a high-fiber diet (63). Moreover, Adlercreutz et al. have found that the intake of total fat and meat and the dietary ratio of fat to fiber correlated with the urinary excretion of equol, and consequently it is hypothesized that subjects consuming large quantities of meat and fat and low quantities of fiber might harbor the gut flora required for equol production (64). However, other studies have shown that equol production is more prevalent in subjects with a high consumption of carbohydrates and dietary fiber and a low dietary fat-to-fiber ratio (52). A recent study has shown that neither plasma levels of daidzein nor urinary excretion are altered by different food matrices (cookies, chocolate bars, and juice). Peak genistein concentrations in blood appear earlier when consumed from a liquid matrix (compared with a solid matrix) but with lower urinary recovery (65).

Difference of isoflavone sources

When comparing soymilk, TVP, and tempeh in men and pre- and postmenopausal women, there has a significantly higher percentage of the genistin dose excreted through urine after ingestion of soymilk than after TVP, an effect that only is detected in women (66). Furthermore, in premenopausal women, an even higher urinary recovery of genistein has been detected after tempeh consumption than after soymilk. However, Xu et al and Tew et al have not agreed with finding of these studies. They have founded that no difference in the bioavailability of daidzein or genistein from cooked soybeans, TVP, tofu, or tempeh in premenopausal women (63,

67). In addition, Hutchins et al have observed a higher bioavailability, assessed as the percent of dose excreted in the urine after ingestion of isoflavones from the fermented soy product tempeh (containing mainly aglycones) than after ingestion of non-fermented soybean pieces (containing the naturally occurring isoflavone glucosides) (68). However, this difference can very well be due to a more difficult release of the compounds from the soybean pieces than from the processed product as observed for most other plant compounds. The solid food matrix of tempeh might protect daidzein from degradation until it reaches the large intestine (66).

Processing and storage

Several factors have been reported to influence the amount of isoflavones actually consumed, therefore, indirectly influencing bioavailability. Surprisingly, storage of soybeans increases the levels of isoflavones; in soybeans stored at room temperature for up to 3 y, all glucosides (daidzin, glycitin, and genistin) are increased, all aglycones are increased but to a lesser extent, and malonylglucosides are decreased (69). However, in soymilk stored at room temperature, genistin is rapidly lost (70). The thermal stability of daidzein is higher than that of glycitein or genistein. For the glycosides, daidzin is more stable than genistin, which is in turn more stable than glycitin (71). Processing can have a direct effect on bioaccessibility. For example, the amount of extractable isoflavones is decreased after extrusion of a corn–soy mixture (72). In a different "biotechnological" approach, the solubility of daidzein is increased by addition of maltose using a microbial enzyme (73).

Gender and age

Several studies have been performed comparing the bioavailability of isoflavones between genders (66, 74, 75). However, in a long-term feeding study by

Lu and Anderson, differences are detected between genders, with an initially higher urinary excretion of isoflavone conjugates in women than in men (57). In the study by Faughnan et al, a tendency is detected for more equol producers among postmenopausal women than among premenopausal women and men which suggest that gender and age might be determinants for the intestinal metabolism of isoflavones (66). However, this tendency is not supported in a study by Lampe et al where no differences are detected in the prevalence of equol excreters between genders (76). One significant age-related difference is lower plasma and urine levels of equol during the first months of life compared with during adulthood, which probably is due to an immature gut flora (60). However, no differences are observed in the single-dose pharmacokinetics of either genistein or daidzein between pre- and postmenopausal women (44, 66).

1.1.5 Pharmacodynamics of isoflavones

Isoflavones concentrations on estrogen receptors

There are two subtypes of estrogen receptor and several isoforms and splice variants of each subtype. The first subtype, the classic ERα (77), was first cloned in 1986; the second subtype, ERβ, was discovered more recently (78). These two receptor subtypes vary in structure, and their encoding genes are on different chromosomes (79, 80).

