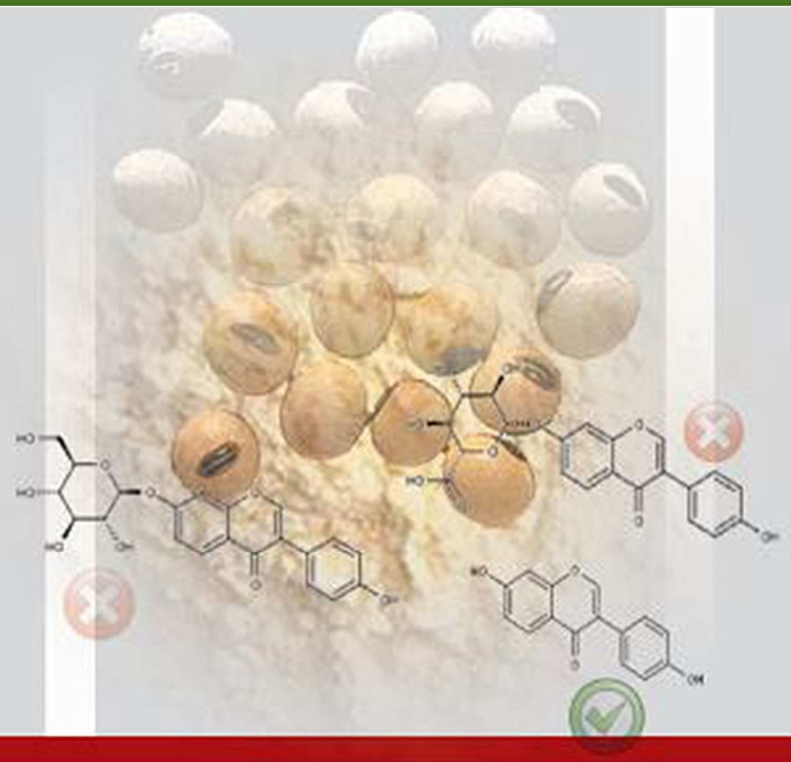
IMPROVEMENT OF RISK ASSESSMENT BY INTEGRATING TOXICOLOGICAL & EPIDEMIOLOGICAL APPROACHES : THE CASE ISOFLAVONES



MOHAMMED ARIFUL ISLAM

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Improvement of risk assessment by integrating toxicological and epidemiological approaches: the case of isoflavones

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Improvement of risk assessment by integrating toxicological and epidemiological approaches: the case of isoflavones

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Thesis

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CONTENTS

1

Introduction

Soybean

Soybean (*Glycine max*) is a leguminous plant probably originating from central China or East Asia. It is economically the most important bean in the world being the main source of vegetable proteins, edible oils and ingredient for hundreds of soy based food products [1]. Currently the United States of America (USA), the People's Republic of China, Brazil and Argentina account for more than 80% of the total production of soybeans, whereas China, the European Union and the USA are the major consumers accounting for respectively about 57 and 30 and 27% of the world soy consumption[2]. Chinese people have been using soybean as food and as constituent of medicines already for about 5,000 years [1]. Soybean based products are widely consumed in many other Asian countries including Japan in various traditional forms [3]. Being a plant based cheap protein source and because of diverse putative beneficial health effects, soybean also gained popularity in Western societies in the last decades [4]. Many of these putative beneficial health effects have been related to the isoflavones present in soybean [5, 6].

Soy isoflavones (SIF)

Isoflavones are biologically-active, non-nutritive compounds that are present in relatively large amounts in soybean and soy foods. Chemically these isoflavones belong to a larger group of plant chemicals called flavonoids which are common in many fruits, vegetables and legumes. Isoflavones are produced by the soybean plant as part of their defence mechanism against insects, diseases and in response to environmental stresses such as drought [7, 8]. They also play an important role in the growth of the soybean plant by stimulating nodule formation [9, 10]. The chemical structure of SIF aglycones resembles that of the natural hormone estradiol (E2). As SIF have weak estrogenic potencies, they are also referred to as phytoestrogens [11-13]. Figure 1.1 shows the common chemical structures of naturally occurring SIF glucosides, their primary metabolites (aglycones), secondary metabolites (i.e. glucuronide and sulphate conjugates) and the natural hormone estradiol (E2). Here the structural similarity of the SIF aglycones with estradiol (E2) is clearly visible.

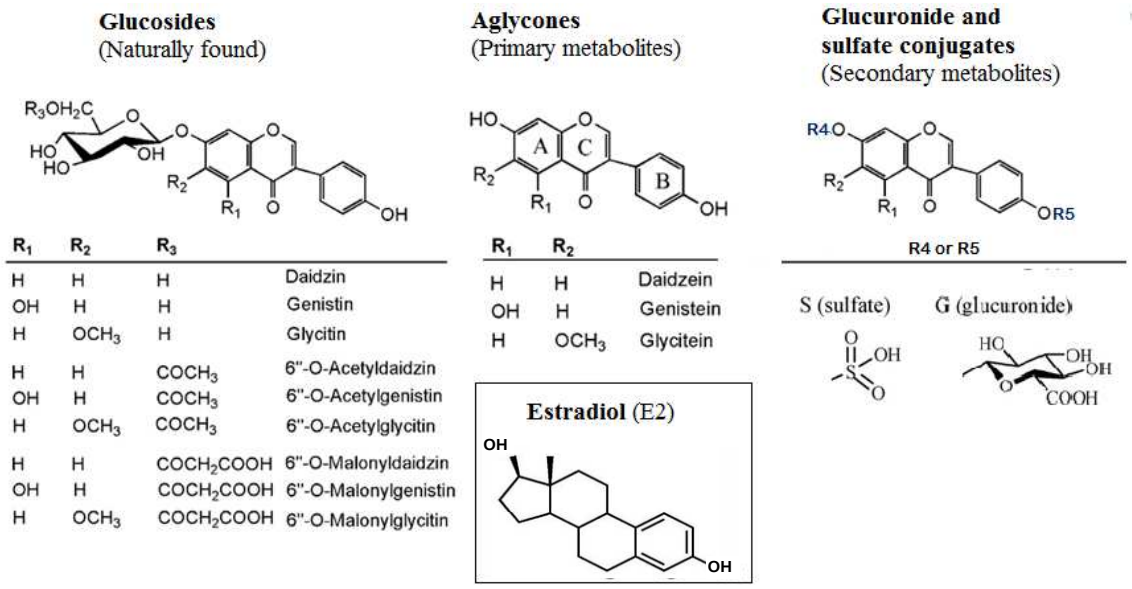

Figure 1.1: Chemical structure of different forms of SIF and estradiol (E2).

Isoflavone content in soy foods

Isoflavones can also be found in small amounts in a number of legumes, grains, and vegetables, but soybean is the most concentrated source of isoflavones in the human diet. Whole (dry) soy bean contains about 200 milligrams of SIF per 100 gram (USDA-Iowa State University Database on the Isoflavone Content of Foods). The concentration and type of SIF present depend on the nature of the food. Whereas in most soy based products SIF glucosides are present, the traditionally fermented soy based foods such as tofu, soymilk, soy nuts, tempeh and miso are generally rich in SIF aglycones [14, 15]. Soy protein concentrates which are a widely used ingredient in burgers, generally do not contain significant amounts of SIF. “Second-generation soy products", such as soy hot dogs and soy-based ice cream, have much lower amounts of SIF because they also contain considerable amounts of non-soy ingredients. Soy oil and soy sauce do not contain any SIF.

Why SIF got scientific attention

Isoflavones first came to the attention of the scientific community in the 1940s when fertility problems were observed in sheep grazing on isoflavone-rich red clover fields in Western Australia [16, 17]. In the nineties Setchell [13] reported that the declining fertility of zoo animals might be due to the presence of SIF in the standard animal diet. As a result, SIF are considered to have effects on the reproductive system. In addition, in the last few decades SIF again got scientific attention because of the so called “Japanese Phenomenon” [4, 18, 19], referring to a lower incidence of certain chronic diseases in the Japanese population compared to Western society due to their high intake of soy foods from the early life onwards. This phenomenon was confirmed by the fact that, after changing their food habits, the prevalence of breast cancer in daughters of migrated Japanese Americans appears to be the same as that of Caucasian Americans. Figure 1.2 shows the significantly lower incidence of

breast, ovary, colorectal and lung cancer in Asian women (A) and prostate, colorectal, lung, and bladder cancer in Asian men (B) compared to the European and American population, respectively. While Figure1.3 shows the breast cancer mortality per 100,000 females in different parts of the world.

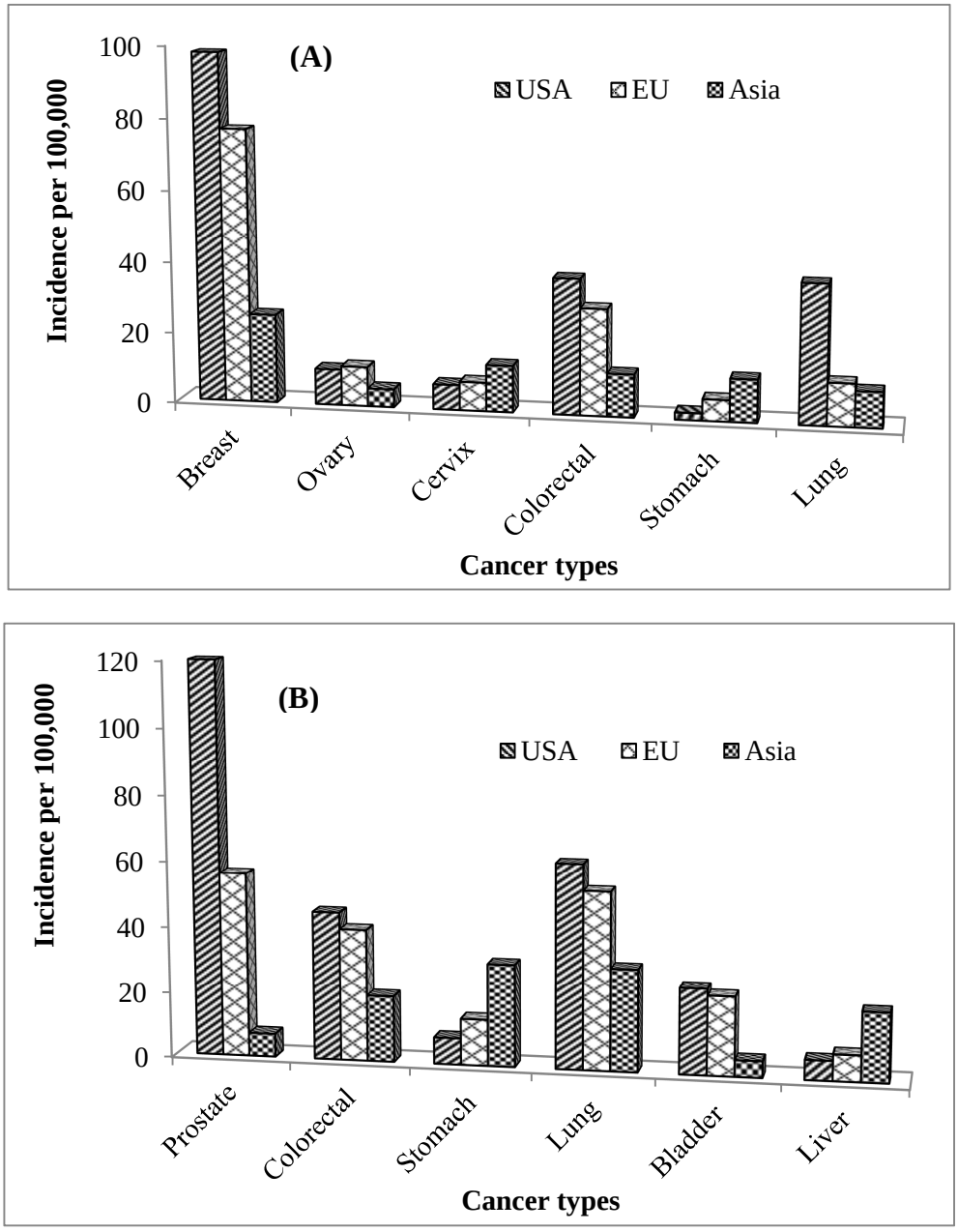

Figure 1.2: Cancer incidence among Asian, European and American populations adapted from Andres and Lampen [20] where the incidences for the EU countries are calculated as the sum of incidences from northern, western, and southern Europe; the incidence for the Asian countries are calculated as the sum of the incidences from China, Japan, southern-eastern and south-central Asian countries.

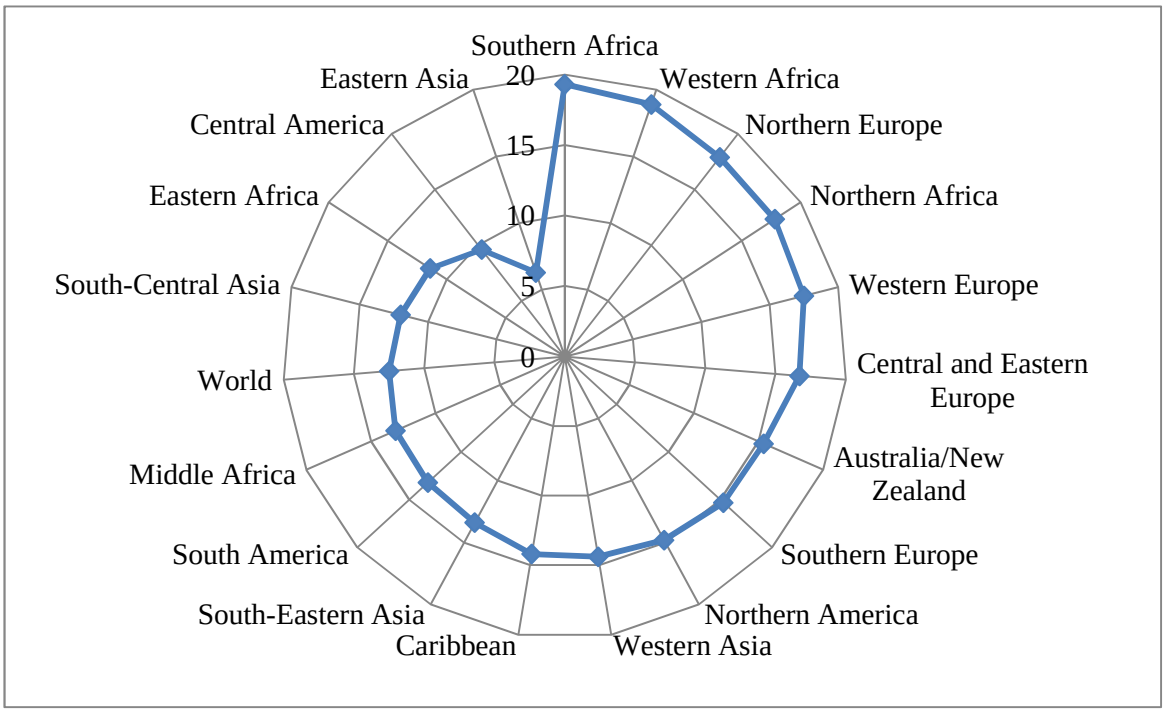

Figure 1.3: Breast cancer mortality per 100,000 females in different parts of the world adapted from Cancer Research UK, [21].

Many recent epidemiological studies reported positive relationships between soy consumption and several health benefits such as lower risks for breast cancer and heart disease, less hot flushes and nocturnal sweating and improved bone density in postmenopausal women, and improved cognitive health [22-24]. In contrast, due to their low estrogenic and anti-estrogenic potency, possible adverse effects of soy consumption or concentrated SIF were also suggested based on data from in vitro and in vivo experiments and from clinical trials [7, 25-27]. Peri- and post-menopausal women with a family history of breast cancer are a sub-population of increasing concern in the Western society, because of their unregulated and self-administrated consumption of soy containing food supplements. These women may show an adverse response after intake of estrogenic supplements, since the effects of long term exposure of concentrated SIF on human health remains unclear [28-30].

Opinion of different governmental and international organizations about the safety of SIF

The Committee On Toxicology of Chemicals in Food, Consumer Products and the Environment (COT, UK) published a report in 2003 [31] to advice on the health implications of dietary phytoestrogens. The COT concluded that evaluation of the public health implications of SIF is complex as these compounds can elicit agonist and antagonist actions *via* the estrogen receptor and non-estrogenic pathways, which are depending on age, tissue, gender, lifestyle, diet and exposure duration. Extrapolation of the effects seen in in vitro and in animal studies to the human situation is

also very complex, for example due to differences in experimental design, exposure type and dose, and because of inter-species differences. Given the level of complexity and paucity of data, the COT considered that it is inappropriate to evaluate the public health implications of phytoestrogens to the population as a whole, or communicate the implications in a single statement. The COT suggested that more long-term prospective human studies with adequate statistical power are necessary to establish the relationship between dietary phytoestrogens and the development of some diseases (for example breast cancer). Similarly, the Senate Commission on Food Safety (SKLM) of the German Research Foundation (DFG) and the Federal Institute for Risk Assessment (BfR) expressed their opinion about the paucity of data and difficulties of extrapolation of data from in vitro and animal studies, hampering definition of a safe human dose [28, 32]. Based on the data provided by Member States, the Scientific Panel on Dietetic Products, Nutrition and Allergies (NDA) of the European Food Safety Authority (EFSA) [33] also concluded that the available data are insufficient to establish a cause and effect relationship between the consumption of SIF and maintenance of bone mineral density, reduction of vasomotor symptoms associated with menopause, protection of DNA, proteins and lipids from oxidative damage, maintenance of normal blood LDL-cholesterol concentrations, maintenance of normal skin tonicity, contribution to normal hair growth and improvement of cardiovascular health [34, 35].

In contrast, the health claim on soy protein food labels that 25 grams of soy per day with a prudent diet may reduce the risk of heart disease was approved by the US Food and Drug Administration (FDA) [36]. The FDA considered soy as supplement and therefore, manufacturers do not need to register their products at the FDA nor do they need an approval before producing or selling dietary supplements. However, the manufacturers are responsible for ensuring the safety before marketing their products. The FDA is responsible for taking action against any unsafe dietary supplement product after it might have reached the market. The French Agency for Food, Environment and Occupational Health and Safety ANSES in their report entitled “Présentation du rapport sur sécurité et bénéfices des phyto-estrogènes apportés par l’alimentation-recommandations”, considered estrogen like effects of phytoestrogen substances from various viewpoints and concluded that 1 mg/kg body weight/day of SIF (as aglycone) is a safe intake level [37]. However, ANSES recommended against this level for breast cancer patients or for women with a breast cancer history within the family. For liquid infant foods containing soy proteins as the main component, the concentration of phytoestrogens in general should be limited to 1 mg/L, which equals a daily intake of 0.15 mg/kg bw assuming a daily milk consumption of 150 mL/kg baby. In Japan the Food Safety Commission, recommended an upper limit for standard daily intake of SIF of 70-75 mg aglycone equivalent/day per person [38]. The Committee suggested that this upper limit is relevant when soy based foods are taken regularly over many years. However, the Committee also indicated that exceeding this upper limit of dietary intake from soy products will not immediately lead to health damage.

Pharmacokinetics (Adsorption, Distribution, Metabolism and Excretion)

For the evaluation of the health benefits or risks of SIF, which are still controversial, a proper understanding of the pharmacokinetics is crucial [4, 20, 25]. These ADME (absorption, distribution, metabolism, excretion) characteristics will determine the ultimate physiological concentrations of the SIF and their metabolites upon intake at relevant dietary levels. These physiological levels may be very different following high dose exposure in rodent models compared to high intake of supplements by humans, thus possibly also resulting in different health effects. Detailed ADME characteristics of SIF have been described in various publications [39-41]. In brief, glucosides are the predominant form of SIF in food (Figure 1). These naturally occurring glucoside forms are generally too polar to cross the intestinal barrier by diffusion [42] which hampers their cellular uptake and bioavailability [43, 44]. Hence, after intake of SIF glucosides hydrolysis is essential to release the biologically active aglycones. Hydrolysis of SIF to their aglycones has previously been assumed to be predominantly catalysed by microflora in the colon [42, 45], but recent human and animal data suggest that it may already occur in the duodenum and the proximal jejunum by different intestinal β-glucosidase enzymes [34, 35, 43, 46, 47]. After deconjugation, the aglycones are conjugated again by the phase II enzymes glucuronyltransferases and sulfotransferases during first pass metabolism, whereby SIF glucuronides and sulfates are produced [48-50]. The major route for elimination of SIF is by renal clearance. The net outcome of these processes will influence the nature and amount of the SIF present in the systemic circulation and their ultimate biological effect in vivo.