Genistein has agonistic activity for both ERα and ERβ, but that genistein's affinity for ERβ is 7-30 folds greater; genistein's affinities for ERβ and ERα are determined to be 8.4 nM and 145 nM, respectively. For daidzein, the corresponding values are 100 nM and 420 nM, respectively, indicative of its much lower affinity for these receptors. At saturating concentrations, both genistein and daidzein could

interact with either of these receptors to activate transcription from estrogen response elements, at least as effectively as the physiological ligand 17β-estradiol (34, 81-86). In general, ERβ-mediated transcription is approximately half-maximal at a genistein concentration of 10 nM, whereas ERα-mediated transcription is minimal at this concentration, only becoming substantial as genistein rises above 100 nM. For genistein, this value has determined to be 30 nM for ERβ and 15 μM for ERα once again indicative of marked selectivity for ERβ. The corresponding values for daidzein are 350 nM for ERβ and >300 μM for ERα.

Some effects of estrogen result not from transcriptional activation at estrogen response elements, but from transcriptional repression of certain promoters that bind NF-kappaB, such as the IL-6 promoter (87). Genistein has been shown to activate ERβ such that it is capable of inhibiting the TNF response element; this effect is half-maximal at a genistein concentration of only 8.5 nM (82). In contrast, in cells over expressing ERα, only a moderate transcriptional repression has been seen with a genistein concentration of 1 μM.

In addition, agonist-activated ERα often increases the transcriptional activation mediated by AP-1, possibly by binding to co-activators that interact with fos/jun (88, 89). However, activated ERβ has the opposite effect, suppressing AP-1- mediated transcription (88, 90-94). Because AP-1 exerts various pro-proliferative effects, these findings may help to rationalize the opposing effects of ERα and ERβ on cell proliferation in certain tissues.

1.1.6 Effects of isoflavones on physiological activity

Effects of isoflavones on breast cell activity

In normal human breast, both types of estrogen receptor are expressed in

epithelial cells (94); ERβ predominates in adult human mammary fibroblasts (95). In ERα knockout mice, the breast has atrophic effect; conversely, in ERβ knockout mice, epithelium is hyperproliferative and the mice are prone to severe cystic breast disease as they age (96, 97). Transfection of ERβ into an ERα-expressing human breast cancer cell line (MCF-7) results in a suppression of proliferation associated with up-regulation of cdk inhibitors p21 and p27, and down-regulation of c-myc and cyclins D1 and A; however, these effects are only partially ligand dependent (98). In the main, these findings suggest that ERα and ERβ may have a ‘‘yin-yang’’ role in breast development, with ERβ opposing the proliferative impact of ERα; however, they do not necessarily imply that ERβ-specific ligands will have an antiproliferative effect. ERβ is expressed by the majority of human breast cancers – even those considered ‘‘estrogen negative’’ (99, 100); the standard assays for breast cancer ‘‘estrogen receptors’’ are ERα-specific. High dose genistein, administered pre-pubertal, induces a premature differentiation of breast tissue that diminishes susceptibility of adult rats to carcinogen-induced breast cancer (101-103); this effect is also seen with estrogen administration, and there is no evidence that physiological levels of genistein can achieve a comparable effect.

However, several studies which conclude that more modest intakes of genistein can favorably influence breast cancer induction. When administered at 250 mg/kg diet, either genistein or daidzein has been found to slow the onset of breast cancer in cancer-prone MMTV-neu mice; however, they can not influence the growth of established tumors (104). On the other hand, a number of studies have shown that dietary genistein can increase the growth of estrogen-dependent MCF-7 tumors in ovariectomized nude mice; this effect appears to hinge on activation of ERα (105-

108). Nonetheless, a much more substantial response is seen with genistein concentrations in the high nanomolar range, consistent with the known affinity of genistein for ERα; responsiveness at 10 nM probable reflects the fact that activation of only a small minority of ERα. Administered at 750 mg/kg in diet to rats pretreated with the carcinogen MNU, genistein increases the size of MNU induced mammary tumors in ovariectomized rats (109); this dose of genistein also increases uterus size, pointing to an ERα -mediated effect on estrogen-sensitive tumors.