Mechanism of action

Interaction with the estrogen receptor

Due to the structural similarity with the natural hormone 17-β-estradiol (E2), SIF aglycones are able to bind to estrogen receptors (ERs). There are two types of ERs, namely ERα and ERβ that have been identified in rats and humans [46, 51]. ERα was the first discovered estrogen receptor. ERβ was discovered later and its discovery led to a new concept of estrogen signalling, especially in pharmacology for the design of drugs for hormone dependent cancer therapy [47]. Activation of ERα promotes cell proliferation and activation of ERβ promotes apoptosis [52, 53]. These two subtypes of ERs also have different relative tissue distributions. ERα is predominant in the uterus, mammary glands, liver, central nervous system (CNS), cardiovascular system, bones and urogenital tract in humans. Whereas ERβ is relatively more present in the cardiovascular system, lungs, kidneys, urogenital tract, mammary glands, colon, immune system, and reproductive organs [8, 40]. SIF interact with both of these ERs, however, ERβ is activated by the major SIFs genistein and daidzein already at lower concentrations than ERα. For E2 this is the other way around. As a result, the

ERβ/ERα activation potency is about 25 times higher for SIF than for E2, although the absolute potency for activation of both receptors is higher for E2 [54].

Interaction with metabolism of steroid hormones

SIF interact with sex steroids in multiple ways. The influence on the metabolism of sex hormones may be quite complex and may depend on several factors including species, sex, age, hormonal status, etc. SIF were found to inhibit the activity of 5α-reductase, which catalyzes the conversion of testosterone to 5α-dihydrotestosterone, and of aromatase P450, which mediates the conversion of testosterone to estradiol [39, 41, 49, 50]. On the other hand, it has been reported that aromatase activity is only inhibited by low concentrations of isoflavone, whereas high isoflavone concentrations increase the activity of this enzyme [48]. In addition to the interactions of SIF with the metabolism of the sex steroids also effects on thyroid hormones were reported. Hampl et al. [55] found that short-term (i.e. 1 week) consumption of a diet with a high amount of soybeans led to a transient increase in concentrations of the thyroid stimulating hormone, thyrotropin. This effect was observed only in men and not in women, and the triiodothyronine and thyroxine serum concentrations were not changed.

Role of epigenetics

Another important mechanism of action of SIF may be through epigenetics, with effects of SIF on DNA methylation, histone modification and miRNA expression patterns. Epigenetics is the study of heritable changes in the phenotype (appearance) or gene expression caused by mechanisms other than changes in the underlying DNA sequence. Rietjens et al. [25] summarized these issues in detail and expressed that science is still at the start of unravelling the complex underlying mode of action for epigenetic effects of SIF. DNA methylation of cytosine bases results in inaccessibility of DNA regulatory elements to bind to their transcription factors and thus inhibition of gene transcription occurs. Effects of genistein on DNA methylation have been studied both in vitro and in vivo. Genistein has been shown to act as a DNA demethylating agent in in vitro studies in which genistein treatment reduces DNA methylation through inhibition of DNA methyltransferase activity [52, 53]. In contrast, several animal studies have observed increased DNA methylation following in vivo genistein treatment [56-58].

Why an integrated Tox-Epi approach is needed

Traditionally the human risk assessment of chemical compounds present in food is based on information from studies in experimental animals, often with unrealistically high exposure levels, and different models and techniques are applied to extrapolate the animal data to the human situation. Due to the inherent differences between human and rat physiologies, rodents appear to be not always adequate models for humans [59, 60], and uncertainties exist about the factors that may play a crucial role in the extrapolation from rodent to human. Therefore there is a chance of under- or overestimation of the risks or the benefits of SIF supplements when extrapolating animal data to humans. Sometimes human epidemiological data are available as basis for the risk assessment. In contrast to animal experiments epidemiological studies evaluate the health effect of exposure to chemical compounds at lower exposure levels representing a "real-life situation". The use of human data could overcome the inherent problems of extrapolating from animals to humans and/or at least facilitate the extrapolation. However, in epidemiological studies the causal relationship between exposure and observed effects is often far less clear than in animal studies, due to many confounding factors that contibute to a high variation. Therefore, the question has been raised, whether a dedicated comparison of comparable human and animal data could improve the current risk assessment of chemical compounds in food.

The aim of the present thesis was to evaluate the use of in vitro and in vivo animal and human models to study early biomarkers of effect, in order to improve the risk and/or benefit assessment of SIF intake for humans. Figure 1.4 presents a so-called parallelogram approach, illustating how a combination of animal and human data for surrogate tissues and animal data for target tissues can be used to predict human effects in target tissues that are experimentally not easily accessible. We hypothesized that an integration of toxicological and epidemiological methodologies, investigating gene expression profiles as early biomarkers of effects in both rat and human tissue samples, would enable a better prediction for effects in human target tissues, and thus would improve the risk-benefit assessment of SIF in humans. To carry out this integrated "tox-epi' aproach, the research project presented in this thesis has been designed in close collaboration with an epidemiological project that studied the effects of the same SIF supplement in human volunteers. SIF were selected as model compounds for this study, because they can be easily studied in human volunteers. In addition, the dissimilarities in the reported toxicological and epidemiological outcomes upon SIF exposure as outlined above, make SIF also an interesting class of compounds for such a study.

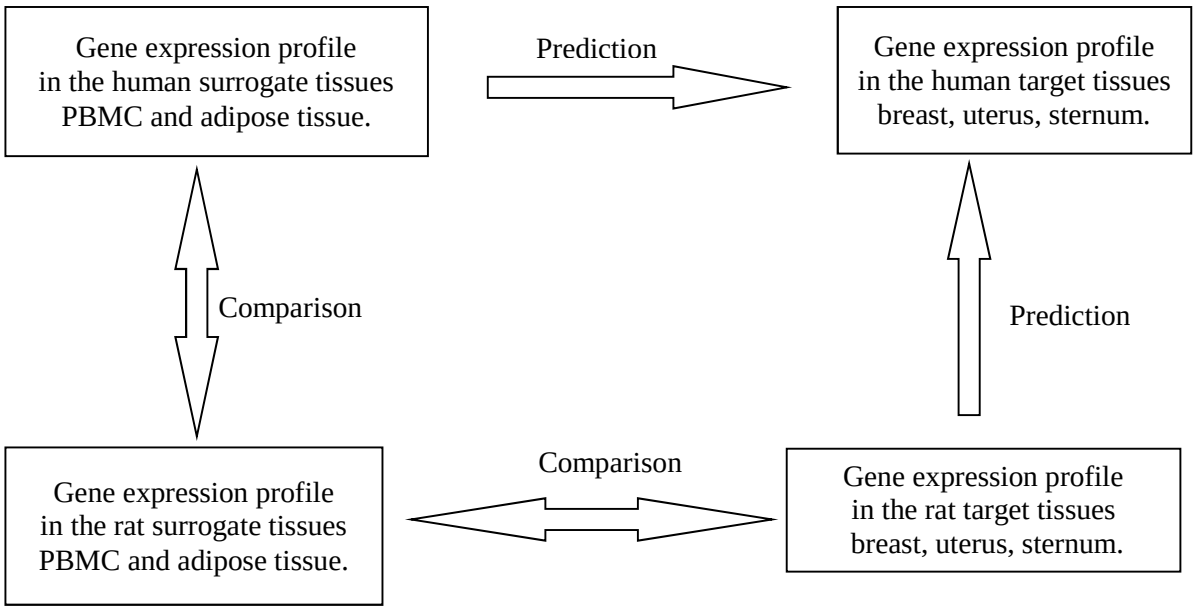

Figure 1.4: Scheme of the integrated "tox-epi" approach combining rat and human transcriptomic data to improve the risk-benefit assessment of soy supplementation for humans. PBMC: peripheral blood mononuclear cell.

Outline of the thesis

This thesis presents a systematic set of in vitro toxicological studies to assess the bioavailability and estrogenic and proliferative potency of SIF using the bioactive SIF aglycones and naturally occurring SIF glucosides. Two commercially available SIF glucuronide metabolites which are found as major metabolites in the systemic circulation were also included in the studies because after consumption of soy food or supplements SIF will become available in the blood circulation as glucuronide metabolites while the estrogenic potency of these metabolites for the target tissues is not yet clear. Two in vivo toxicological studies using F344 rats were conducted to assess the bioavailability of the SIF and the gene expression induced in various tissues upon SIF exposure. The gene expression data of animal studies were compared to gene expression data obtained for peripheral mononuclear blood cells (PBMC) from human intervention studies performed by the department of Human Nutrition of Wageningen UR [61, 62]. The same commercial supplement with a similar daily dose per kg body weight was used in the rat studies and in the human intervention studies.

In *Chapter 1* (the current chapter) of this thesis, a general introduction is provided and background information is given that sets the scene for the research problem that is addressed. In addition, the aims and a short outline of this thesis are presented.

Given that the biological effects of SIF are highly dependent on their kinetics (absorption, distribution, metabolism, and excretion) *Chapter 2* describes the results of studies performed to compare the kinetics of three major soy isoflavone glucosides and their aglycones in a series of in vitro rat and human intestinal models. Studies with a human digestion model, a Caco-2 transwell model, and enzymatic kinetic studies applying specific inhibitors were conducted. Moreover, the outcomes of in vitro studies were compared with available in situ and ex vivo data.

In *Chapter 3*, rat and human in vitro models characterizing relevant biological effects of SIF and their major metabolites were applied. The estrogenic potency of two major and commercially available glucuronide metabolites (i.e. genistein-7-O-glucuronideand daidzein-7-O-glucuronide) which are predominantly present in the systemic circulation were compared with the potency of their corresponding bioactive aglycones using different cellular in vitro models. 17-β-Estradiol (E2) was used as a positive control and standard. Moreover, to study the deconjugation of circulating glucuronides in target tissues (i.e. production of the bioactive aglycones), incubations with breast S9 fractions of rat and human origin were performed.

Chapter 4 describes a 2-day study in rats characterizing the plasma bioavailability and the gene expression profiling induced in peripheral mononuclear blood cells (PBMC) by a commercial soy supplement. The rats were fed a dose of 2 mg SIF/kg bw, in order to provide a dose equivalent to that of an adult who consumes a soy based supplement. In addition, a tenfold higher dose (i.e. 20 mg SIF/kg bw) was tested as well.

Chapter 5 presents the results of an 8 week rat study using the same commercial soy supplement, focussing on gene expression profiling in six different tissues. The rats were fed a dose of 2 mg SIF/kg bw/day and gene expression profiling in PBMC, adipose tissue, liver, breast, sternum and uterus were studied. To assess the relevance of gene expression profiles in PBMC and adipose tissue as early biomarkers, the results were compared with those of the same human tissues from an epidemiological experiment carried out by the department of Human Nutrition of Wageningen UR, using the same source (commercial supplement), dose and exposure period.

In *Chapter 6*, the results obtained in this thesis are discussed, as well as the lessons learned from the different studies conducted, also in comparison with the available literature. Finally, the future perspectives of the work that has been carried out are presented, including the most relevant topics for further research.

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2

Conversion of major soy isoflavone glucosides and aglycones in in vitro intestinal models

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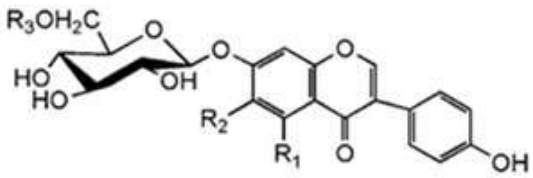
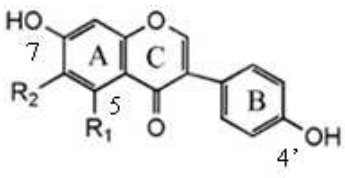
Abstract

This study compares conversion of three major soy isoflavone glucosides and their aglycones in a series of in vitro intestinal models. In an in vitro human digestion model isoflavone glucosides were not deconjugated, whereas studies in a Caco-2 transwell model confirmed that deconjugation is essential to facilitate transport across the intestinal barrier. Deconjugation was shown upon incubation of the isoflavone glucosides with rat as well as human intestinal S9. In incubations with rat intestinal S9 lactase phlorizin hydrolase, glucocerebrosidase, and cytosolic broad-specificity β-glucosidase (BSβG) all contribute significantly to deconjugation, whereas in incubations with human intestinal S9 deconjugation appeared to occur mainly through the activity of BSβG. Species differences in glucuronidation and sulfation were limited and generally within an order of magnitude with 7-*O*-glucuronides being the major metabolites for all three isoflavone aglycones and the glucuronidation during first pass metabolism being more efficient in rats than in humans. Comparison of the catalytic efficiencies reveals that deconjugation is less efficient than conjugation confirming that aglycones are unlikely to enter the systemic circulation. Altogether, the data point at possible differences in the characteristics for intestinal conversion of the major soy isoflavones between rat and human, especially with respect to their deconjugation.

Introduction

Isoflavones are naturally occurring ingredients predominantly found in soybean and food products. They can be present as esterified forms (i.e. malonyl glucoside, acetyl glucoside or glucoside) [1-3]. In whole soybeans and other soy protein products, 97-98% of the isoflavones are present as their esterified conjugated forms predominantly as glucosides, whereas fermented products mostly contain aglycones [1, 4]. The chemical structures of the major isoflavone glucosides and aglycones are shown in Figure 2.1. Because of several proposed beneficial health effects including lower incidences for a number of chronic diseases such as mammary, prostate and colon cancer, osteoporosis and cardiovascular diseases [5-7], a variety of food supplements containing soy isoflavones are commercially available and the consumption of these food supplements in Western countries increased markedly in the last decades [8]. Being derived from a natural source, consumers often consider soy supplements a safe alternative for "hormone replacement therapy" for menopausal women [9-11] to supplement the decreasing E2 levels and to prevent adverse effects in the body during that period. However, likely adverse effects related to possible promotion of different types of cancer such as breast, endometrial, cervical and ovarian cancer have also been reported [12-14] and may be related to their estrogen receptor agonist activity [8, 13]. Exposure to isoflavones may also result from the use of isoflavone-containing products as part of the regular diet, such as the use of fermented soy products including tempeh, miso, or natto, which are part of the traditional diet in many Asian countries and now popular in Western societies especially for vegetarians [15]. The health benefit or risk of isoflavones are still controversial [8, 13, 16] and in order to perform an adequate evaluation of their health effects, it is crucial to have knowledge of the biological fate of dietary isoflavones, in particular of their absorption, distribution, metabolism and excretion characteristics. These absorption, distribution, metabolism and excretion characteristics will determine the ultimate physiological concentrations of the isoflavones and their metabolites upon intake at dietary relevant levels, but also the health effects upon high dose exposure in rodent models or upon human intake of highly dosed supplements. The glucoside forms of the soy isoflavones are generally too polar to cross cellular membranes by diffusion [17], and this hampers their cellular uptake and bioavailability [18, 19]. Hence, after intake of soy isoflavone glucoside hydrolysis is essential to release the biologically active aglycones.





Chemical composition		Glucosides	Aglycones
R1	R2	(naturally found in soy but biologically inactive)	(primary metabolite(s) which are biologically active)
H	H	daidzein-7-O-glucoside	daidzein
OH	H	genistein-7-O-glucoside	genistein
H	OCH_3	glycitein-7-O-glucoside	glycitein

[1] R3 represents H, $COCH_3$ or COCH2COOH for glucosides, acetyl-glucoside and malonyl-glucosides for soy isoflavones, respectively.

Figure 2.1: Chemical structures of soy isoflavone glucosides and their corresponding aglycones.

Hydrolysis of isoflavones to their aglycones has previously been assumed to be predominantly catalysed by microflora in the colon [17, 20], but recent in vivo human and animal data suggest that it may already occur in the duodenum and proximal jejunum by different intestinal β-glucosidase enzymes [19, 21-24]. Using saliva from human volunteers Allred et al. [25], reported that genistin can deconjugate to its aglycone genistein already in the mouth. In addition, it is also interesting to know whether the low pH in the stomach can chemically deconjugate the isoflavone glucosides since a considerable number of analytical procedures use acid hydrolysis of these glucosides [2, 26, 27]. Following deconjugation, the aglycones may become conjugated again by first pass metabolism along their transport over the intestinal wall and/or in the liver, before entering the systemic circulation. Clearly the outcome of these processes will influence the nature and amount of the soy isoflavones entering the systemic circulation and exerting a biological effect in vivo. In addition, possible species differences between rats and humans might determine to what extent the rat can be considered an adequate model to predict bioavailability of isoflavones in humans. Therefore, the aim of the present study was to characterize possible differences between rats and humans in the conversion of the three major soy isoflavones in the intestine and the liver using S9 fractions. To this end conversion of the major soy isoflavones was quantified using well-designed in vitro model systems.

Materials and methods

Chemicals and materials

Genistin, daidzin, glycitin (all of >99 % purity) and their corresponding aglycones, i.e. genistein, daidzein and glycitein, were purchased from LC Laboratories (Woburn, MA, USA). Two glucuronide metabolites, namely genistein-7-O-glucuronide and daidzein-7-O-glucuronide were obtained from

Extrasynthese (Genay, France). Analytical grade ethanol, NaCl, KSCN, $NaH_2PO_4.H_2O$, Na_2SO_4, KCl, $CaCl_2$, $NaHCO_3$, KH_2PO_4, $MgCl_2.6H_2O$, NaOH, 37 % HCl, HNO_3, NH_4Cl, urea, pepsin, D-glucose monohydrate, D-glucosamine hydrochloride, TFA and $ZnSO_4$ were purchased from VWR International (Darmstadt, Germany). N-(n-Butyl)deoxygalactonojirimycin (NB-DGJ) was obtained from EMD Chemicals (Darmstadt, Germany); uric acid, D-glucuronic acid, α-amylase, mucin, pancreatin (pig), lipase (pig), bile (bovine), BSA, ascorbic acid and gluconolactone were obtained from Sigma-Aldrich (Steinheim, Germany). ACN (ULC/MS) and methanol (HPLC Supra-gradient) were purchased from Biosolve BV (Valkenswaard, Netherlands). Conduritol B epoxide was from Santa Cruz Biotechnology (CA, USA) and DMSO from Acros Organics (NJ, USA). Fetal bovine serum was purchased from PAA Laboratories (Pasching, Austria). All cell culture reagents were purchased from Gibco (Paisley, UK). The pooled human intestinal S9 was ordered from Biopredic International (Rennes, France; batch FRA318008), whereas pooled Sprague Dawley (SD) male rat intestinal S9 was ordered from Xenotech (Kansas, USA; batch 1010434). Pooled human liver S9 and SD male rat liver S9 were obtained from BD Biosciences (MA, USA; batch 22877 and batch 88875, respectively). Fresh nanopure water was collected from an Elga ultra-high quality water purification system (High Wycombe, Buckinghamshire, UK).

Isoflavone content of commercial soy supplements

To select a suitable supplement for the in vitro human digestion model isoflavone supplements were collected from the local market (Wageningen, the Netherlands) and analyzed for their isoflavone content. Extraction was done following a method described by Fiechter et al. [28]. In brief, 300 mg of the supplement were placed in a 30 mL glass extraction tube. To prevent possible hazy extracts, 150 mg $ZnSO_4$ was added from a 0.3 g/mL stock solution in nanopure water. After that, 20 mL of 80% (v/v) methanol/water were added as extraction medium. The mixture was placed in an orbital shaker at 550 rpm for 1 h at room temperature followed by centrifugation at $2000 \times g$ for 15 min at 10^0C. After transferring the supernatant to a 100 mL volumetric flask, the remaining pellet was extracted once more using the same procedure with fresh 80 % methanol, but without $ZnSO_4$. The supernatants thus obtained were combined and the volume was adjusted to 100 mL with 80 % methanol. The extract was subsequently stored at 4^0C until analysis. Before ultra-performance liquid chromatography (UPLC) analysis, the extract was diluted ten times in nanopure water followed by centrifugation at 16.000 *g* for 5 min and filtrated using a 0.2 µm cellulose acetate filter from VWR (West Chester, PA, USA). Identification of the different soy isoflavones and the corresponding aglycones was done by comparison of their retention times and UV spectra to those of commercially available standards. The compounds were quantified based on comparison of the area under the curve of their peaks in the UPLC chromatograms to those of calibration curves made using commercially available standards. The recovery factors for genistein, daidzein and glycitein, of 94.5, 94.7 and 93.8%, respectively [28]

were taken into account to calculate the total amount of isoflavones in the supplement. The amount of isoflavones claimed to be present in the supplements as well as the resulting estimated daily intake were established using the information provided by the supplier on the label or the relevant Internet site, usually recommending two capsules per day.