Effects of isoflavones on endometrial cell activity

Both types of estrogen receptor are expressed in endometrial of human and soy isoflavones at physiological concentrations may be safe to for the uterine endometriam because of the uterotrophic effect of estrogens appears to be mediated solely by ERα. Soy isoflavones have been shown to have no impact on endometrial proliferation in clinical studies (110-114). In women, soy isoflavones do not suppress the endometrial proliferate response to estrogen (115). Based on these observations, ingestion of genistein within the nutritional range would not be expected to either increase or decrease endometrial cancer risk. However, when rats are fed doses of genistein 750 mg/kg in diet, resulting in a free serum genistein level of 400 nM, uterotrophic activity is indeed seen (116). This finding is consistent with the responsiveness of ERα to high nanomolar concentrations of genistein. The implication is that genistein doses which greatly exceed the nutritional range should not be presumed to be safe from the standpoint of endometrial cancer risk.

Effects of isoflavones on hepatocytes cell activity

Studies in rats, primates, and humans demonstrate that hepatocytes express ERα, but not ERβ (117). Many of the notable physiological effects of oral estrogen are

mediated in the liver. Thus, oral estrogen lowers LDL cholesterol, raises serum triglycerides, boosts synthesis of angiotensinogen and sex hormone-binding globulin, and decreases hepatic production of IGF-1 – effects which are thought to reflect direct estrogenic activity in hepatocytes (118-125). Moreover, oral estrogen up regulates thrombotic mechanisms by modulating hepatic production of a range of plasma proteins which regulate thrombosis. Thus, oral estrogen increases plasma concentrations of clotting factors VII and IX, activated protein C, and C-reactive protein, while decreasing those of antithrombin, proteins C and S, and tissue factor pathway inhibitor (126-130). Since physiological concentrations of genistein can be expected to have only a modest impact on ERα activity, it is not surprising that none of these effects are observed when soy isoflavones are ingested (131-136).

Gallstone risk is higher for women than men, and this has been traced to the fact that activated ERα boosts cholesterol output to the bile. It can be deduced that soy isoflavones will not increase risk for gallstones. Isoflavones may be disappointing to concede that soy isoflavones cannot lower LDL cholesterol, or diminish cancer risk by suppressing plasma IGF-1, it is nonetheless comforting to realize that isoflavones will not increase thrombotic risk in the way that oral estrogens do. Conceivably, the prothrombotic hepatic effects of oral estrogen are largely if not wholly responsible for the unanticipated increase in risk for myocardial infarction that has been observed during recent prospective trials of oral hormone replacement therapy, despite the favorable influence of estrogen activity on vascular endothelium and LDL cholesterol (137).

Effects of isoflavones on vascular endothelial cell activity

Vascular endothelium expresses both ERα and ERβ (138). The one reason for

doubt that hormone replacement therapy would decrease cardiovascular risk is that estrogens have a favorable impact on endothelial function, promoting the activity of the endothelial nitric oxide synthase (eNOS). Thus the result both from increased transcription, and also from extranuclear effects of both activated ERα and ERβ estrogen receptors exerted at the plasma membrane (139-144). Estrogen can also enhance the bioactivity of nitric oxide (NO) by down-regulating NADPH oxidase expression – more specifically, that of its gp91phox subunit in human endothelial cells (145). Studies of the role for ERα in the induction of eNOS and/or NO production in the endothelial cells of individual species (146-151) and the impact of ERβ in this regard is less clear. Whereas estrogen increases the expression of eNOS in the coronary arteries of ovariectomized ERα knockout mice (152), consistent with a role for ERβ in eNOS induction, no such effect has been seen in their cerebrovascular arteries (148). Induction of eNOS by ERβ in rat cardiac myocytes and in human myometrium has been reported (153, 154). In the vascular smooth muscle cells of rodents, ERβ exerts both antihyperplastic and antihypertensive effects (155, 156). Clinical observations appear consistent with the possibility that ERβ supports endothelial eNOS activity in at least some vascular beds.

Suitable oral intakes of genistein have been reported to improve endothelium-dependent vasodilation as well as other markers for endothelial NO production in postmenopausal women (157-159). The favorable effects of genistein on endothelial function might be operative in men as well, in light of a report that intrabrachial administration of genistein lads to a NO-mediated increase in forearm blood flow in male volunteers; infusion of 17β-estradiol – but not daidzein – has a comparable effect (160). However, one study has reported a modest reduction in endothelium

dependent vasodilation in men ingested isoflavone-rich soy protein for three months (161). The impact of dietary genistein on endothelial function in males should be studied further. Estrogen can boost endothelium-dependent vasodilation in males (162-164).

The role of endothelial NO production plays in preservation of vascular health is well known. It seems likely that genistein's ability to up-regulate eNOS function could decrease vascular risk similarly to the relative protection from cardiovascular disease effect of estrogen that may be largely responsible for the enjoyed by premenopausal women (165-167). It would be a fortunate development indeed if men could use genistein to achieve at least a portion of this benefit without incurring typical estrogenic side effects.

Effects of isoflavones on osteoblast and osteoclast activity

The increase in bone osteoclastic activity and bone resorption ushered in by menopause is thought to stem primarily from an alteration of osteoblast function; soluble mediators produced by osteoblasts have a major impact on the functional status of nearby osteoclasts. Estrogen inhibits osteoblast production of IL-6, an important tropic factor for osteoclasts; it also boosts production of osteoprotegerin, a soluble ''false receptor'' which inhibits activity of RANKL, another important trophic factor for osteoclasts (87, 168, 169). In physiological concentrations, genistein has been shown to suppress IL-6 production, and boost osteoprotegerin production (170, 171). These findings strongly suggest that genistein can interact with ERβ to achieve transrepression of the IL-6 promoter – as it does with the TNF promoter (82). Genistein also has the potential to improve bone metabolism through its impact on

vascular eNOS, in light of recent evidence that this enzyme is a mediator of the osteogenic impact of estrogen on osteoblasts (172-174).

Effects of isoflavones on mesangial cells

Mesangial cells express both ERα and ERβ (175, 176). Physiological concentrations of estrogen have shown to suppress the response of mesangial cells to transformine growth factor-b (TGF-b) (177, 178). Moreover, estrogen can also suppress glomerular production of TGF-b by boosting the NO production of glomerular endothelial cells (179). NO's suppressive impact on TGF-b production is poorly understood; the fact that eNOS inhibitors up-regulate glomerular TGF-b synthesis suggests that this mechanism is of physiological significance. A further theoretical possibility is that activated ERβ could inhibit transcription of TGF-b by interfering with AP-1 activity in its promoter while ERα would be expected to enhance AP-1 activity (88, 91).

The high-physiological concentrations of genistein could activation of ERβ and inhibit the effects of TGF-b on mesangial cells. Thus, genistein could help to prevent glomerulosclerosis. Whether activation of ERβ could also increase glomerular NO production and/or interfere with AP-1 activity – resulting in suppression of glomerular TGF-b production – is more speculative. The possibility that phytochemical components of soy such as isoflavones – contribute to the nephroprotection afforded by soy-based diets has been suggested by Valasquez and Bhathena (180, 181).

1.1.7 Clinical use of isoflavones

Bone mineral density (BMD)

Bone health is a major concern as women age, and femur or vertebral fractures

may severely affect the quality of life. HRTs have been the first line of treatment of hormone-related osteoporosis, but major side effects preclude their whole use. The observation that Southeast Asian women reported a lower occurrence of osteoporosis suggestion that phytoestrogens are a possible alternative choice for the prevention of osteoporosis.