In vitro human digestion model

The in vitro human digestion model used was the one developed by the National Institute for Public Health and the Environment (RIVM, Bilthoven, the Netherlands). Figure 2.2 presents a schematic overview of this digestion model described in detail by Oomen et al. [29]. In accordance with this publication, different digestion juices (i.e. saliva, gastric, duodenal and bile juice) were prepared 1 day before the experiment. Three hundred milligrams of a selected isoflavone supplement (containing genistin, daidzin and glycitin at levels quantified (see Results)) without capsule were placed in a 30 mL glass tube and 6 mL of saliva juice (pH 6.0 ± 0.5) were added. After a quick shaking a 0.1 mL sample was collected immediately. The mixture was then rotated with an upside-down rotor for 5 min at 40 rpm at 37^0C. After this another 0.1 mL sample was collected and subsequently 12 mL of gastric juice were added and the pH was adjusted to 1.5 ± 0.5 with 37 % HCl. Then the mixture was placed in the rotor for 2 h at the same speed and temperature, followed by collection of again 0.1 mL sample. Finally, 12 mL of duodenal juice and 6 mL of bile juice were added to the mixture, the pH was adjusted to 6.0 ± 0.5 with 37 % HCl and the sample was placed in the rotor for another 2 h. After that, the last sample of 0.1 mL was collected. Before starting the experiment, all the digestive juices were pre-incubated at 37 ± 2^0C and the incubations were performed at the same temperature. Collected samples were stored at -80^0C until analysis by UPLC.

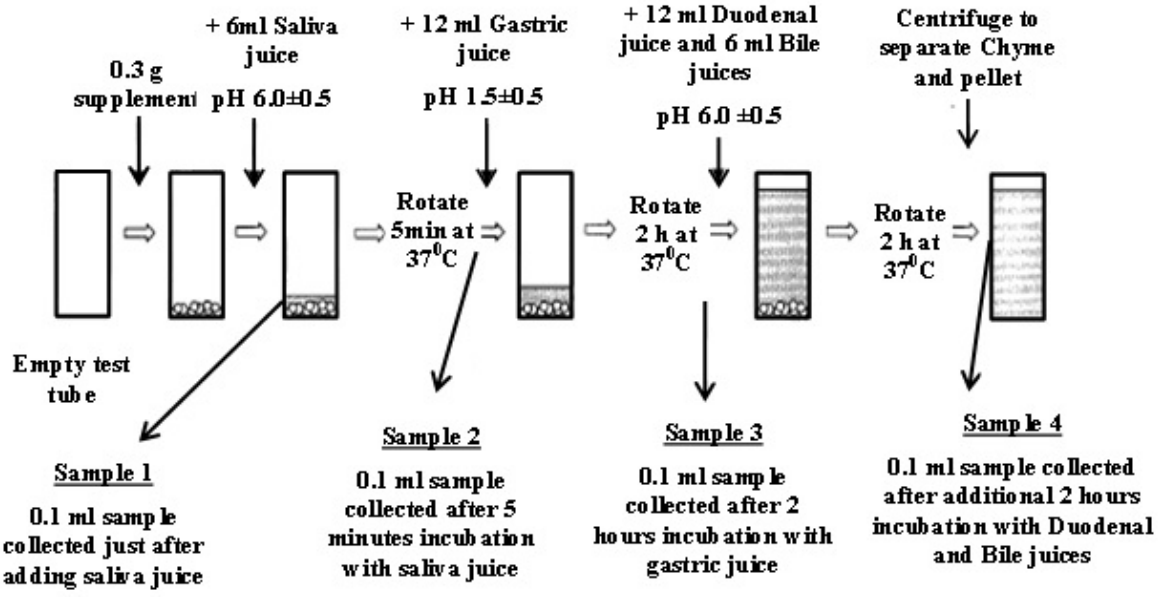


Figure 2.2: Schematic presentation of the different steps of the human in vitro digestion model, indicating the collection of the different samples to be analyzed including samples representing the situation 1) after ingestion, 2) after passing the oral cavity, 3) after passing the stomach and 4) after passing the intestinal compartment, respectively (amended from Omen et al. [29]).

Intestinal Caco-2 cell model

Caco-2 cells from ATCC (Manassas, VA, USA) were cultured in a humidified atmosphere of 5% CO_2 at 37^0C in Dulbecco's modified eagle's medium (DMEM) supplemented with 10% fetal bovine serum, 1% minimum essential media nonessential amino acids and 0.1% mg/mL gentamicin as described by Brand et al. [30]. For transport experiments, 1×10^5 cells/well were seeded from a stock of 2×10^5 cells/mL in a Corning Costar 12-well transwell plate with an insert membrane. This insert membrane has a pore size of 0.4 μm and growth area of 1.12 cm^2. During formation of the monolayer, the medium was changed three times a week and 18 or 19 days post-seeding the experiments were performed. The passage number of the cells used in the experiments was between 45 and 51. Before exposure, monolayers were washed with DMEM (without phenol red) and the integrity of the monolayers was checked by measuring transepithelial electrical resistance (TEER) values using a Millicell ERS volt/ohmmeter from Millipore (Bedford, MA). Only monolayers demonstrating a TEER value between 500 and 1000 $\Omega\cdot cm^2$ were used. Transport experiments were carried out with medium consisting of DMEM (without phenol red) supplemented with 1% (v/v) minimum essential media nonessential amino acids. To all media 1 mM ascorbic acid (final concentration) was added to prevent auto-oxidation of the isoflavones.

Cells were exposed to 50 µM of the isoflavone glucosides and/or their corresponding aglycones at the apical side and 100 µL sample was collected at eight time points (0, 10, 20, 40, 60, 80, 100 and 120 min) both from the apical and the basolateral compartments. A complementary volume (i.e. 100 µL growth medium containing 50 µM soy isoflavones) was added every time only in the apical compartment just after sample collection. In all experiments, the concentration of DMSO was kept < 0.5 %. Collected samples were kept on ice during the experiment and subsequently stored at -80^{0}C until analysis by UPLC. At the end of the experiment, the TEER values were checked again to confirm the integrity of the monolayers.

Enzymatic deconjugation of isoflavone glucosides by rat and human intestinal S9

To obtain the kinetic constants for deconjugation of the isoflavone glucosides, incubation mixtures (total volume 100 µL) contained 100 mM potassium phosphate (pH 7.0), increasing concentrations of substrate (1 to 200 µM for genistin and daidzin and 1 to 500 µM for glycitin, respectively) all added from 200 times concentrated stock solutions in DMSO, and 0.15, 0.40 or 0.10 mg protein/mL rat intestinal S9 or 0.13, 0.40 or 0.10 mg protein/mL human intestinal S9 for genistin, daidzin, and glycitin, respectively. Incubations of genistin, daidzin and glycitin with rat intestinal S9 were performed for 15, 10 and 40 min, respectively and with human intestinal S9 for 10, 10 and 15 min, respectively. The reactions were terminated by adding equal volumes of ice-cold methanol containing 0.8 mM ascorbic acid to stabilize the sample as described by Day et al. [24]. To identify the role of different glucosidases present in intestinal S9 fractions, incubations were also performed in the presence of specific inhibitors including 250 µM NB-DGJ, 5 mM conduritol B epoxide and 10 mM ẟ-gluconolactone that were all used at concentrations selected based on literature information [24, 31, 32]. Table 2.1 presents an overview of the different enzyme inhibitors used and their specificity for the intestinal enzymes at the concentrations generally used in literature. Kinetic parameters were measured in duplicate.

Table 2.1: Different enzyme inhibitors with their specific inhibition potency for various intestinal enzymes as obtained from literature.

Inhibitor	Exposure conc. (mM)	Inhibited enzyme(s)	% inhibition	Reference
NB-DGJ	0.25	LPH	60	[31]
	10	Glucocerebrosidase	51	[32]
Conduritol B epoxide	5	Glucocerebrosidase	100	[24]
	5	LPH	87	[31]
	6.20	Glucocerebrosidase	100	[32]
Gluconolactone	10	BSβG	93	[24]
		Glucocerebrosidase	51	[24]

Enzymatic conjugation of isoflavone aglycones by rat and human intestinal and liver S9

To study glucuronidation of aglycones, incubation mixtures (total volume 99.5 µL) were prepared containing (final concentrations) 10 mM $MgCl_2$, 25 µg/mL alamethicin added from a 200 times concentrated stock solution in methanol and 10 mM uridine 5-diphosphoglucuronic acid trisodium salt in 50 mM Tris-HCl (pH 7.4) [33]. The reactions were started by adding 0.5 µL of the substrate genistein, daidzein or glycitein (final concentration ranges were between 0.5 to 400 µM added from 200 times concentrated stock solutions in DMSO) to incubation mixtures containing 0.05, 0.25, or 0.25 mg protein/mL rat intestinal S9, respectively, and incubated for 15, 10 or 10 min, respectively, with rat liver S9 were carried out using 0.5, 0.5, or 0.02 mg protein/mL for 5, 5 or 3 min, respectively. Incubations of genistein, daidzein and glycitein were carried out with 0.05, 0.25, or 0.25 mg protein/mL human intestinal S9 for 10, 10 or 10 min, respectively and with 0.5, 0.5, or 0.5 mg protein/mL human liver S9 for 10, 10 or 10 min, respectively. The reactions were terminated by adding 25 µl ice-cold ACN. Under these conditions, metabolite formation was linear with time and with the amount of protein added (data not shown). All incubations were conducted at 37^0C and activities were expressed in nmol min^{-1} mg $protein^{-1}$. Samples were stored at -80^0C until analysis by UPLC. Kinetic parameters were measured in duplicate.

To study sulfation of the aglycones, incubation mixtures (total volume 99.5 µL) contained (final concentrations) 100 µM 3'-phosphoadenosine 5'-phosphosulfate in 50 mM potassium phosphate (pH 7.4) containing 5 mM $MgCl_2$ and S9 fractions. The reactions were started by addition of 0.5 µL genistein, daidzein or glycitein (final concentrations ranging from 0.25 to 400 µM added from 200-fold concentrated stock solutions in DMSO) to incubation mixtures containing 0.5 mg protein/mL rat intestinal S9 and incubated for 90, 120, 30 min, respectively. Incubations with rat liver S9 were carried

out using 0.2, 0.2, 0.1 mg protein/mL for 60, 15, 5 min, respectively. Incubation of genistein, daidzein and glycitein was also carried out with 0.2, 0.2 and 0.5 mg protein/mL human intestinal S9 for 30, 30 and 10 min, respectively and with 0.2, 0.2 and 0.2 mg protein/mL human liver S9 for 10, 30 and 10 min, respectively. These reactions were terminated by adding 25 µL ice cold ACN. Under these conditions, metabolite formation was linear with time and with the amount of protein added (data not shown). Samples were stored at -80^{0}C until analysis by UPLC.

UPLC analysis for identification and quantification of isoflavones

All samples were analysed using a Waters Acquity^{TM} UPLC, (Milford, MA, USA) that consists of a binary solvent manager, sample manager and photodiode array detector, equipped with a Waters Ethylene Bridged Hybrid (BEH) C18 1.7 µm 2.1 × 50 mm column (Waters, Ireland). Nanopure water with 0.1% TFA and 100 % ACN were used as solvent A and B, respectively. After centrifugation at 16.000 × *g* for 4 min, samples were analysed. In case of samples collected from supplement extraction and the in vitro human digestion model, an extra filtration step (0.2 µm filter, VWR, PA, USA) was performed before UPLC analysis. The injection volume for UPLC analysis was 3.5 µL and the flow rate was 0.6 mL/min. Elution was started with 0% of solvent B followed by an increase in % of solvent B from 0 to 10, 15, 50 and 80% at 0.58, 2.85, 4.28 and 4.40 min, respectively. The 80% solvent B condition was kept until 4.52 min, and thereafter the % of solvent B was reduced to 0 %at 4.63 min and maintained at that percentage until 5.80 min. Photodiode array spectra were detected between 200-360 nm and chromatograms acquired at 260 nm were used for quantification of the amount of isoflavone glucosides and aglycones using calibration curves of their commercially available reference compounds. Using the above mentioned UPLC conditions the retention times of the different test compounds were as follows; genistein 3.71 min (UV_{max} 260 nm); daidzein 3.36 min (UV_{max} 249 nm); glycitein 3.46 min (UV_{max} 257 and 319.8 nm); genistin 2.56 min (UV_{max} 259.4 nm); daidzin 1.76 min (UV_{max} 249 and 301.9 nm) and glycitin 1.92 min (UV_{max} 257.6 and 320.4 nm). The 7-O-glucuronides of genistein and daidzein are commercially available and the monoglucuronides were quantified based on calibration curves made with these available standards. The other monoglucuronide and monosulfate conjugates were quantified using the standard curve of their corresponding glucosides. This was possible because the 7-O-glucuronides of genistein and daidzein were shown to have similar UV spectra and calibration curves as their corresponding glucosides (data not shown).

Analysis of kinetic parameters

The maximum velocity (V_{max}), expressed in nmol/min/mg S9 protein, and Michaelis-Menten constant (K_m) expressed in micromoles, were determined by fitting the data to the Michaelis-Menten equation: $v = V_{max} * [S] / (K_m + S)$, with [S] being the concentration of the substrate, using Graphpad Prism (version 5.02) from Graphpad Software (San Diego, CA, USA). Based on the kinetic constants

obtained, the catalytic efficiency (V_{max}/K_m), expressed as mL/min/mg S9 protein or µL/min/mg S9 protein, for the different reactions was calculated.

Results

Isoflavone content in commercial soy supplements

Since we aimed at investigating the species differences between rat and human for conversion of isoflavones from soy supplements as a possible important source for current dietary exposure in the Western world, nine different supplements were randomly chosen from the local market. Figure 2.3a and 3b present the UPLC chromatograms of two analysed commercial supplements. These chromatograms reveal that the isoflavone content and composition of different supplements varies considerably. Supplement number 1 (Figure 2.3a) contains mainly the three major soy isoflavone glucosides daidzin, glycitin and genistin, whereas supplement no 5 (Figure 2.3b) contains only small amounts of daidzin and genistin and relatively larger amounts of their corresponding aglycones daidzein and genistein along with several minor unidentified ingredients. Figure 2.3c presents the overall isoflavone content determined for the different soy supplements compared to the amount claimed to be present on the labels or on the specified websites. For all supplements, except for supplement 1, the actual amounts detected appeared to be significantly lower than the amounts expected based on information provided by the supplier. Based on these results supplement number 1 was selected as the supplement to be used in the subsequent in vitro human digestion model study since it appeared to contain all three model isoflavone glucosides in substantial amounts.

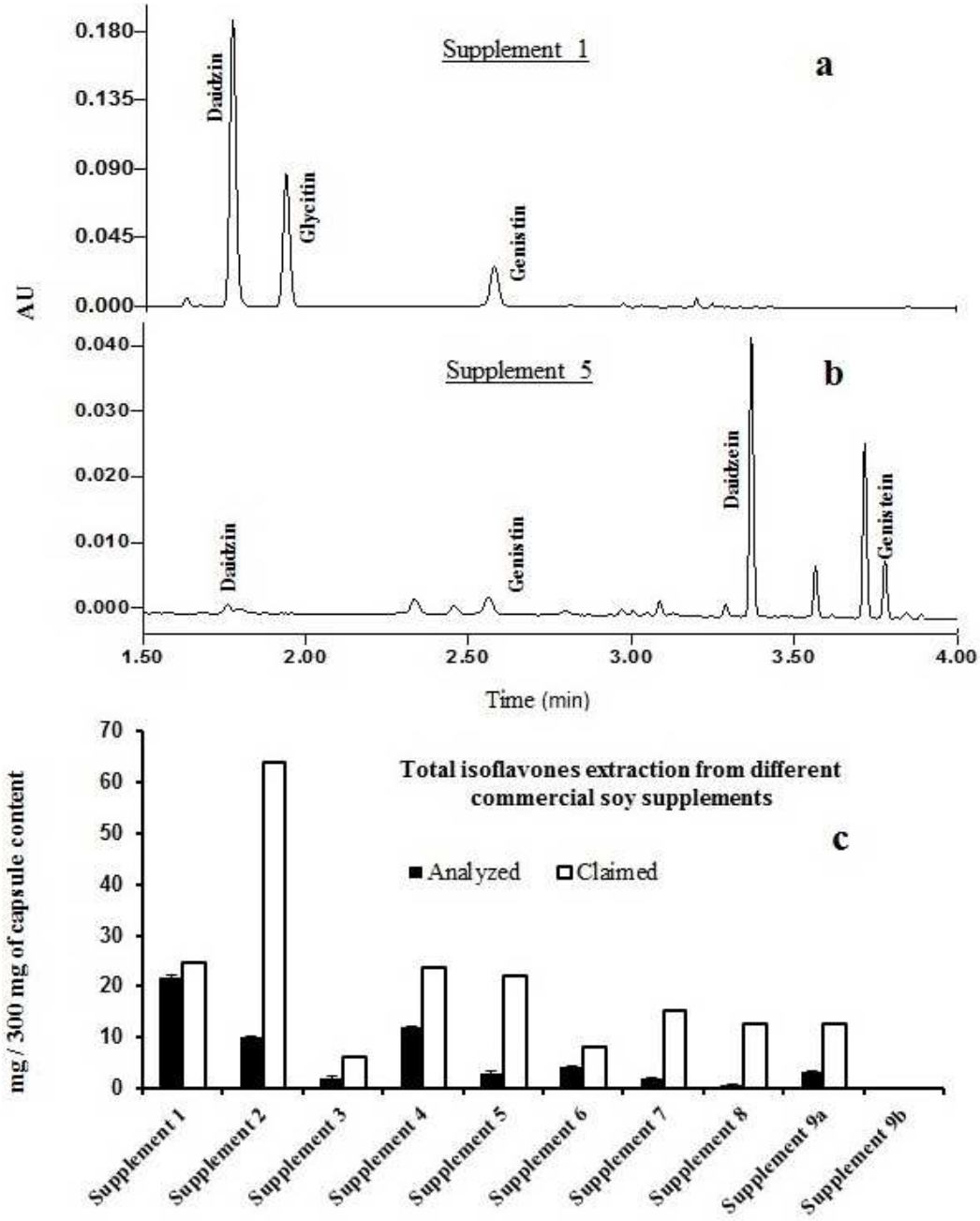


Figure 2.3: Representative UPLC chromatograms of commercially available supplements containing especially (a) isoflavone glucosides or (b) aglycones, and (c) a comparison of the total isoflavone content (i.e. glucosides and aglycones) as detected in different commercial soy supplements and the amounts claimed to be present in the manufacturer's information. The values are given in mg/300mg capsule content (each data point represents the average value of three independent experiments).

Deconjugation of soy isoflavone glucosides in an in vitro human digestion model

Table 2.2 shows the amount of total isoflavones (i.e. glucosides plus aglycones) and aglycones detected in different samples from the human digestion model. The results obtained reveal that incubation with different human model digestion juices does not convert the isoflavone glucosides to their respective aglycones to any significant extent, not even after 2 h incubation at low gastric pH (1.5 ± 0.5). No deconjugation was detected even when the experiment was repeated with modified gastric juices that did not contain BSA, mucin and pepsin, thereby eliminating the possible protection against deconjugation by these proteins.

Table 2.2: Amount of isoflavones in mg (total i.e. glucosides plus aglycones and aglycones detected in the human digestion model, as detected in A) a digestion model with normal gastric juice and B) a digestion model with modified gastric juice (without BSA, mucin and pepsin). The values for total isoflavones and aglycones are presented in mg per 300 mg of commercial supplement.

Type of gastric juice	Sample no	Situation represented	Incubation time	Total isoflavones (mg)	Aglycones (mg)
A	1	just after oral ingestion	0	12.7 ± 1.3	0.35
	2	after passing oral cavity	5 min	13.2 ± 3.5	0.26
	3	after passing stomach	2 h	14.6 ± 3.5	0.35
	4	after passing small intestine	2 h	16.1 ± 6.1	0.34
B	1	just after oral ingestion	0	13.6 ± 3.2	0.4
	2	after passing oral cavity	5 min	12.8 ± 2.8	0.4
	3	after passing stomach	2 h	14.9 ± 2.9	0.4
	4	after passing small intestine	2 h	15.2 ± 2.9	0.5

Transport of isoflavones in a Caco-2 cell model

In a next step, it was investigated to what extent the soy isoflavone glucosides would be able to pass, at least to some extent, the intestinal barrier themselves. This was done by using a Caco-2 cell monolayer two-compartment transwell system, an in vitro model often applied to study transport across the intestinal barrier [30, 33, 34]. As can be seen in Figure 2.4, no transport of the soy isoflavone glucosides across the Caco-2 monolayer was observed, whereas addition of the corresponding aglycones to the apical side of the monolayers resulted in a time dependent increase in the amount of these isoflavone aglycones in the basolateral compartments. No metabolite formation was observed when Caco-2 cells were exposed to the aglycones (UPLC chromatograms not shown). The transport efficiency of three aglycones decreased in the order genistein > daidzein > glycitein, with the amount of genistein transported after 120 min being 2.3 and 4.1 fold higher than that of daidzein and glycitein, respectively.