The increase in bone osteoclastic activity and bone resorption ushered in by menopause is thought to stem primarily from an alteration of osteoblast function; soluble mediators produced by osteoblasts have a major impact on the functional status of nearby osteoclasts. Estrogen inhibits osteoblast production of IL-6, an important tropic factor for osteoclasts; it also boosts production of osteoprotegerin, a soluble ‘‘false receptor’’ which inhibits activity of RANKL, another important trophic factor for osteoclasts (87, 168, 169).

Clinical studies of supplementation with soy isoflavones or isoflavone-rich soy protein in postmenopausal women have reached different conclusions: some find that such supplementation has a favorable effect on markers of bone metabolism and on preservation of bone density (13, 18, 182-186), whereas others do not (187-189). Since East Asian women often consume ample amounts of soy isoflavones in their habitual diets, several studies have attempted to correlate habitual isoflavone intake or serum isoflavone level with postmenopausal bone density and/or bone metabolism in such women. Two studies – one each from Japan and China – have reported that women in the highest quartile of soy intake, as contrasted to the lowest quartile, have higher bone density (190, 191). Several other studies, including those in the US or Europe, where soy intake is comparatively low, did not observe such a correlation (192-194), albeit a Southern California study has a positive outcome (195).

Hot flashes

A recent review notes that 4 of soy isoflavones on postmenopausal hot flashes studies have a positive outcome, 5 are negative, and one has shown a positive trend that missed statistical significance (196). A number of clinical studies have assessed the impact of supplemental soy isoflavones (with or without soy protein). The contradiction of the results of clinical studies may reflect the fact that the benefit to be expected is modest, as well as the likelihood that some of the isoflavone regimens tested failed to achieve adequate free genistein concentrations in some subjects. It appears highly unlikely that about half of the clinical studies find statistically significant benefit, if in fact isoflavones have no genuine potential for controlling hot flashes. Soy isoflavones have access to the brain, and certain regions of the hypothalamus express ERβ (197-199); conceivably, some of these receptors mediate the impact of genistein on hot flashes.

Lipid profiles

The relationship between soy products and lipid profiles has been investigated for many years. Animal studies as early as the 1940s demonstrate the beneficial effect of soy proteins on lipid profiles (200). Recent animal studies also have demonstrated the benefits of soy isoflavones on total cholesterol and LDL (201). In addition, various cross-sectional analyses demonstrate an inverse relationship between soy intake and cholesterol levels (202).

A meta-analysis of 38 clinical trials that examined the relationship between soy protein intake and serum lipids have shown that the consumption of soy in men and women is associated with a significant decrease in serum cholesterol, LDL and triglyceride levels (203). This association is strongest for subjects with higher initial

levels of cholesterol. In 34 out of the 38 studies, serum cholesterol levels decrease in treatment subjects following the consumption of soy. The four trials that find no effect from the consumption of soy consisted of women with fairly low initial cholesterol levels. Thus, the authors of this meta-analysis reported that an average intake of 47 g of soy protein per day results in statistically significant reductions in total cholesterol, LDL and triglyceride levels. In a recently published randomized controlled cross-over trial that examined the effects of isoflavones in 18 postmenopausal women with either normal or mildly elevated cholesterol levels, LDL is found to be 6.5% lower in the high-isoflavone treatment group, and is also significantly lower in the low isoflavone treatment group. The LDL/HDL ratio also significantly decreases in the high isoflavone group (204). Similar results that isoflavones decrease triglyceride and LDL levels have also been demonstrated in other studies either a randomized, double-blind, parallel trial (205) or a randomized, double-blind, and cross-over trial of 6 weeks (206). In a randomized, double-blind, placebo-controlled trial that examined the effects of dietary soy supplements containing 118 mg isoflavones on the lipid profiles of men and postmenopausal women with relatively normal cholesterol levels, the LDL/HDL ratio decreases in the isoflavone treatment groups without any change in total cholesterol (161). However, some studies have demonstrated that isoflavone treatment groups show no change in any of the lipid parameters (207).