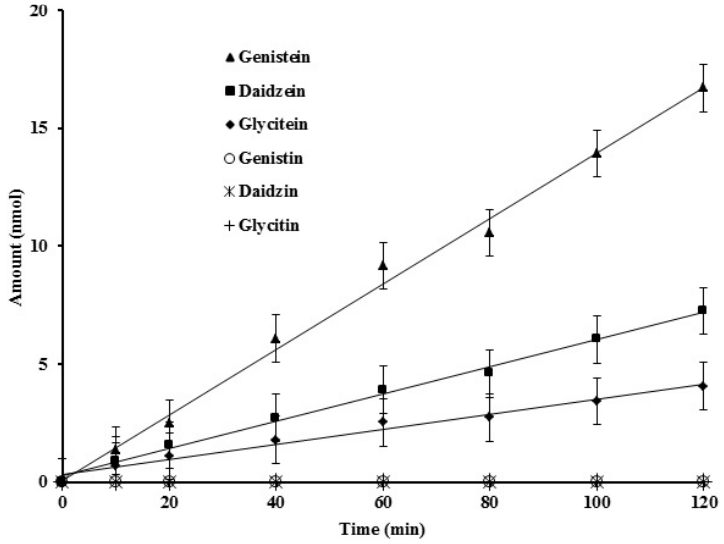


Figure 2.4: Time dependent increase of the amount (nmol) of three soy glucosides and the corresponding aglycones in the basolateral compartment of a Caco-2 transwell culture experiment in which the cells were exposed to the isoflavones (50 µM) at the apical side. Data points represent the mean of triplicate determinations ± standard deviation.

Enzymatic deconjugation of soy isoflavone glucosides by rat and human intestinal S9

Since naturally existing soy isoflavone glucosides were not deconjugated by the acidic stomach pH (see Section 3.2) and also were not able to cross the Caco-2 monolayer barrier (see Section 3.3), human and rat intestinal S9 fractions were tested for their soy isoflavone glucoside deconjugating ability. Table 2.3 depicts the kinetic parameters (V_{max} and K_m) for deconjugation by rat and human intestinal S9 as well as the catalytic efficiencies (V_{max}/K_m) derived from these values. Compared to the catalytic efficiencies for deconjugation by rat intestinal S9, catalytic efficiencies for deconjugation by human intestinal S9 were 3.9 and 6.5 fold higher for genistin and daidzin and almost similar for glycitin.

To investigate possible further differences between rat and human intestinal deconjugation of the soy isoflavone glucosides, inhibition of the reaction by selected glucosidase inhibitors (Table 2.1) was investigated. Figure 2.5 shows the inhibition of the dose-dependent deconjugation of the isoflavone glucosides in the absence and presence of the different inhibitors. The results presented point at a

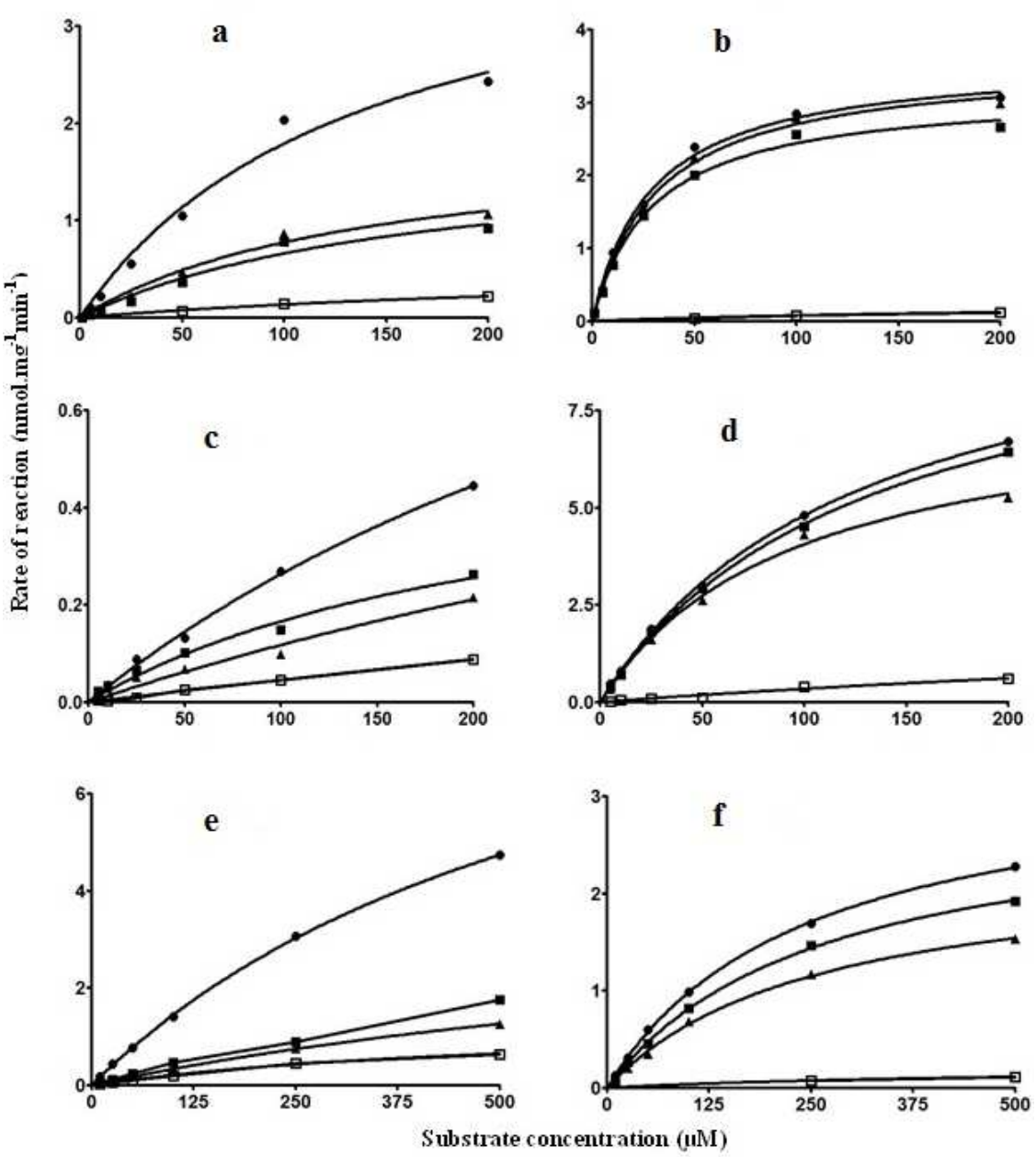


Figure 2.5: Dose dependent deconjugation of genistin (upper panel), daidzin (middle panel) and glycitin (lower panel) glucosides after incubation with rat intestinal S9 (left panel) and human intestinal S9 (right panel) in the absence (●), or presence of the following inhibitors: 250 µM NB-DGJ (▲) as a lactase domain selective inhibitor of LPH; 5 mM of Conduritol B epoxide (■) as an inhibitor of both glucocerebrosidase and LPH; 10 mM gluconolactone ( ) as an inhibitor of cytosolic BSβG and glucocerebrosidase.

significant species dependent difference between rat and human with regard to the type of enzymes involved in deconjugation. Figure 2.5a and 5b show that deconjugation of genistin by rat intestinal S9 was inhibited by all inhibitors tested, whereas in incubations with human intestinal S9 especially

gluconolactone inhibited the deconjugation. For the inhibition of the deconjugation of daidzin (Figure 2.5c and 2.5d) and glycitin (Figure 2.5e and 2.5f) similar species dependent differences were observed. These results suggest that in human intestinal S9 samples especially broad-specificity β–glucosidase (BSβG) is involved in the deconjugation of the isoflavone glucuronides, whereas in rat intestinal S9 there is an additional contribution from lactase phlorizin hydrolase (LPH) and/or glucocerebrosidase.

Evaluation of the use of intestinal S9 as a model for intestinal deconjugation

Given that part of the glucosidases present in intestinal S9 are intracellular enzymes with only LPH being located on the brush border of the enterocytes [31, 35], it was of interest to evaluate the possible overestimation of the level of deconjugation of isoflavone glucosides obtained when using the cell-free S9 samples. To obtain some insight in this possible overestimation the in vitro catalytic efficiency for deconjugation of genistin by rat intestine S9 was compared to the catalytic efficiency calculated based on literature studies reporting intestinal perfusion data for the same compound. To allow comparison the in vitro catalytic efficiency of 0.036 mL/min/mg S9 protein (Table 2.3) was first scaled to the whole organ by multiplying with an S9 protein yield of 11.4 mg S9/g intestine [36, 37]. At organ level the catalytic efficiency for deconjugation is then 0.4 mL/min/g intestine. In three published perfusion studies with genistin [19, 38, 39] the clearance or catalytic efficiency of the intestine (CE expressed as mL/min) for deconjugation was calculated based on the reported inlet (Cin expressed as µmol/mL) and outlet concentrations (Cout expressed as µmol/mL) of genistin and the applied perfusion flow rate (Qp expressed as mL/min) with the following equation [40-42] :-

$$CE = Q_p\,(C_{in} - C_{out})/C_{in}$$

In this equation, the fraction of genistin that does not appear in the outlet ($(C_{in}-C_{out})/C_{in}$) is considered to completely correspond to the fraction of genistin that is deconjugated, since loss of genistin between the inlet and outlet due to possible uptake is also assumed to reflect deconjugation given that the aglycone is the preferential form for uptake [19, 23, 38]. In a study of Andlauer et al. [38] small intestines of male Sprague Dawley rats were ex vivo perfused with a flow rate of 0.5 mL/min. Experiments were performed at three different inlet concentrations of 5.9, 12.0, and 23.8 µmol/L genistin. The reported outlet concentrations of genistin were 2.4, 7.0, and 10.2 µmol/L, respectively. Based on these data deconjugation catalytic efficiencies were calculated to range from 0.2 to 0.3 mL/min for the three different inlet concentrations. In a study reported by Steensma et al. [39] the small intestines of male Wistar rats were in situ perfused with a flow rate of 1 mL/min at an inlet concentration of 55 µmol/L genistin. The average reported outlet concentration of genistin was 26 µmol/L, resulting in a deconjugation catalytic efficiency of 0.5 mL/min. In the study of Liu and Hu [19] different segments of the small intestines of male Sprague Dawley rats were separately perfused in situ with a flow rate of 0.191 mL/min and the reported inlet concentration of genistin was 100 µmol/L and outlet concentrations of genistin were 43, 67 and 87 µmol/L for duodenum, jejunum and

ileum, respectively. Based on these values the sum of the deconjugation efficiency by the different intestinal segments can be calculated to be 0.2 mL/min. Overall, the calculated deconjugation efficiencies for the in situ and ex vivo perfusion studies are comparable ranging from 0.2 to 0.5 mL/min for the whole small intestine corresponding to 0.06 to 0.14 mL/min/g intestine (calculated based on an average small intestine weight of 3.5 g for rats [43]). The scaled catalytic efficiency for deconjugation as determined with the incubations with rat intestine S9 fractions that amounted to 0.4 mL/min/g intestine, was three to sevenfold higher than these ex vivo and in situ perfusion values.

Table 2.3: Kinetic parameters for deconjugation of three soy isoflavone glucosides as determined in in vitro incubations using human or rat intestine S9 fractions.

Source of enzyme	Glucosides	K_m (μM)	V_{max} (nmol min^{-1} mg $protein^{-1}$)	V_{max}/K_m (μL min^{-1} mg $protein^{-1}$)
rat intestine S9	Genistin	133.3 ± 7.8	4.8 ± 0.8	36.6
	Daidzin	507.2 ± 77.2	2.0 ± 0.6	3.8
	Glycitin	656.3 ± 6	11.2 ± 0.4	17.1
human intestine S9	Genistin	28.7 ± 0.5	4.1 ± 0.7	142.5
	Daidzin	114.4 ± 21.0	2.8 ± 0.0	24.7
	Glycitin	185.1± 79.5	3.1 ± 0.4	18.1

Based on the incubations with the different inhibitors of glucosidase enzymes, an estimation can be made of the contribution of LPH to the catalytic efficiency for deconjugation by rat intestine S9. From Figure 2.5a it can be derived that the maximum deconjugation activity of genistin is about 60% lower in the presence of NB-DGJ, which inhibits LPH (Table 2.1). This suggests that 60% of the total S9 catalytic efficiency of 0.036 mL/min/mg S9 protein (Table 2.3) may be attributed to LPH activity, which corresponds to ~0.022 mL/min/mg S9 protein or 0.25 mL/min/g intestine when scaled from S9 to the intestine. This latter value is quite comparable to the catalytic efficiencies derived from the data in literature on in situ and ex vivo perfusion studies ranging from 0.06 to 0.14 mL/min/g intestine. This confirms that the primary site for deconjugation is at the brush border in a reaction catalyzed by LPH. In incubations with human intestine S9 (Figure 2.5b) the maximum deconjugation of genistin is only about 16% lower in the presence of NB-DGJ. Combining this result with the catalytic efficiency observed in incubations with human intestine S9 of 0.14 mL/min/mg S9 protein (Table 2.3), one could calculate that the deconjugation efficiency by LPH is ~0.022 mL/min/mg S9 (i.e. 16% of the total deconjugation catalytic efficiency of 0.14 mL/min/mg S9) or 0.25 mL/min/g intestine when scaled from S9 to the intestine. This suggests that despite a relative lower contribution of LPH to

deconjugation, the absolute deconjugation efficiency by LPH may still be comparable between humans and rats.

Enzymatic conjugation of isoflavone aglycones by rat and human intestinal and liver S9

Table 2.4 presents the different kinetic parameters of glucuronidation by small intestinal and liver S9 for both rat and human. Formation of 7-O- as well as 4'-O-glucuronide metabolites was observed, although for both rat and human intestine and liver catalytic efficiency for formation of the 7-O-glucuronides was generally one to two orders of magnitude higher than that for formation of 4'-O-glucuronides. This was due to lower K_m in combination with higher V_{max} values and indicates that formation of 7-O-glucuronides is preferred over formation of 4'-O-glucuronides in both rat and human. Furthermore the catalytic efficiencies for the production of 7-O-glucuronides by rat intestinal S9 were 10.1, 2.3 and 2.4 fold higher than those for human intestinal S9 and those for rat liver S9 were 1.4, 4.9 and 11.7 fold higher than those for human liver S9 and genistein, daidzein and glycitein, respectively. This suggests glucuronidation to be more efficient in rat than in human first pass metabolism, although the differences seem small and are generally less than one order of magnitude.

Table 2.4: Apparent V_{max} and K_m values of aglycones and catalytic efficiencies (V_{max}/K_m) derived from these values for glucuronidation as determined in in vitro incubations using human and rat, small intestinal and liver S9 fractions.

Source of enzyme	Aglycones	7-O-glucuronide			4'-O-glucuronide		
		K_m (µM)	V_{max} (nmol min^{-1} mg $protein^{-1}$)	V_{max}/K_m (µL min^{-1} mg $protein^{-1}$)	K_m (µM)	V_{max} (nmol min^{-1} mg $protein^{-1}$)	V_{max}/K_m (µL min^{-1} mg $protein^{-1}$)
Rat intestine S9	Genistein	2.8 ± 2.8	3.6 ± 0.1	1290	26.4 ± 18.1	0.15 ± 0.01	6
	Daidzein	7.1 ± 2.8	1.3 ± 0.1	190	49.2 ± 22.3	0.15 ± 0.02	3
	Glycitein	3.0 ± 1.4	3.2 ± 0.6	1070	ND	ND	ND
Rat liver S9	Genistein	51.6 ± 3.0	5.1 ± 1.3	100	38.1 ± 2.8	0.3 ± 0.1	7
	Daidzein	5.6 ± 3.7	1.4 ± 0.3	250	77.7 ± 14.5	0.3 ± 0.1	3
	Glycitein	1.8 ± 1.5	5.3 ± 4.3	2980	ND	ND	ND
Human intestine S9	Genistein	5.8 ± 6.7	0.7 ± 0.7	130	18.9 ± 12.3	0.2 ± 0.1	11
	Daidzein	2.7 ± 1.2	0.2 ± 0.1	80	2.9 ± 1.1	0.1 ± 0.1	40
	Glycitein	3.4 ± 1.5	1.5 ± 0.5	450	4.2 ± 0.5	0.1 ± 0.01	17
Human liver S9	Genistein	22.2 ± 15.1	1.5 ± 0.7	70	31.9 ± 18.5	0.1 ± 0.01	3
	Daidzein	18.9 ± 14.1	1.0 ± 0.0	50	72.1 ± 61.1	0.2 ± 0.01	2
	Glycitein	7.5 ± 5.6	1.9 ± 0.4	250	64.3 ± 22.5	0.1 ± 0.02	1

* ND, not detectable because of substrate inhibition at higher concentrations and too low conversion at lower concentrations

Upon incubation of the isoflavone aglycones with rat or human intestinal or liver S9 and 3-phosphoadenosine 5-phosphosulfate formation of only 7-O sulfate metabolites was observed (Table 2.5) with human intestinal and liver S9 showing higher catalytic efficiencies than the corresponding rat S9 fractions. The catalytic efficiencies for sulfation by human intestinal S9 were 558 and 1.3 fold higher than those of rat intestinal S9 to conjugate daidzein and glycitein, while for human liver S9 they were 29, 2.2 and 18 fold higher than those of rat liver to conjugate genistein, daidzein and glycitein, respectively. Due to substrate inhibition at higher concentrations and too low conversion at lower concentrations, the kinetic parameters of genistein sulfation by human intestinal S9 fraction could not be quantified.

Table 2.5: Apparent V_{max} and K_m values of aglycones and catalytic efficiencies (V_{max}/K_m) derived from these values for sulfation as determined in in vitro incubations using human and rat, small intestinal and liver S9 fractions (based on results from single experiments).

S9 fractions	aglycones	K_m µM	V_{max} nmol min^{-1} mg $protein^{-1}$	V_{max}/K_m µL min^{-1} mg $protein^{-1}$
Rat Intestine S9	Genistein	35.44	0.01	0.28
	Daidzein	98.17	0.01	0.10
	Glycitein	1.56	0.01	7
Rat Liver S9	Genistein	33.04	0.13	4
	Daidzein	1.82	0.02	11
	Glycitein	10.17	0.20	20
Human Intestine S9	Genistein	ND	ND	ND
	Daidzein	0.35	0.02	57
	Glycitein	16.88	0.13	8
Human Liver S9	Genistein	0.44	0.05	114
	Daidzein	0.82	0.02	24
	Glycitein	0.23	0.08	348

* ND, not detectable because of substrate inhibition at higher concentrations and too low conversion at lower concentrations.

Discussion

Knowledge on the conversion of soy isoflavones is crucial to understand and evaluate their possible health benefits and risks. Furthermore, quantification of possible species differences in kinetics of isoflavones is essential for adequate extrapolation of animal experimental data to the human situation. In the present study different in vitro models were used to investigate the conversion of the major soy isoflavones and possible species differences between rat and human in this conversion. Apart from genistin and daidzin, an important soy isoflavone glycitin was included in the studies because

information on the absorption and metabolism of this isoflavone is still lacking. Thus the present study provides a comparative investigation of metabolic characteristics in rat and human of the three major soy isoflavones and their corresponding aglycones in a series of in vitro models.

Before starting the metabolism studies, a series of commercially available soy supplements was analyzed for their isoflavone content to select a suitable supplement to be tested in the human digestion model and to obtain some insight in the nature of the isoflavones generally present in this possibly important dietary source of isoflavones in the Western diet. To this end, nine isoflavone supplements were collected from the local market (Wageningen, the Netherlands) and analysed for their isoflavone content and composition. Results obtained reveal that eight out of nine supplements analyzed did not contain the amount of isoflavones as indicated on the labels or relevant websites. This illustrates that it is difficult to predict the exposure or intake by a target population based on the information presented by the suppliers. The calculated estimated daily intake of total isoflavones (glucosides plus aglycones) calculated based on the analytical results presented ranges from 17 to 88 mg per day which is in accordance with estimates presented by Eisenbrand et al. [44]. Several other groups also noticed such unexpected low yields compared to the labelled specifications for isoflavone supplements [2, 26, 28]. The results also indicated variation in the relative level of isoflavone glucosides or aglycones in the supplements, a factor that may affect bioavailability since plasma serum levels upon intake of genistein and daidzein have been shown to be much higher than those upon intake of the corresponding glucosides [45-47]. Based on the results obtained, the soy supplement with high levels of the major three soy isoflavone glucosides was selected to be tested in the human digestion model.

The in vitro human digestion model used was the model developed by the National Institute for Public Health and the Environment [29]. The results obtained clearly indicate that digestive juices applied are not able to deconjugate the isoflavone glucosides. This finding indicates that although concentrated acid hydrolysis at elevated temperature ($\geq 80^0$C) is frequently used in analytical methods for quantification of isoflavones, physiologically relevant acidic pH values combined with a physiologically relevant temperature (37^0C) are not sufficient to deconjugate the isoflavone glucosides. Our results also indicate that the 70% deconjugation of genistin after 90 min incubation with human saliva reported by Allred et al. [25] may be due to prolonged incubation time (90 min instead of the physiologically relevant 5 min incubation) in combination with the microbial activity present in the saliva juice applied in their in vitro digestion model.

Since isoflavone glucosides are thus likely to end up intact in the small intestine their transport over an in vitro intestinal barrier model was investigated using the Caco-2 transwell model. Isoflavone glucosides were not able to cross the Caco-2 monolayer whereas the corresponding aglycones were

translocated in a time-dependent manner. These results are in line with other studies reporting that initial hydrolysis is essential for absorption of related flavonoid glucosides [23, 34, 45]. The present study reveals differences in transport efficiency of the three major soy isoflavone aglycones, with transport decreasing in the order genistein > daidzein > glycitein. The transport of genistein is 2.3 and 4.1 fold faster than that of daidzein and glycitein, respectively. This difference between translocation of genistein and daidzein across the Caco-2 model layer is in line with the about 1.3 to 3.0 fold difference in rat plasma metabolite concentrations of genistein and daidzein upon dosing 7.9 μmol isoflavone aglycones per kg bw to rats [45] and with the about 1.3 fold faster transport of genistein compared to daidzein after 2 h incubation of Caco-2 cell monolayers with 50 μM isoflavone aglycones as reported by Steensma et al. [48]. The Caco-2 cells appeared unable to deconjugate the isoflavone glucosides and/or to metabolize the isoflavone aglycones to their corresponding glucuronide and sulfate metabolites, confirming the absence of LPH and phase II conjugating enzymes in these cells as reported before [19, 31]. Liu en Hu [19] and also Chen et al. [49] studied the transport of isoflavone glucosides or aglycones using cloned Caco-2 TC7 cells . Chen et al. [49] reported transport of especially isoflavone aglycones. Liu and Hu [19] reported limited transport of the glucoside genistin to the basolateral side, whereas in our studies this transport was not observed. This difference might be due to the fact that the studies of Liu and Hu [19] used the cloned Caco-2 TC7 cell line isolated from a late passage of the parental Caco-2 cells to provide a more homogeneous population, somewhat different pH values and longer incubation times whereas they did not report the TEER values of their monolayer after the prolonged 4 h incubation, leaving the possibility that the limited transport observed may have been due to some loss in monolayer integrity. Results from other studies using parental Caco-2 cells are in line with our results reporting that glucosides are not transported across the Caco-2 monolayer to any significant extent [34, 48]. Our results are also in line with human data reporting that isoflavone glucosides are not absorbed as such across the enterocytes of healthy adults and that initial hydrolysis by intestinal enzymes is essential for their transport across the enterocytes [46].

Given that isoflavone glucosides will not be hydrolyzed before entering the small intestine deconjugation by rat and human intestinal samples was investigated. There is evidence in literature that some flavonoid glucosides can be hydrolyzed in the small intestine [23, 50]. They may be hydrolyzed by LPH, located in the brush border of the small intestine [31, 35] and/or by a BSβG and/or glucocerebrosidase upon entering the intestinal epithelial cells via specific transporters [24, 51, 52]. In subsequent experiments of the present study incubations with intestinal S9 fractions from human and rat were used to study the deconjugation of the isoflavone glucosides in the absence or presence of specific inhibitors of LPH, glucocerebrosidase and BSβG (Table 2.1). The results obtained point at a significant species dependent difference between rat and human, since incubations with rat intestinal samples reveal inhibition by all inhibitors tested, whereas in incubations with human

intestinal S9 predominantly gluconolactone inhibited the deconjugation almost completely. This indicates that in human intestinal S9 samples especially intracellular BSβG is involved in deconjugation, whereas in rat intestinal S9 there is a significant contribution from LPH and glucocerebrosidase. This finding together with the outcome of the Caco-2 study might lead to the conclusion that in human, deconjugation is largely carried out by gut microbiota as reported in the literature [17, 20]. However, Walsh et al. [23] reported that isoflavonoid glucosides are deconjugated and absorbed in the small intestine of human subjects with ileostomies, indicating that deconjugation can take place in the small intestine where the microbial contribution is very limited.

To further evaluate the appropriateness of the use of cell free S9 samples to study intestinal deconjugation, the catalytic efficiency for deconjugation calculated based on our in vitro data was compared to the in vivo catalytic efficiencies calculated based on data from intestine perfusion studies reported in literature. This comparison revealed that in vitro S9 clearance as determined by the intestinal S9 rat incubations results in three- to seven fold overestimation compared to intestinal perfusion studies. The presence of all cytosolic and membrane-bound enzymes in intestinal S9 fractions (http://biofocus.com/offerings/adme-pk-laboratory/s9fraction) might be the cause of this overestimation in the in vitro model since in the perfusion studies glucocerebrosidase and BSβG may only be able to deconjugate glucosides to a limited extent since in this model they can only contribute after intracellular uptake of the isoflavone glucosides by specific transporters.

The results from subsequent conjugation studies revealed that glucuronidation of these isoflavone aglycones, especially at the 7-hydroxyl moiety, is the main metabolic route for glucuronidation. This efficient conjugation explains the low bioavailability of the free aglycones as reported in several studies [46, 53, 54] and indicates that 7-O-glucuronides are the major metabolites to be expected in the systemic circulation, with 4'-O-glucuronides being the least important isoflavone glucuronide expected in the systemic circulation. The additional contribution of sulfotransferases to the phase II metabolism of the isoflavone aglycones (see Table 2.5) will contribute to the conjugation of the isoflavone aglycones with relatively lower catalytic efficiencies than the glucuronidation [55, 56]. In this aspect it is also of interest to notice that in vivo upon prolonged circulation of the isoflavones also combined metabolites are formed including 7-glucuronide 4-sulfate metabolites that have been reported to be found as major metabolites in human plasma after administration of a traditional Japanese roasted soy product called Kinako that contains considerable amount of soy isoflavone aglycones [53, 57]. This confirms that the 7-position is the preferred site for glucuronidation. Relatively modest species differences in the plasma levels of glucuronides and sulfates for rats and humans are in line with literature data reporting the proportion of isoflavone glucuronide and sulfates conjugates in serum of women and female rats after consumption of dietary containing soy protein isolate [58]. Finally, comparison of the catalytic efficiencies for deconjugation and conjugation reveals

that deconjugation is less efficient than conjugation corroborating that isoflavones in their unconjugated form are unlikely to enter the systemic circulation. This is in line with the relatively low percentage of isoflavones in plasma present in the aglycone form [46, 53, 58]. Altogether the data point at possible differences in characteristics for intestinal conversion of the major soy isoflavones between rat and human, especially with respect to their deconjugation.

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3

Deconjugation of soy isoflavone glucuronides needed for estrogenic activity

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Abstract

Soy isoflavones (SIF) are present in the systemic circulation as conjugated forms of which the estrogenic potency is not yet clear. The present study provides evidence that the major SIF glucuronide metabolites in blood, genistein-7-O-glucuronide (GG) and daidzein-7-O-glucuronide (DG), only become estrogenic after deconjugation. The estrogenic potencies of genistein (Ge), daidzein (Da), GG and DG were determined using stably transfected U2OS-ERα, U2OS-ERβ reporter gene cells and proliferation was tested in T47D-ERβ cells mimicking the ERα/ERβ ratio of healthy breast cells and in T47D breast cancer cells. In all assays applied, the estrogenic potency of the aglycones was significantly higher than that of their corresponding glucuronides. UPLC analysis revealed that in U2OS and T47D cells, 0.2-1.6% of the glucuronides were deconjugated to their corresponding aglycones. The resulting aglycone concentrations can account for the estrogenicity observed upon glucuronide exposure. Interestingly, under similar experimental conditions, rat breast tissue S9 fraction was about 30 times more potent in deconjugating these glucuronides than human breast tissue S9 fraction. Our study confirms that SIF glucuronides are not estrogenic as such, and that the small % of deconjugation in the cell is enough to explain the slight bioactivity observed for the SIF-glucuronides. Species differences in deconjugation capacity should be taken into account when basing risk-benefit assessment of these SIF for the human population on animal data.

Introduction

Isoflavones are naturally occurring dietary compounds mostly found in soy. Due to their structural similarity with the natural hormone estradiol, they have weak estrogenic potencies and are referred to as phytoestrogens [1-3]. Many studies reported positive relationships between soy consumption and several important health benefits like lower risks for breast cancer and heart disease, less hot flushes and nocturnal sweating, improved bone density and cognitive health [4-6]. Peri- and post-menopausal women with a family history of breast cancer are a sub-population in Western society that increasingly is considered vulnerable for unregulated and self-administrated consumption of dietary soy supplements [7-9]. Despite their beneficial effects for human health and large societal acceptance by consumers, potential adverse effects as suggested in in vitro and in vivo experiments, and in clinical trials, raise questions about the safety of high dosages of phytoestrogens [10-13]. However, most of the in vitro estrogenicity experiments used biologically active aglycones, which generally make up <1-2% of the total isoflavone levels present in blood [14-16]. Glucuronides (Figure 3.1) are the predominant metabolites reported in blood plasma (around 75%, depending on the species studied), followed by sulfates (24%) and aglycones (<1%) [14-16]. This means that estrogen sensitive tissues will predominantly be exposed to conjugated metabolites via the systemic circulation.

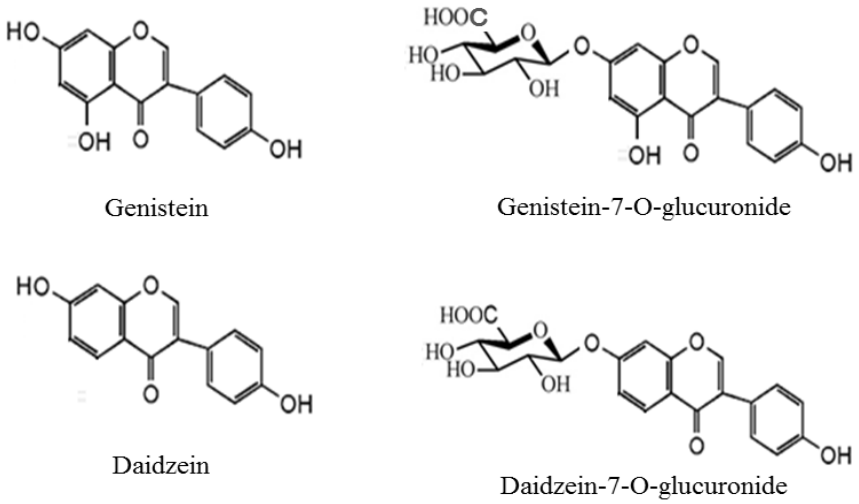

Figure 3.1: Molecular structure of genistein, daidzein and their corresponding 7-O-glucuronides, the commercially available and predominant metabolites in the systemic circulation.

The hydrophilic nature of these circulating glucuronide metabolites probably retards their cellular uptake, unless mechanisms exist for uptake and/or hydrolysis of these conjugates. Furthermore, it is not clear whether these glucuronides are biologically active or not. For example, Bolca et al. [17] concluded from a human intervention study that after consumption of dietary supplements rich in soy

isoflavone (SIF), 98% of SIF reached the breast tissue as glucuronide, whereas Yuan et al. [18] using different breast cancer cell lines concluded that glucuronides are not active themselves. Some studies also suggested that these glucuronides act as precursor of bioactive aglycones and are bioactivated in the target tissues [15, 19, 20]. In estrogen sensitive tissues, the estrogenic responses are mediated by estrogen receptors (ERs) of which an alpha and beta form exist. The relative occurrence of ERα and ERβ is tissue dependent, for example, ERα is predominant in the mammary gland, epididymis, testis, uterus, kidney and pituitary gland whereas more ERβ than ERα is present in the prostate, bladder and lungs [21-24]. In certain types of breast cancer, the ERα/ERβ ratio is significantly increased compared to healthy tissue [25, 26], mainly due to a decrease of the ERβ levels [27, 28]. It has been shown that ERα activation stimulates cell proliferation in breast tissue, whereas ERβ is involved in inhibition of proliferation and stimulation of apoptosis [25, 29, 30]. Phytoestrogens such as genistein have been shown to activate the two ERs with a greater relative potency compared to estradiol (E2) for ERβ than for ERα [30, 31]. As a consequence the potency of aglycones such as Ge or Da to suppress or induce cell proliferation depends on the specific ratio between ERα and ERβ present in the cells studied. However, in the reported in vitro studies, no attention was paid to use ERα and ERβ ratios reflecting the ratios present in specific estrogen sensitive tissues. In the present study an in vitro cell model was used where in T47D-ERβ breast cancer cells the genetically enhanced ERβ levels could be suppressed by tetracycline to achieve an ERα/ERβ ratio which according to Evers et al. [21] is similar to that in normal human breast tissue. This is important, given that due to differences in ERα/ERβ ratios, the effect of estrogen active compounds on inhibition or stimulation of cell proliferation can vary significantly between different estrogen sensitive tissues and cell models. In addition, also the metabolic forms in which the SIF are present in the systemic circulation and the respective tissues will determine their ultimate biological effect. Giving the relevance of deconjugation as bioactivation step in tissues, it is important to know the capacity for deconjugation of glucuronides by breast tissue as a target organ as well as species specific differences in deconjugation capacity. The aim of the present study was to investigate to what extent SIF glucuronide metabolites are estrogenic themselves or need to be deconjugated to the corresponding biologically active aglycones to become estrogenic, and to study to what extent normal rat and human breast tissue is able to carry out this deconjugation of the SIF glucuronides. To address these research questions the U2OS-ERα and U2OS-ERβ reporter gene assays, and T47D wild type (wt) and T47D-ERβ proliferation assays were used to quantify the estrogenicity of selected SIF glucuronides and their aglycones. Genistein-7-O-glucuronide (GG) and daidzein-7-O-glucuronide (DG) were selected as model compounds because they are commercially available and occur quite predominantly in the systemic circulation [14, 15]. The production of SIF aglycones in the cell culture experiments was determined as well.

Materials and methods

Chemicals

17β-estradiol (E2), genistein (Ge), daidzein (De) (with purity>99%) were purchased from LC Laboratories (Woburn, USA). Genistein-7-O-glucuronide (GG) and daidzein-7-O-glucuronide (DG) were obtained from Extrasynthese (Genay, France). Dimethyl sulfoxide (DMSO (>99%) was purchased from Acros Organics (Geel, Belgium). Geneticin (G418) and fetal calf serum (FCS) (Australian origin, 1099) were purchased from Invitrogen Life Technologies (Paisley, Scotland). Dextran-charcoal-treated FCS (DCC-FCS, SH30068.05) was obtained from Perbio Science NV (Etten-Leur, the Netherlands). Non-essential amino acids (NEAA-100x, 11140-035), phosphate-buffered saline (PBS) (without Ca^{2+} and Mg^{2+}) and all the culture media were supplied by Gibco (Paisley, Scotland). Trypsin 0.25 g/100 mL in PBS was obtained from Difco (Detroit, USA). Sodium hydroxide (NaOH), ethylenedinitrotetraacetic acid ($EDTA.2H_2O$; Titriplex), magnesium sulphate ($MgSO_4.7H_2O$), 37% HCl, and KCl were purchased from Merck (Darmstadt, Germany). Magnesium carbonate ($(MgCO_3)4Mg(OH)_2.5H_2O$), phenyl methyl sulfonyl fluoride (PMSF) were obtained from Aldrich (Saint Louis, MO, USA) and dithiothreitol (DTT), Tris from Sigma (Steinheim, Germany). *Trans*-1,2-diaminocyclohexane-N,N,N′,N′-tetraacetic acid monohydrate (CDTA) and glycerol was obtained from Fluka (Buchs, Switzerland). Hygromycin and D-luciferin were obtained from Duchefa (Haarlem, the Netherlands). Trifluoro acetic acid (TFA) was purchased from VWR International (Darmstadt, Germany) and acetonitrile (ULC/MS) was purchased from Biosolve BV (Valkenswaard, Netherlands). The 5-bromo-2'-deoxyuridine (BrdU) kit (colorimetric, 11647229001) was obtained from Roche Diagnostics (Mannheim, Germany) and the Pierce BCA protein assay kit (product number 23227) from Thermo Scientific (Rockford, IL, USA). All other cell culture reagents from Gibco (Paisley, UK). Pooled SD male rat liver S9 was obtained from BD Biosciences (MA, USA; batch 88875).

Cell culturing

The human osteosarcoma (U2OS) cell lines stably expressing ERα or ERβ, linked to the 3xERE-tata-luciferase gene, were provided by the Hubrecht Institute, Utrecht. They were cultured and used as described before [31]. Briefly cells were cultured in a 1:1 mixture of F12 and DMEM (31331-028) supplemented with 7.5% FCS and 0.5% NEAA. U2OS-ERα growth medium was supplemented with selective antibiotics geneticin (200µg/mL) and hygromycin (50µg/mL), while U2OS-ERβ growth medium was supplemented only with geneticin (200µg/mL) as selection antibiotic. U2OS-ERα cells were used in passages 10-25 and U2OS-ERβ cells in passages 12-29 for the reporter gene assay and analysis of metabolite formation.

T47D human breast cancer cells (T47D-wt) were purchased from the American Type Culture Collection (Manassas, VA, USA) and cultured in a 1:1 mixture of F12 and DMEM (31331-028) supplemented with 5% FCS. T47D-wt cells were used in passages 10-21 for the cell proliferation assay and analysis of metabolite formation.

The T47D-ERβ cell line, stably transfected to display tetracycline-dependent ERβ-expression was provided by Ström et al. [29] and used as described before [30]. These cells were also grown in a 1:1 mixture of F12 and DMEM (31331-028) supplemented with 5% FCS plus 100 ng tetracycline/mL. With this amount of tetracycline the ERα/ERβ ratio becomes comparable with that of normal human breast tissue based on Western blot analysis carried out by Evers et al. [21]. The cells were reselected (with 0.5 µg/mL puromycin) every 10 passages (about 3 weeks) to prevent loss of ERβ and its related expression of enhanced green fluorescent protein (EGFP) activity [30]. For cell proliferation experiments, cells were incubated in a 1:1 mixture of DMEM and F12 without phenol red, supplemented with 5% DCC-FCS, 0.5% NEAA and the amount of tetracycline needed to obtain the desired ERα/ERβ ratio (100 ng/mL to mimic normal human breast cells).T47D-ERβcells were used in passages 33-49 for the cell proliferation assays.

Reporter gene assays

Reporter gene assays were performed using human osteosarcoma cells (i.e. U2OS-ERα and U2OS-ERβ). These cells are devoid of endogenous ERs but have been transfected with either any of the ER and stably express 3xERE-tata-Luc [32]. As a result, the amount of luciferase produced is a direct indication of the amount of estrogen-mediated gene expression via ERs [31, 33]. These two cell lines are ideal models for measuring ER-isotype specific transcriptional activity. Reporter gene assays were performed using the method described before [30, 31]. In brief, 80-90% confluent ERα/β-U2OS-Luc cells were washed with PBS, trypsinized and transferred to phenol-red free assay medium, and seeded in the inner 60 wells of a 96-well plate at 100 µl/well at a density of 10^5cells/mL (ERα-U2OS-Luc) or 7.5×10^4 cells/mL (ERβ-U2OS-Luc). Plates were incubated at 37˚C and 5% CO_2. The assay medium was renewed after 24h of incubation. At 48h, 100 µl of assay medium containing the test compounds (E2, Ge, Da, GG or DG at indicated concentrations) were added. All test compounds were dissolved in DMSO and the final concentration of DMSO was 0.4% in each well. Compounds were tested in triplicate; outer rows of the 96-well plate were filled with 200 µl of sterile PBS to prevent evaporation from the inner 60-wells of the plate. The exposure concentrations were 0.03-400 µM for both U2OS-ERα and U2OS-ERβ cells. After 24h exposure, cells were washed with 100 µl diluted PBS (1/2 × PBS in demineralised water) which was subsequently replaced by 30 µl of a hypotonic low-salt buffer (10mM Tris-HCl pH 7.8 containing 2mM DTT and 2mM CDTA). To lyse the cells the plate was then placed on ice for 10 minutes to allow swelling and subsequently frozen at -80^{0}C for at least 30

minutes. Before measuring, the plates were thawed and briefly shaken until reaching room temperature. Using a luminometer (Thermo LabSystems Luminoskan Ascent) estrogen-mediated luciferase production in the ERα-U2OS-Luc and ERβ-U2OS-Luc cells was measured at room temperature as described before [31].