The mechanism by which soy isoflavone is thought to alter lipid profiles remains unknown, but the proposed mechanisms may be due to an alteration in LDL receptor quantity or quality (208), enhancement of bile acid excretion, increased hepatic metabolism of cholesterol, and altered variations in hormone secretion (209). In

addition, there is evidence to indicate that isoflavones reduce lipid peroxidation and increase resistance of LDL to oxidation *in vivo*. This effect may be beneficial in reducing atherosclerosis.

Glomerulosclerosis

Chronic renal disease tends to progress less rapidly in women than in men (175, 210, 211) because of estrogen (212). Indeed, estrogen improves the progression of glomerulosclerosis in various rodent models of this disorder (213-219). The various agents and conditions which provoke glomerulosclerosis by boosting glomerular production of TGF-b by activation of AP-1 response elements in the TGF-b promoter (220-222). This increased production of TGF-b leads to increased production of various ground substance proteins including collagen types I and IV, laminin, and fibronectin and decreased production of the collagenases MMP-2 and MMP-9 as well as increased production of protease inhibitors such as tissue inhibitor of metalloproteinase 1 (TIMP-1) (223-226). However, the net effect is an accumulation of mesangial ground substance and a thickening of glomerular basement membranes. TGF-b is also a mediator of the proteinuria characteristically seen in glomerular disorders (227-231).

A subsequent study with hypercholesterolemic rats prone to glomerulosclerosis has demonstrated that addition of an isoflavone-rich soy ethanol extract to their casein-based diets ameliorates subsequent renal damage; the interpretation of this study is complicated by the fact that serum lipids decline in the isoflavone-supplemented rats (232). Neugarten and colleagues have demonstrated that low concentration at 1–10 nM of genistein markedly inhibits synthesis of both type I and type IV collagen by murine mesangial cells. Conceivably, this finding simply reflects

the fact that genistein-activated ERβ can block the impact of autocrine TGF-b on mesangial cells (233). Of related interest is a report that a diet supplemented with red clover isoflavones decreases production of TGF-b by prostatic epithelium in mice (234). Even if the studies prove that soy isoflavones can indeed reduce risk or slow the progression of glomerulosclerosis, it would not necessarily follow that a diet high in soy protein should be recommended.

Cardiovascular disease

Premenopausal women are relatively protected from left ventricular hypertrophy (LVH), and estrogen replacement has been shown to limit expansion of ventricular mass in postmenopausal women and ovariectomized rodents at risk for this disorder (235-238). The intracellular signaling mechanisms which mediate LVH appear to be very similar to those that evoke glomerulosclerosis (239, 240). NO has an antagonistic impact on development of LVH analogous to its impact on glomerulosclerosis (241). A recent study examining ERα - and ERβ knockout mice demonstrates that ERβ mediates the protective effect of estrogen on cardiac hypertrophy. Moreover, cardiac myocytes and fibroblasts express both isoforms of the estrogen receptor, but, in neonatal rat cardiac myocytes, only ERβ has an inductive effect on eNOS (242). Thus, it is reasonable to suspect that genistein has the potential to protect postmenopausal women from LVH.

Prostate cancer

The reasons to believe that ERβ activity has an antiproliferative impact both in healthy prostate and in prostate cancers (243-246) because human prostatic epithelium expresses ERβ, but not ERα. (245, 246). Prostatic hypertrophy is common in aging ERβ-knockout mice, whereas knockout of ERα has no such effect (247, 248).

Genistein has been shown to decrease expression of the androgen receptor in the human prostate cancer-derived LNCaP cell line, an effect mediated by activation of ERβ (249). Moreover, genistein feeding down-regulates androgen receptor expression in rat prostate (250). Pilot clinical studies evaluating the impact of oral genistein on early stage prostate cancer have achieved a moderate reduction of prostate specific antigen (PSA) in a minority of patients, and an apparent reduction in cancer growth rate in others (251-254). The studies from Asia are reasonably compatible that diets high in soy products have been associated with lower risk for prostate cancer (253, 254).