Cell proliferation assay

As functional measure of estrogenicity, the estrogen-dependent cell proliferation of T47D-wt breast cancer cells and T47D-ERβ cells was used. The T47D-ERβ cells with tetracycline dependent inhibition of expression of ERβ allowed mimicking the ERα/ERβ ratio of normal rat and human breast tissue[21]. The cell proliferation assay was performed using the method described by Sotoca et al. [34]. In brief, 80-90% confluent T47D cells were washed with PBS, trypsinized and transferred to phenol-red free assay medium supplemented with 5% DCC-FCS. Cells (1.8 x 10^5 cells/mL) were seeded in the inner 60 wells of a 96-well plate at 100 µl/well and incubated overnight at 37˚C plus 5% CO_2. The assay medium was renewed after 24h of incubation. At 48h, 100 µl of assay medium containing the test compounds in DMSO (final conc. 0.4%) was added. Compounds were tested in triplicate and outer rows of the 96-well plate were filled with 200 µl of sterile PBS. After 48h of exposure of cells with different concentrations of SIF, cell proliferation was determined following the BrdU Roche's colorimetric protocol using a multi-mode microplate reader (SpectraMax® M2). Based on the DNA synthesis, the BrdU method provides an indirect and precise indication of cell proliferation.

Measuring deconjugation of glucuronides in cell models

Exposure of the cells in phenol red-free assay medium was performed according to exposure protocols mentioned earlier in the cell proliferation assay section, with the exception that 24 well plates were used, and that exposurewas limited to 20 µM and 400 µM GG or DG for 24h. After the exposure, the medium was collected and cells were washed with PBS. Then cells were harvested and lysed in cold65% aqueous methanol [35]. All samples were stored at -20°C until analysis.

Preparation of rat and human breast tissue S9 fractions

As breast tissue S9 fractions of rat or human are not commercially available, these S9 fractions were prepared from available tissue samples. Four samples of anonymous human breast tissue medically judged to be normal were kindly provided by the Maastricht University Medical Center under ethical approval. Rat breast tissues were pooled from 5 control female F344 rats in a study approved by the Animal Welfare Committee of Wageningen University (Wageningen, the Netherlands). Frozen breast tissue samples (about 1.0 ± 0.2g of human or 0.23 g of pooled rat breast tissue) were thawed in a Petri dish on ice and cut into small pieces. The tissues were placed in a 12 mL Greiner tube and immersed

with three times the tissue volume of ice-cold 100 mM Tris-HCl (pH 7.4) containing 20% glycerol, 150 mM KCl, 1 mM EDTA, 0.2 µM DTT and 0.2 mM PMSF. The buffer was stored at -20^{0}C because of the limited stability of DTT and PMSF. Homogenization was done with an Ultra-Turrax T25 homogenizer (Janke & Kunkel IKA® Labortechnik, Germany**)** followed by centrifugation (Sigma 4K10, Osterode, Germany) at 9000g for 15 min at 4^{0}C. After removing the fat layer from the top, the supernatant was collected as subcellular S9 fractions and stored at -80^{0}C in small aliquots until use. Taking one aliquot of each S9 sample, the protein concentration was determined with a Pierce BSA protein assay kit (Thermo Scientific, Rockford, USA). As commercial human or rat breast tissue S9 is not available, we validated our S9 preparation procedure by comparing the deconjugation activity of the S9 fraction we prepared from F344 ovariectomized rat liver with that of commercially available SD male rat liver S9 (supplementary document 1).

Glucuronide deconjugation by breast tissue S9 fractions

Rat and human breast tissue S9 fractions were incubated with GG and DG to study their deconjugation potency. To this end 40 µM of each glucuronide was incubated for 24h at 37^{0}C with 0.1 mg/mL S9 protein. The reaction was started by adding 0.4 µl of GG or DG from a 200 times concentrated stock solution (prepared in DMSO) in 99.6 µl of 100 mM Tris-HCl (pH 7.4) buffer (total volume 100 µl), and terminated by adding 25µl ice-cold acetonitrile. Samples were analyzed immediately.

UPLC analysis of SIF glucuronides and aglycones

All samples of the deconjugation experiments were analysed using a Waters Acquity^{TM} UPLC (Ultra Performance Liquid Chromatography system; Milford, MA, USA) which consists of a binary solvent manager, sample manager and photodiode array (PDA) detector, equipped with a Water Ethylene Bridged Hybrid (BEH) C18 1.7 µm 2.1 × 50 mm column (Water, Ireland). Nanopure water with 0.1% TFA and 100% acetonitrile were used as solvent A and B, respectively. After thawing and centrifugation at 13,000 × *g* for 5 min at 4^{0}C, samples were pipetted (70-80 µl) in special UPLC vials and placed in a plate sampler. The injection volume for UPLC analysis was 3.5 µl and the flow rate was 0.6 mL/min. Elution was started with 0% solvent B followed by an increase of solvent B from 0 to 10%, 15%, 50% and 80% at 0.58, 2.85, 4.28 and 4.40 min, respectively. The 80% solvent B condition was kept until 4.52 min, thereafter the % of solvent B was reduced to 0% at 4.63 min and maintained at that percentage until 5.80 min. PDA spectra were analysed between 200-360 nm and the chromatograms acquired at 260 nm were used for quantification of the amount of isoflavone aglycones and glucuronides using calibration curves of commercially available reference compounds. Using the above mentioned UPLC conditions the retention times of different test compounds were as follows: genistein 3.78 min (UV_{max} 260 nm); daidzein 3.41 min (UV_{max} 249 nm); GG 2.81 min (UV_{max} 259.4 nm) and DG 1.96 min (UV_{max} 249 nm).

Data analysis

The plate to plate variation in the E2-control responses within and between experiments (<1.4 fold difference) was corrected based on the response of 10 pM E2 for U2OS-ERα and 200 pM E2 for U2OS-ERβ cells. Plate to plate variation for the proliferation assays with T47D-wt and T47D-ERβ cells was corrected based on the response induced by the vehicle control (0.4% DMSO) which was set at 100% proliferation. Dose-response curves were fitted using Graphpad Prism version 5.02, Graphpad Software (San Diego, CA). The relative light units (RLUs) in every well of the U2OS-Luc reporter gene assays were standardised by setting the response of the vehicle control (DMSO 0.4%) at 0% induction and the maximum luciferase induction obtained by E2 at 100%. The concentration of the test chemical eliciting transcriptional activity equivalent to 10% of the positive control value, was defined as PC10 and that equivalent to 50% as PC50 [36]. For the more potent compounds like Ge and Da, also the PC50 values were determined. Estradiol equivalency factors (EEF) were calculated based on the PC10 or PC50 values as EEF(PC10) = PC10(E2)/PC10 (compound) and EEF(PC50) = PC50(E2)/PC50 (compound). Unless stated otherwise, the effect concentrations were calculated from three independent experiments in triplicate.

Results

Estrogenic potency of aglycones and glucuronides

Figure 3.2 shows the dose response curves for the estrogenic activity of genistein (Ge), daidzein (Da) and their corresponding 7-O-glucuronides (GG and DG) in the U2OS-ERα and U2OS-ERβ reporter cell lines together with E2. The potency order was the same in both the U2OS-ERα and U2OS-ERβ cell line, namely E2 > Ge > Da > GG > DG (Table 3.1). The effective concentration of E2 was lower in the U2OS-ERα than in theU2OS-ERβ, whereas for the SIF this was the opposite. This result is in line with other reports [30, 31, 37]. The estradiol equivalency factor (EEF) values for PC10 and PC50 obtained with both cell lines (Table 3.1) show that Ge was 5-9 fold more potent than Da, and GG was 10-18 fold more potent compared to DG. The SIF and their corresponding glucuronides yield a higher maximum activity than E2 (Table 3.1), which in both ER-transfected cells was up to >200% of the maximum induction by E2.

Figure 3.2

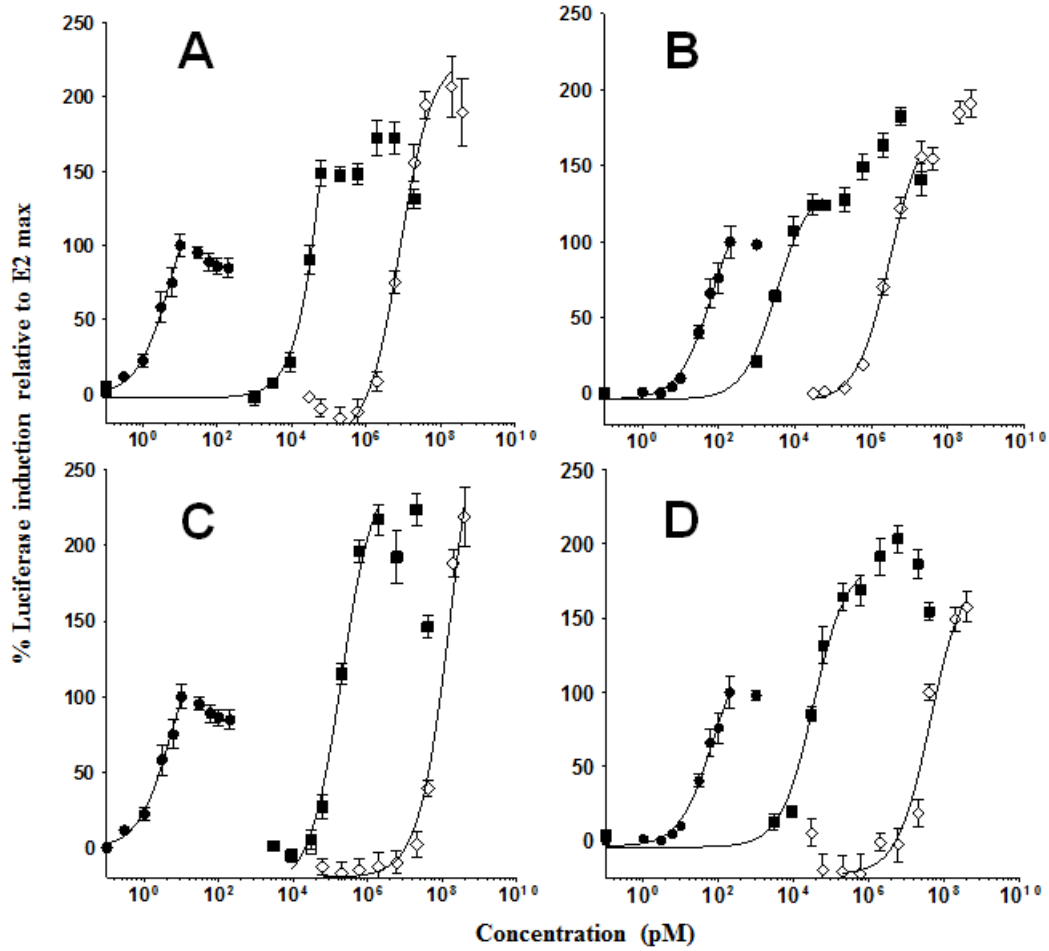

Figure 3.2: Induction of ERE-mediated Luc activity in the U2OS-ERα (A and C) and U2OS-ERβ (B and D) cells upon exposure to increasing concentrations of E2 (•), Ge (■) and GG (◊) (A and B) and of E2 (•), Da (■) and DG (◊) (C and D). Induction was expressed relative to the maximum E2 response set at 100%. Data points represent the mean of triplicate determinations ± standard deviation.

Table 3.1: The estrogenic potency of genistein (Ge), genistein-7-O-glucuronide (GG), daidzein (Da) and daidzein-7-O-glucuronide (DG) compared to E2 in the U2OS-ERα and U2OS-ERβ cells. Also the concentrations for maximum estrogenic effect and the maximum induction relative to E2 (max set at 100%) are presented.

	Compound	PC10*	EEF **at PC10	PC50*	EEF **at PC50	Conc. giving maximum level of induction (µM)	Maximum induction relative to E2 (%)
U2OS ERα	E2	0.4 pM	1	2.6 pM	1	$1*10^{-5}$	100
	Ge	2.9 nM	1.4E-04	16.2 nM	1.6E-04	6.3	172
	GG	1.3 µM	3.1E-07	3.9 µM	6.7E-07	400	206
	Da	25.7 nM	1.6E-05	75.9 nM	3.4E-05	73	224
	DG	12.6 µM	3.2E-08	38.9 µM	6.7E-08	400	219
U2OS ERβ	E2	7.6 pM	1	40.7 pM	1	$2*10^{-4}$	100
	Ge	0.4 nM	1.9E-02	1.9 nM	2.1E-02	6.0	182
	GG	0.3 µM	2.5E-05	1.3 µM	3.1E-05	400	191
	Da	3.2 nM	2.4E-03	13.8 nM	3.0E-03	6.0	203
	DG	5.5 µM	1.4E-06	22.9 µM	1.4E-06	400	157

* PC10 and PC50 = The concentration of the test chemical eliciting transcriptional activity equivalent to 10% and 50% of the positive control value in the U2OS-ERα and U2OS-ERβ reporter cell line.

**EEF= estradiol equivalency factor calculated as PC10(E2)/PC10 (compound) and EEF(PC50) = PC50(E2)/PC50 (compound).

Proliferation effects of soy isoflavones (aglycones and glucuronides)

Figure 3.3 presents the dose-dependent induction of cell proliferation in the T47D-wt human breast cancer cell line, by the glucuronides and their corresponding aglycones, compared to estradiol (E2). Interestingly, low concentrations of compounds consequently reduced the proliferation compared to that of the DMSO control, and at higher exposure concentrations the proliferation increases in a dose-related manner. Low concentrations of the SIF reduce the proliferation more than E2, and at high concentrations the maximum proliferation induced by the aglycones is higher than that of E2 (Table 3.2).

Figure 3.3

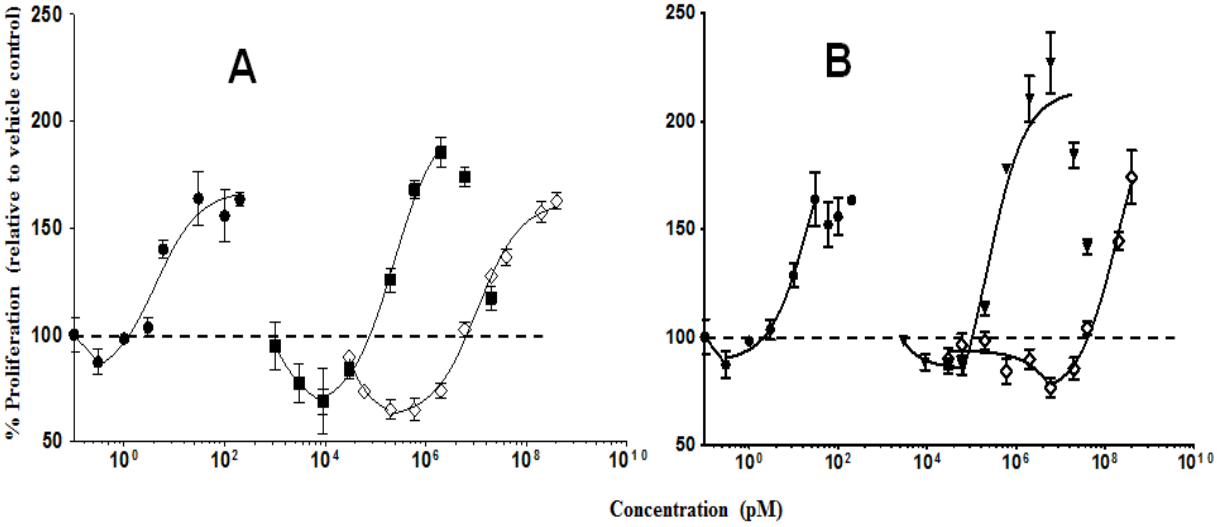

Figure 3.3: Proliferation effect of E2 (•), Ge (■) and GG (◊) (Figure 3.3A) and E2 (•), Da (▼) and DG (◊) (Figure 3.3B) in T47D-wt type cells. Data are presented after normalization with respect to the proliferation of control cells set at 100%. Data points represent the mean of triplicate determinations ± standard deviation.

Table 3.2: Potency for induction of cell proliferation by genistein (Ge), genistein-7-O-glucuronide (GG), daidzein (Da) and daidzein-7-O-glucuronide (DG) compared to E2 in T47D-wt cells. Also the concentrations inducing maximum proliferation and the maximum proliferation to the vehicle control (DMSO 0.4%) set at 100% are presented.

Compound	PC10*	EEF** at PC10	PC50*	EEF** at PC50	Conc. of maximum proliferation (µM)	Maximum proliferation relative to vehicle control (%)
E2	0.8 pM	1	4.2 pM	1	$2*10^{-5}$	163
Ge	60.3 nM	1.3E-05	191 nM	2.2E-05	2	185
GG	7.4 µM	1.1E-07	21.4 µM	2.0E-07	400	162
Da	61.7 nM	1.3E-05	186 nM	2.3E-05	6	227
DG	32.4 µM	2.5E-08	107 µM	3.9E-08	400	174

* PC10 and PC50 = The concentration of the test chemical eliciting proliferation equivalent to 10% and 50% of the positive control value in the T47D-wt cells.

**EEF= estradiol equivalency factor calculated as PC10(E2)/PC10 (compound) and EEF(PC50) = PC50(E2)/PC50 (compound).

The proliferation potencies, expressed as estradiol equivalency factors (EEF), of Da and Ge in the T47D-wt cells, were similar, but the potency of GG was about a factor of 5 higher than that of DG.

Figure 3.4 presents the dose-dependent increase in proliferation of T47D-ERβ cells with an ERα/ERβ ratio mimicking the ERα/ERβ ratio in normal rat and human breast tissue by addition of 100nM

tetracycline [21]. Again, low concentrations of the SIF reduced the proliferation as compared to E2, and at high concentrations the maximum proliferation induced by the aglycones is higher than that of

E2 (Figure 3.4 and Table 3.3), although the differences were less obvious than with the T47D-wt cells. All the aglycones and their glucuronides show lower proliferation potency (higher PC10 values) compared to E2. The proliferation potency of Da expressed as EEF, was 6-29 fold higher than that of Ge. This difference was not observed in T47D-wt cells and in the reporter gene assay (Table 3.3). As with the reporter gene assays, the glucuronides were about 100-fold less potent than their respective aglycones (Table 3.2 and 3.3).

Figure 3.4

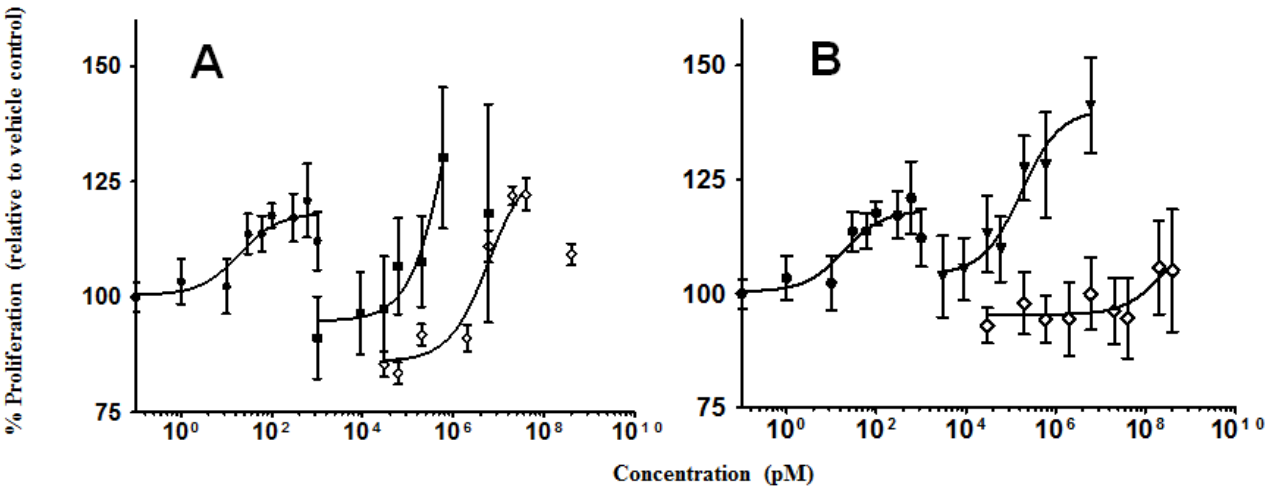

Figure 3.4: Proliferation effect of E2 (•), Ge (■) and GG (◊) (Figure 3.4A) and E2 (•), Da (▼) and DG (◊) (Figure 3.4B), in T47D-ERβ cells when exposure medium contains 100 ng/mL tetracycline to mimic the ERα/ERβ ratio in normal breast tissue. Data are presented after normalization with respect to the proliferation of control cells set at 100%. Data points represent the mean of triplicate determinations ± standard deviation.

Table 3.3: Potency for induction of cell proliferation by genistein (Ge), genistein-7-O-glucuronide (GG), daidzein (Da) and daidzein-7-O-glucuronide (DG) compared to E2 in T47D ERβ cells. Also the concentrations inducing maximum proliferation and the maximum proliferation to the vehicle control (DMSO 0.4%) set at 100% are presented.

Compound	PC10*	EEF** at PC10	PC50*	EEF** at PC50	Conc. giving maximum level of proliferation (µM)	Maximum proliferation relative to vehicle control (%)
E2	1.6 pM	1	24.5 pM	1	$6*10^{-5}$	120
Ge	81.3 nM	2.0E-05	200 nM	1.2E-04	0.6	131
GG	3.0 µM	5.3E-07	7.8 µM	3.1E-06	40	122
Da	2.8 nM	5.7E-04	35.5 nM	6.9E-04	6	141
DG	126 µM	1.3E-08	>400 µM	>4.0E-09	400	105

* PC10 and PC50 = The concentration of the test chemical eliciting proliferation equivalent to 10% and 50% of the positive control value in the T47D-wt cells.

**EEF= estradiol equivalency factor calculated as PC10(E2)/PC10 (compound) and EEF(PC50) = PC50(E2)/PC50 (compound).

In vitro deconjugation study

a) Cell lines

The UPLC chromatograms (Figure 3.5 a- c) show the deconjugation of GG to Ge as detected by analysis of the cell culture medium and the intracellular content after incubation for 24h with U2OS (ERα or ERβ) and T47D-wt cells. Incubation of 400 µM GG results in the formation of 2.4 – 6 µM of Ge (0.6 - 1.5%). A similar deconjugation pattern was found when incubated with DG (data not shown). T47D-wt cells were 1-3 fold less efficient in deconjugation of glucuronide metabolites than U2OS (ERα or ERβ) cells. The chromatograms reveal that glucuronide metabolites remain the predominant compounds (i.e. ≥ 99%) and upon incubation with the cells only a small portion (maximum about 1.6%) of the SIF glucuronides were converted to the corresponding aglycones (Table 3.4). At similar incubation conditions the concentration of genistein produced was 4-7 fold higher compared to that of daidzein. A small portion (0.1-0.2% i.e. 0.1 to 0.7 µM) of glucuronide conjugates was detected in the intracellular extract, where no aglycones were detected (Figure 3.5 and Table 3.4).

Incubation of 20 µM of GG or DG SIF with the same cell lines yielded comparable results, where the glucuronide conjugates were the predominant compounds (~98%) and the deconjugated aglycones were hardly detectable (<2%), close to the limit of detection (0.1µM) (data not shown).

Figure 3.5

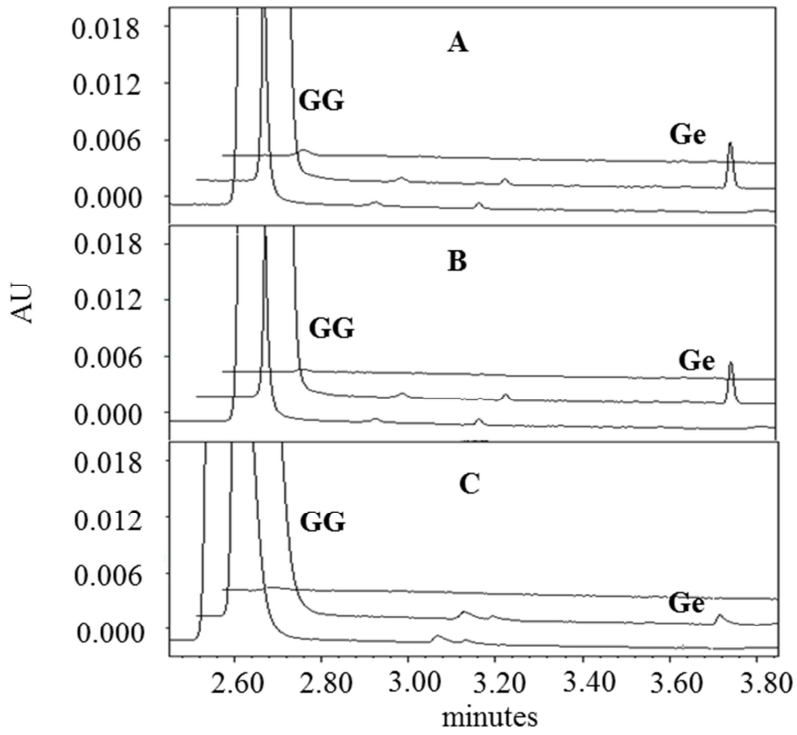

Figure 3.5: UPLC chromatograms of culture medium and intracellular content after incubations of GG with **A**) U2OS-ERα, **B**) U2OS-ERβ and **C)** T47D-wt cells. Chromatogram sequences (front to back) are- GG at T0; GG and Ge at T24 h; and intracellular GG at T24 h.

Table 3.4: Deconjugation of 400 µM genistein-7-O-glucuronide (GG) into genistein (Ge) and daidzein-7-O-glucuronide (DG) into daidzein (Da) after 24h incubation with U2OS-ERα, U2OS-ERβ and T47D wild type cells (concentration and % of the total amount of compound). Also the intracellular concentrations of the glucuronides is presented.

Cell line	Compound	Concentration (µM)	% of total
U2OS-ERα	GG	393.50	98.2
	Ge	6.50	1.6
	Intercellular GG	0.68	0.2
	DG	399.11	99.6
	Da	0.89	0.2
	Intercellular DG	0.54	0.1
U2OS-ERβ	GG	394.70	98.6
	Ge	5.30	1.3
	Intercellular GG	0.29	0.1
	DG	398.93	99.5
	Da	1.07	0.3
	Intercellular DG	0.61	0.2
T47D wt	GG	397.56	99.2
	Ge	2.44	0.6
	Intercellular GG	0.77	0.2
	DG	399.35	99.6
	Da	0.65	0.2
	Intercellular DG	0.62	0.2

b) Tissue S9 model

Figure 3.6 shows the fate of GG and DG when incubated for 24h with S9 fractions prepared from normal human or rat breast tissues. Results obtained under similar incubation conditions reveal a large inter-species difference with a 24-32 fold larger deconjugation into bioactive aglycones by rat than human breast tissue S9. Under the conditions applied, the average total deconjugation after 24h by human breast tissue S9 was 2.4% and 2.0% or 0.007 and 0.005 nmol/min/mg S9 protein or 0.0044 and 0.0035 nmol/min/g breast tissue for GG and DG, respectively (Table 3.5). For rat these values were 69% and 58% or 0.19 and 0.16 nmol/min/mg S9 protein or 0.013 and 0.011 nmol/min/g breast tissue for GG and DG, respectively (Table 3.5).

Figure 3.6

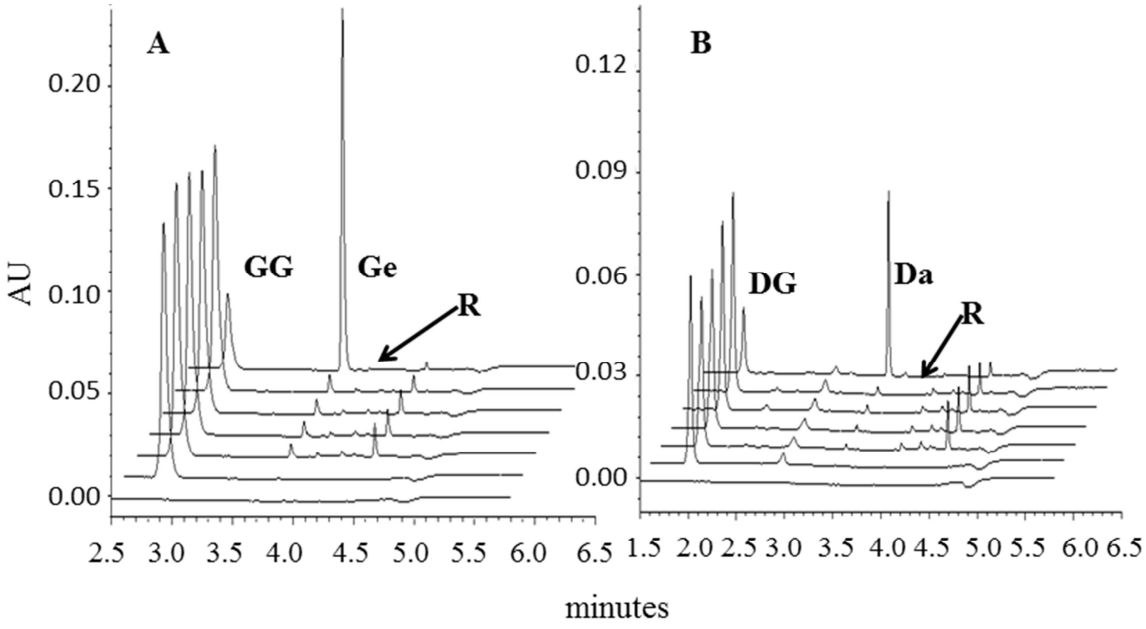

Figure 3.6: UPLC chromatograms showing deconjugation of SIF glucuronides (i.e. formation of aglycones) by S9 fractions prepared from four normal human breast tissue samples and 1 pooled rat sample (indicated with R) after 24h incubation with the glucuronide GG (A) and DG (B). In all the incubations, final S9 protein concentration was 0.1 mg/mL. The chromatograms are given at 260nm wavelength and presented front to back in the following order: nanopure water, blank incubation (i.e. only substrate without S9 protein), incubations with four human samples and one rat sample (R), for both GG (A) and DG (B).

Table 3.5: Deconjugation of genistein-7-O-glucuronide (GG) into genistein (Ge) and daidzein-7-O-glucuronide (DG) into daidzein (Da) upon incubation with normal human (1-4) and rat (5) breast tissue S9 fractions. Incubations were performed with 0.1 mg/mL S9 protein for 24h using a substrate concentration of 40 µM, and analysed with UPLC.

Substrate	Breast tissue S9 sample	% deconjugation after 24h	Deconjugation rate	
			(nmol/ min/mg S9 protein)	(nmol/min/g breast tissue)
GG	1	2.1	0.0058	0.0030
	2	2.4	0.0067	0.0026
	3	2.4	0.0067	0.0051
	4	2.9	0.0081	0.0068
	5 (rat)	69.3	0.1925	0.0132
DG	1	1.8	0.0050	0.0026
	2	1.9	0.0053	0.0020
	3	2.0	0.0056	0.0042
	4	2.2	0.0061	0.0051
	5 (rat)	58.3	0.1619	0.0111

Discussion

Supplements containing SIF are frequently used by peri- and post-menopausal women for their putative beneficial health effects. As a result levels of isoflavone metabolites in the blood of these women can reach up to ~2 µM, especially in the form of glucuronide conjugates [18, 38, 39]. However, the estrogenicity of these circulating glucuronide metabolites is not clear. In the present study the intrinsic estrogenicity and effects on breast cancer cell proliferation of SIF glucuronides and aglycones were measured in cellular models, including the T47D-wt cells in which the expression of ERβ is hardly present, and the T47D-ERβ cell model in which the cellular ERα/ERβ ratio can be modulated to reflect the level in normal human breast tissue [21]. To the best of our knowledge this is the first in vitro study quantifying the proliferative effect of SIF glucuronide metabolites and aglycones on T47D-wt and T47D-ERβ cells that mimic in their ERα/ERβ ratio in cancer and normal human breast tissue, respectively. In addition, the species dependent deconjugation potency of glucuronide metabolites was compared using S9 fractions, prepared from normal rat and human breast tissue.

The intrinsic estrogenic potency of Ge, Da and their corresponding 7-O-glucuronide metabolites was quantified in reporter gene assays using ERα or ERβ specific human osteoblast (U2OS) cell models. The intrinsic estrogenic potency of the glucuronide metabolites was 450-1730 fold lower than that of

the corresponding aglycones (Table 3.1). In the proliferation assay with T47D-wt cells the proliferative potency of the glucuronides was also 123-525 fold lower than that of the corresponding aglycones (Table 3. 2). This result is in line with the considerably lower relative binding affinity to ERs for Ge and Da glucuronides as reported by Zhang et al. [20], and is also expected because in many cases conjugation (i.e. glucuronidation) acts as a detoxification reaction. The PC10 and PC50 concentrations for both aglycones and glucuronides were 5-8 and 2-4 fold lower in the U2OS-ERβ compared to U2OS-ERα reporter gene assay, respectively, confirming their ERβ selectively in contrast to the ERα selectivity of E2. The dose response curve of Ge and Da were biphasic and the second part of the curve reaches a higher maximum than the E2-curve. This phenomenon was previously suggested to be related to multiple ERE's in the reporter construct or to stabilization of luciferase at higher concentrations of Ge [35, 40].

The cell proliferation induced by SIF was tested in T47D-ERβ cells and T47D-wt cells. The ERα/ERβ ratio of the T47D-ERβ cells has been made comparable with normal breast tissue by incubating them with the proper amount of tetracycline [21]. In T47D-wt cells, on the other hand, the ERβ expression is negligible resulting in a much higher ERα/ERβ ratio. In both cell lines the order of estrogenicity was the same (Table 3.2 and 3.3) as in the reporter gene assays (i.e. E2 > Ge > Da > GG > DG) (Table 3.1). Again, the aglycones showed much higher (36->4500 fold) potencies than their corresponding glucuronides (Table 3.3). Interestingly, the maximum proliferation relative to control was 50% less in the T47D-ERβ cells with the ERα/ERβ ratio resembling normal breast tissue than in the T47D-wt cells with insignificant amounts of ERβ (Table 3.2 and 3.3 and Figure 3.3 and 3.4). This result is in accordance with the earlier reported decrease in proliferation with increasing ERβ levels [30]. It is an important finding that breast cell proliferation induced by SIF is much (50%) less in cells that mimic the ERα/ERβ ratio of normal breast tissue.

Interestingly, lower concentrations of SIF reduced the cell proliferation up to 35% below those of the solvent control (Table 3.6). Depending on the nature of the soy supplement used (e.g. fermented products provide higher plasma concentration), plasma concentrations of SIF in women taking these supplements can raise up to 2 µM [18, 38, 39]. In this concentration the percentage of aglycones could be 1-4%, which corresponds to 20-80nM. Our results (Figures 3.3 and 3.4 and Table 3.6) indicate that if this concentration of 20-80 nM is in the form of genistein, which is the most active isoflavone reported so far, this would lead to an inhibition or no proliferation of breast cancer cells rather than to a stimulating effect. As can be seen in Table 3.6, also the glucuronide concentrations found in blood inhibited cell proliferation in our in vitro experiments. Thus based on the outcome of our experiments in vitro cellular models, no proliferative effects of SIF are to be expected at physiologically relevant concentrations, concentrations that could occur in women taking soy supplementation. Surprisingly, even T47D wt cells with a higher than normal ERα/ERβ ratio did not show proliferation when exposed

to physiologically relevant SIF concentrations. This finding, together with the other positive health effects of SIF such as reducing the blood lipid levels [41-43], might contribute to the beneficial instead of adverse effects of soy supplementation observed in epidemiological and clinical trials.

Table 3.6: Concentrations of genistein (Ge), genistein-7-O-glucuronide (GG), daidzein (Da) and daidzein-7-O-glucuronide (DG) at which inhibition of proliferation is observed in human breast cancer cell lines.

Cell lines	Compound	Concentration inhibiting proliferation (nM)	Concentration giving highest inhibition (nM)	Maximum inhibition to vehicle control (%)
T47D wt	Ge	0-30	9	32
	GG	0-2000	200	35
	Da	0-60	30	20
	DG	0-20000	6000	24
T47D-ERβ	Ge	0-9	1	10
	GG	0-2000	60	16
	Da	ND	ND	0
	DG	0-40000	200	6

ND = not detected

To detect the fate of the genistein or daidzein 7-O-glucuronides in the in vitro cell models, U2OS-ERα, U2OS-ERβ and T47D-wt cells were exposed to 20 and 400 µM of each glucuronide for 24h These concentrations were chosen as 20 µM is the average PC50 concentration and the highest stimulation of cell proliferation was found at 400 µM of the glucuronides (Table 3.1-3.3). The results indicate that upon exposure of the cells to 400 µM glucuronides only 0.2-1.6 % of these conjugates is converted to the corresponding aglycones resulting in a final aglycone concentration of about 0.7- 6.5 µM (Table 3.4). These aglycone concentrations are higher than their PC50 values observed in the reporter gene assay (Table 3.1) and high enough to induce maximum cell proliferation (Table 3.2). As the absence of traces of aglycone impurities in the glucuronide stocks has been confirmed in advance (see supplementary document 2 & 3), it is concluded that in cellular reporter gene assays or in proliferation assays with the SIF glucuronides, the low percentage of the glucuronides converted into the corresponding aglycones can account for the effects observed. This finding is important and according to our knowledge this has not been reported so far. This is also in line with the results of Yuan et al. [18] who exposed MCF-7 and T47D cells to genistein-7-O-glucuronide and found that the proliferative effects correlated with the degree of deconjugation of genistein-7-O-glucuronide.

Deconjugation can be an important factor in bio-activation of circulating SIF glucuronides in target tissues. In a previous study we demonstrated natural deconjugation and conjugation of major SIF by intestinal and liver S9 fractions of rat and human [44]. In this study, we focus on the deconjugation of

SIF glucuronides by rat and human normal breast tissue S9. Although not enough rat breast tissue was available to allow for detailed kinetic studies as done before for deconjugation of the isoflavone glucuronides by rat and human intestinal and liver S9 fractions [44], the HPLC chromatograms of the resulting incubations (Figure 3.6) revealed that limitation of the substrate concentration under the experimental condition applied was not the cause of the marked difference in overall deconjugation between human and rat tissue. Under the experimental conditions applied rat breast S9 appeared to be highly capable to deconjugate about 60-70% of GG and DG within 24h In contrast to this, under the same incubation conditions human breast S9 deconjugated only ~2-3% of the SIF glucuronide. For both GG and DG the deconjugation capacity of rat breast tissue S9 appeared to be about 30 fold higher than that of human breast S9. This finding is in line with some recent reports where higher levels of genistein (around 10% of the level of the glucuronide) were observed in rat plasma [45]compared to human (about 1-2%) [14, 15]. Only one publication [46] reports that the circulating concentrations of IF aglycones in rats were markedly higher than those in human volunteers. However, the rats and humans in this study were dosed with IF aglycones from different sources, following different administration schedules and the animals were treated with a 4–30 fold higher dose level than the human volunteers. Therefore, the higher IF-aglycone concentrations in the plasma of rats than of humans may be due to the higher dose levels used in the rat studies, and from these data no conclusion can be drawn about the relative presence of the glucuronide vs the aglycone in blood under comparable dosing conditions. Thus species differences in the deconjugation activity should be taken into account when risk and/or benefit assessment of these SIF for the human population is based on animal data. In this respect it can be suggested that if the deconjugation reaction in the enzymatic micro-environment is not active enough, potential health effects of SIF may not take place. It was reported by Bartholome et al. [47] and Shimoi [48] that the production of β-glucuronidase, which is involved in deconjugation, is higher in inflammatory sites than in normal tissue. The results of the present study suggest that normal healthy breast tissue may be relatively insensitive to the estrogenic effects of SIF because deconjugation by β-glucuronidase is relatively low compared to inflammatory tissues and the level of ERβ is relatively high as compared to cells from tumour tissues. Although the breast tissues used in this study were judged to be normal by the Maastricht University Medical Center, we did not quantify the relative and absolute specific ER levels. It is therefore important to further study the ERα/ERβ ratio as well as the absolute ER-levels in a more elaborate series of breast tissue samples from women with a variety of breast health conditions and to optimize the kinetic parameters.