Brest cancer

The moderate intake of genistein may slow the onset or progression of certain mammary tumors, but that very high intake can be expected to boost the growth of estrogen-dependent tumors, and even moderate intake may have the potential to at least modestly influence the growth of certain tumors that are highly sensitive to ERα activation. One recent prospective Japanese study has found that high intake of miso or of isoflavones are associated with decreased risk for pre- or postmenopausal breast cancer (183). Other prospective studies have a negative outcome. One case–control study in Asians and Americans focused on soy consumption during adolescence, and found that high soy intake during this time predicted a lower risk for postmenopausal breast cancer; such risk is lowest for those who maintained high soy consumption in adult life (14). Given the fact that most Asian diets provide suboptimal isoflavone intake from the standpoint of ERβ activation, these findings are rationally according with the possibility that somewhat higher supplemental intake of genistein might be

protective in regard to breast cancer risk; however, this proposition has not been confirmed.

There is little reason to doubt that genistein intake in the high nutritional range would increase breast cancer risk and some reason to doubt that such a measure might decrease this risk. However, in women who have been diagnosed with estrogen-sensitive breast cancers, the possibility cannot be excluded that nutritional intake of genistein will modestly boost cancer growth by promoting low-level activation of ERα. Hypothetically, selective activation of ERβ with moderate-dose of genistein might slow the growth of certain estrogen-sensitive mammary cancers which express this receptor, but this is speculative. Until further evidence is available, it might be prudent for women with estrogen-sensitive breast cancer to refrain from frequent soy ingestion or isoflavone supplementation (255).

1.1.8 Toxicity and safety of isoflavones

The isoflavones, individually or combined, are considered to be safe up to quite high doses. Human toxicity studies of isoflavones by Busby et al., currently in publication, suggest that doses ranging from 1 to 16 mg/kg body weight are reasonably safe. At the doses being recommended for prevention of bone loss in postmenopausal women, little concern has been raised about deleterious effects. A few studies of rodents and isolated cells, however, suggest that isoflavones may not be totally safe. Genistein at high doses has been shown *in vitro* to inhibit cell growth and induce apoptosis (256, 257). In addition, some reproductive disturbances, such as uterotropic effects, have been reported in animals fed a diet rich in isoflavones or other phytoestrogens (258, 259). Therefore, toxicity concerns from studies of rodents

and *in vitro* cells must be resolved before isoflavones can be recommended as therapeutic agents.

1.2 Vitamin D

Vitamin D is a hormone that controls phosphorus, calcium, and bone metabolism and neuromuscular function. The two main forms of vitamin D are: vitamin D_3 or cholecalciferol, which is formed in the skin after exposure to sunlight or ultraviolet light, and ergocalciferol or vitamin D_2 which is obtained by irradiation of plants or plant materials or foods.

1.2.1 Sources of vitamin D_2 and D_3

The physiological source of vitamin D is 7-dehydrocholesterol in the skin which becomes activated to form vitamin D_3 on exposure to UV radiation of an appropriate wavelength, normally in the form of sunlight. The availability of vitamin D from this source depends upon season and latitude as well as the extent of skin exposure, its pigment and thickness (260). Pigmented skins (261), and aging skin (262), are less efficient at producing vitamin D_3. Both vitamin D_3 and vitamin D_2, derived by UV irradiation of the plant sterol, ergosterol, may occur in the diet, either naturally or as a result of supplementation; both forms are metabolized in a similar manner in humans.

1.2.2 Metabolism and calcium homeostasis

Vitamin D_2 (ergocalciferol) and D_3 (cholecalciferol) is hydroxylated in the liver to 25-hydroxyvitamin D_3 (25(OH)D) and subsequently in the kidney into 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$). This is the active metabolite, which stimulates the calcium absorption from the gut (263). When $1,25(OH)_2D$ is sufficiently

available, 24,25-dihydroxyvitamin D (24,25$(OH)_2$D) is formed in the kidney, which is further catabolized. The vitamin D metabolites are bound in the circulation to vitamin D binding protein which has a high affinity to 25(OH)D, 24,25$(OH)_2$D and 1,25$(OH)_2$D and has a high homology to albumin.

The active metabolite 1, 25$(OH)_2$D enters the cell and binds to the vitamin D receptor. This complex forms a heterodimer with the retinoid receptor and binds to a vitamin D responsive element on a responsive gene, such as that of osteocalcin, calcium binding protein or 24-hydroxylase. This is followed by transcription and translation and proteins are formed such as the calcium binding protein or osteocalcin. The classic effect of 1,25$(OH)_2$D on active calcium transport occurs in the intestinal cell. Calcium enters the cell through membrane proteins. In the intestinal cell, 1,25 $(OH)_2$D binds to the vitamin D receptor and the calcium binding protein is synthesized and this regulates the active transport through the cell. The calcium is transported to the extracellular fluid by an ATP dependent mechanism. There also is passive transport through paracellular diffusion of calcium. The vitamin D dependent calcium absorption has a maximum, but depends on the calcium gradient. The 1,25$(OH)_2$D has its effect on the classic target organs bone, intestine and kidney and stimulates calcium transport from these organs to the blood. The production of 1,25$(OH)_2$D is stimulated by parathyroid hormone (PTH). There is a negative feedback through calcium which decreases PTH and a direct negative feedback from 1,25$(OH)_2$D to PTH. The active metabolite 1,25$(OH)_2$D also shows rapid actions through a membrane receptor (264).

1.2.3 Vitamin D and calcium on postmenopausal osteoporosis

Vitamin D and calcium supplement is the cornerstone of prevention and

treatment of osteoporosis. Vitamin D supplementation alone does not appear to reduce the incidence of hip or a vertebral fracture, but use of vitamin D in combination with calcium has been shown to be effective in reducing the risk of vertebral and nonvertebral fractures, including hip fractures (265). In a randomized, placebo-controlled study, elderly ambulatory women who are followed for 18 months, 1,200 mg/day of elemental calcium and 800 units/day of vitaminD_3 provided a 43% reduction in the number of hip fractures and a 32% reduction in the number of nonvertebral fractures (266). A reduction in nonvertebral fractures is observed over a 3-year period from the use of calcium 500 mg/day and vitamin D 700 units/day (267). Recently published data from the Women's Health Initiative, postmenopausal women aged 50 to 79 years, has demonstrated that the use of elemental calcium 1,000 mg/day and vitamin D_3 400 units/day over an average of 7 years result in a 1.06% increase in hip BMD compared with the placebo group. However, calcium plus vitamin D_3 supplementation do not significantly reduce the incidence of hip fractures, and it increases the risk of kidney stones. But, when the analysis is limited to adherent patients, the reduction in risk for hip fracture is significant (268). Vitamin D supplementation appears to reduce the risk of falls in ambulatory older individuals. Data from 5 studies have shown that vitamin D reduces the corrected odds ratio of falling by 22% (269). Vitamin D supplements include vitamin D_2 and vitamin D_3. Vitamin D_3 is often preferred because it has greater potency than vitamin D_2. In addition, intake recommendations for vitamin D are based on vitamin D_3 not D_2. Calcitriol is the active form of vitamin D (i.e., 1,25-dihydroxycholecalciferol). It has a narrow therapeutic index (4, 5). Calcitriol is a prescription medication and is often

reserved for patients with renal impairment who cannot create the active moiety. Alfacalcidol is a safer analogue that recently became available.

1.3 The scope and aims of this research

The objective of the present study was to determine the effect of D_3 and calcium supplement on isoflavone pharmacokinetics in Thai postmenopausal women.