In conclusion, our results show that SIF glucuronides are not estrogenic as such, but have to be deconjugated to become bioactive. Under the experimental condition applied we also show that species differences may play an important role in deconjugation of SIF glucuronides. These findings should be taken into account during risk-benefit assessment of these SIF together with the other

bioactive metabolites such as equol produced by some specific gut microbes and by equol producing (post)menopausal women. Of course the exposure to SIF in the present in vitro studies are relatively short compared to the long term human exposure to the same SIF supplement, and the ultimate internal exposure concentration and resulting health effect may be influenced by for example accumulation of the SIFs in fatty tissue. Nevertheless, our in vitro results suggest that soy supplement intake by post-menopausal women will not induce proliferation of normal breast tissue and may even inhibit proliferation. Of course further in vivo research is needed to confirm this hypothesis.

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The authors have declared no conflict of interest.

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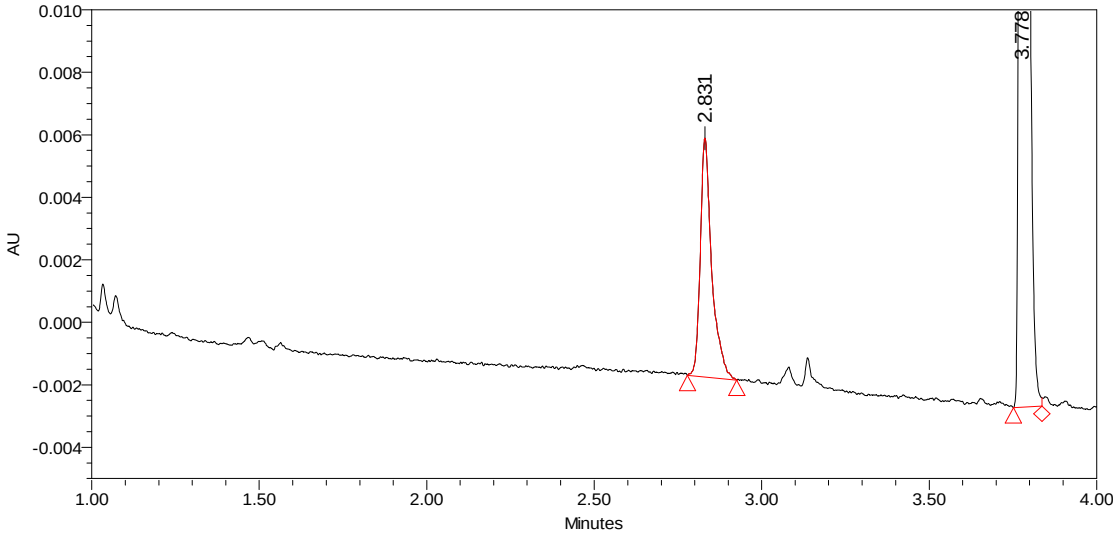
Supplementary document 1

Comparison of commercial SD male RLS9 (CRLS9) with prepared F344 female RLS9 (PRLS9)

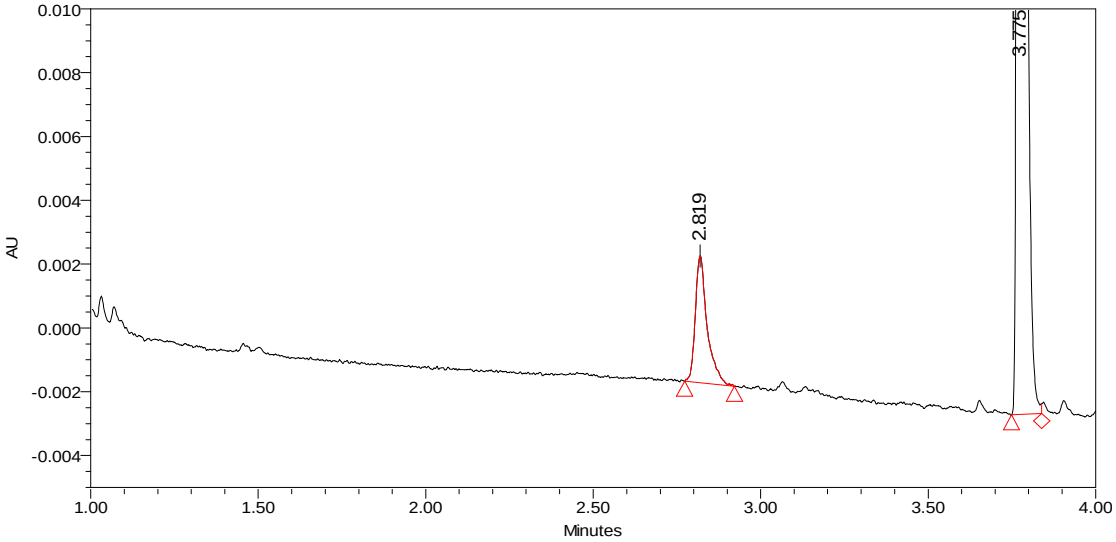
Incubation time 5 minutes, Ge-aglycone conc 20uM

1) CRLS9: 0.5mg/ml CRLS9 (commercial rat liver S9)



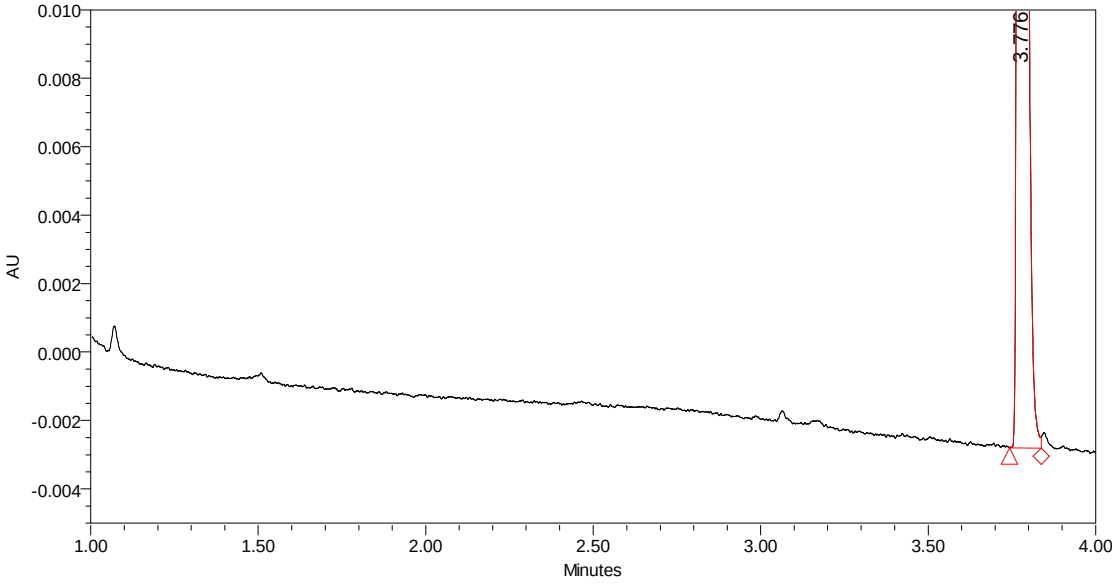
	Name	RT	Area	% Area	Height
1	Ge-glucuronide	2.831	18210	9.67	7645
2	Ge-aglycone	3.778	170164	90.33	147836

2) PRLS9: 0.5mg/ml PRLS9 (prepared rat liver S9)



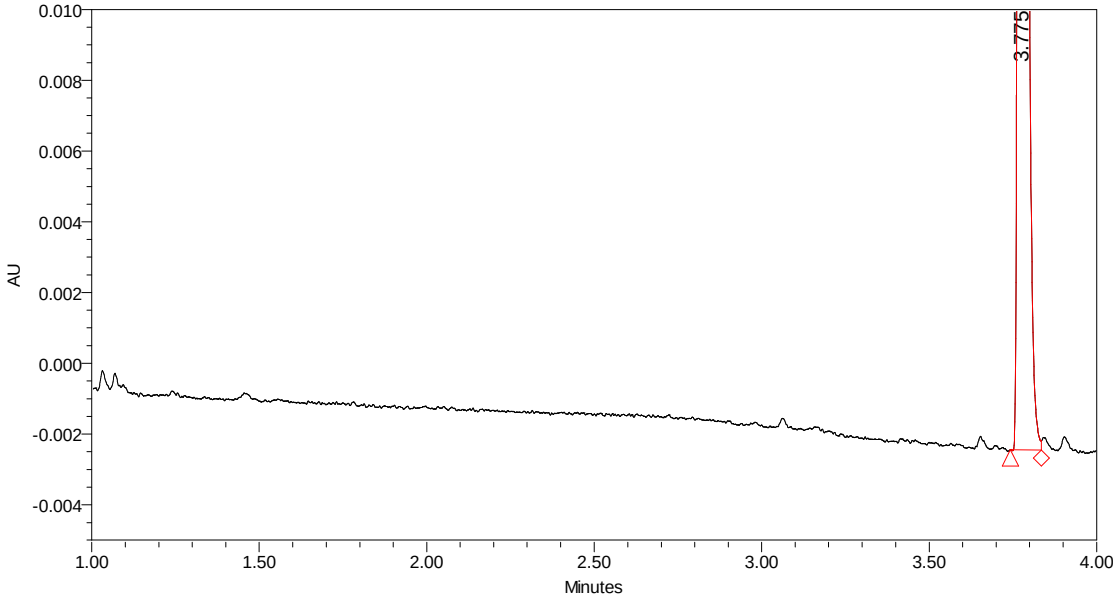
	Name	RT	Area	% Area	Height
1	Ge-glucuronide	2.819	9806	5.14	3978
2	Ge-aglycone	3.775	181091	94.86	157814

3) CB1 (No S9) (Blank)



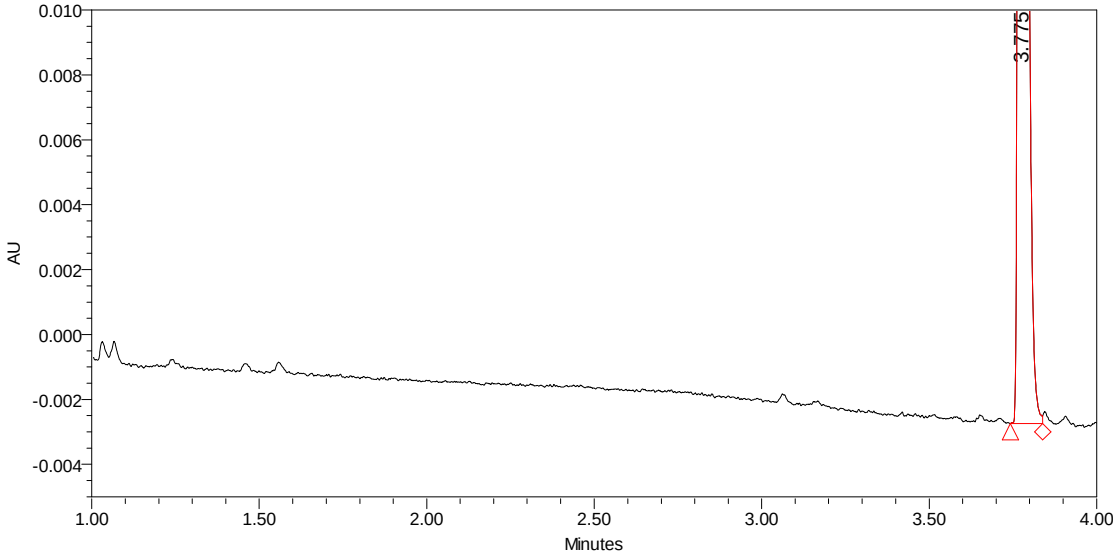
	Name	RT	Area	% Area	Height
1	Ge-aglycone	3.776	211232	100.00	183672

4) PB1 (No S9) (Blank)



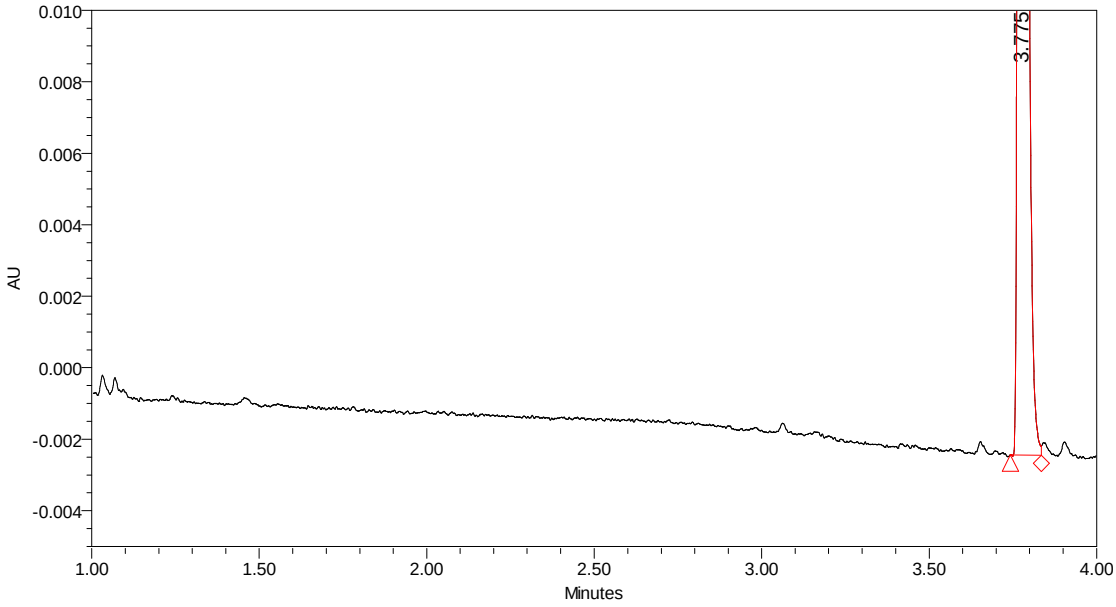
	Name	RT	Area	% Area	Height
1	Ge-aglycone	3.775	187702	100.00	163588

5) CB2 (No UDPGA) (Blank for commercial S9)



	Name	RT	Area	% Area	Height
1	Ge-aglycone	3.775	184126	100.00	159923

6) PB2 (No UDPGA) (Blank for prepared S9)

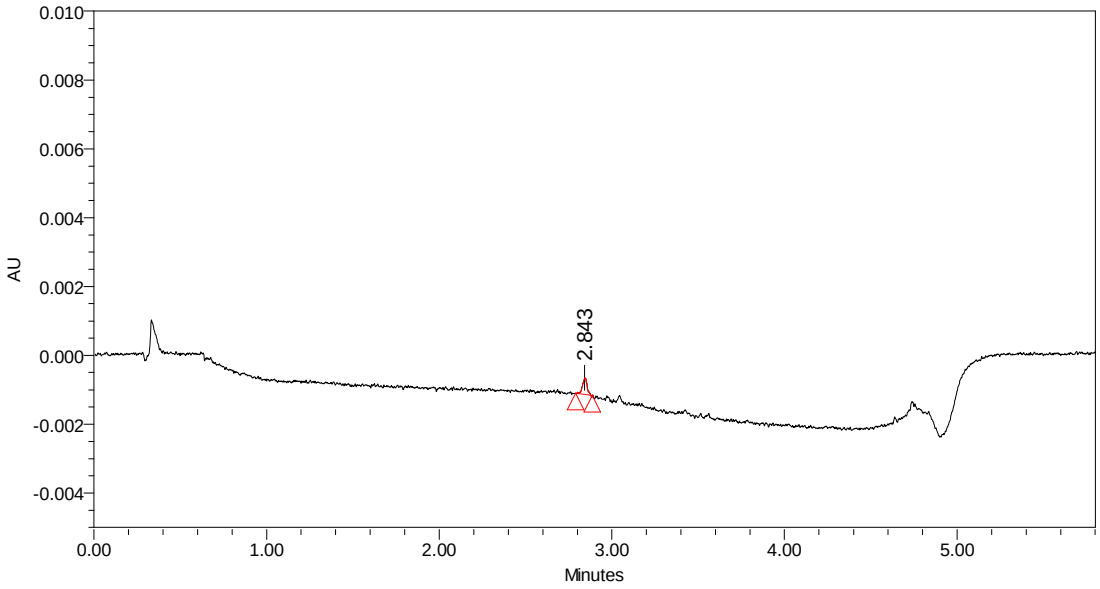


	Name	RT	Area	% Area	Height
1	Ge-aglycone	3.775	187694	100.00	163587

Supplementary document 2

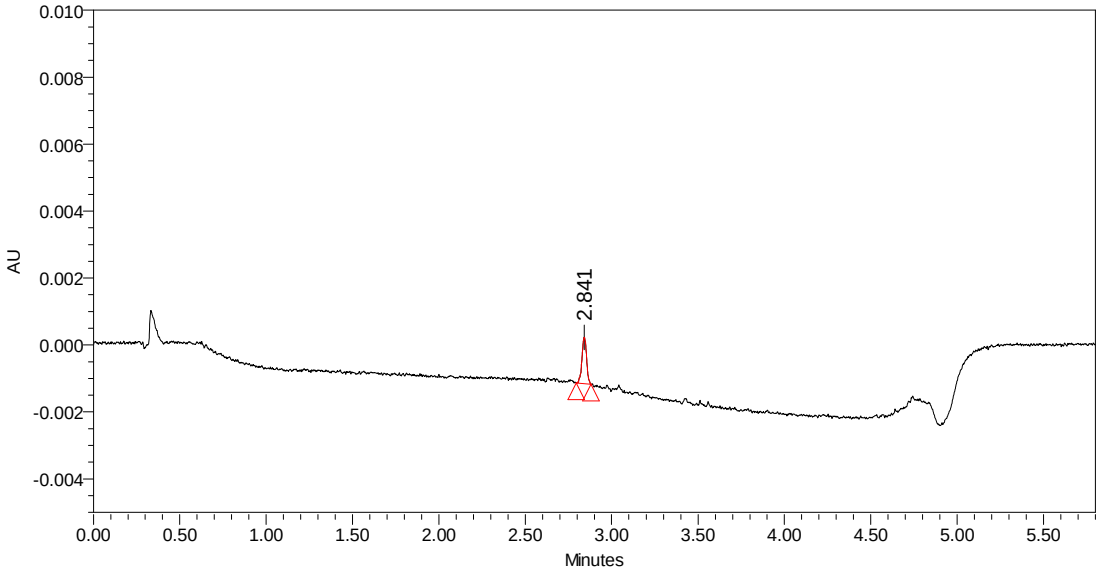
Std series of Genistein glucuronide (GG)

GG (0.2 µM)



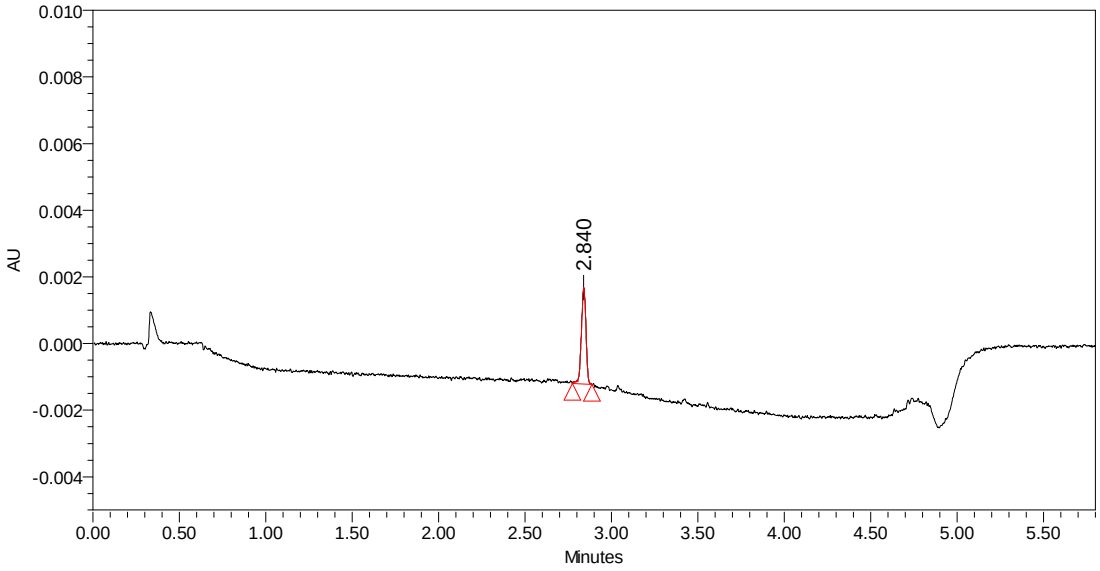
	Name	Retention Time	Area	% Area	Height
1	GG	2.843	890	100.00	482

GG (0.5 µM)



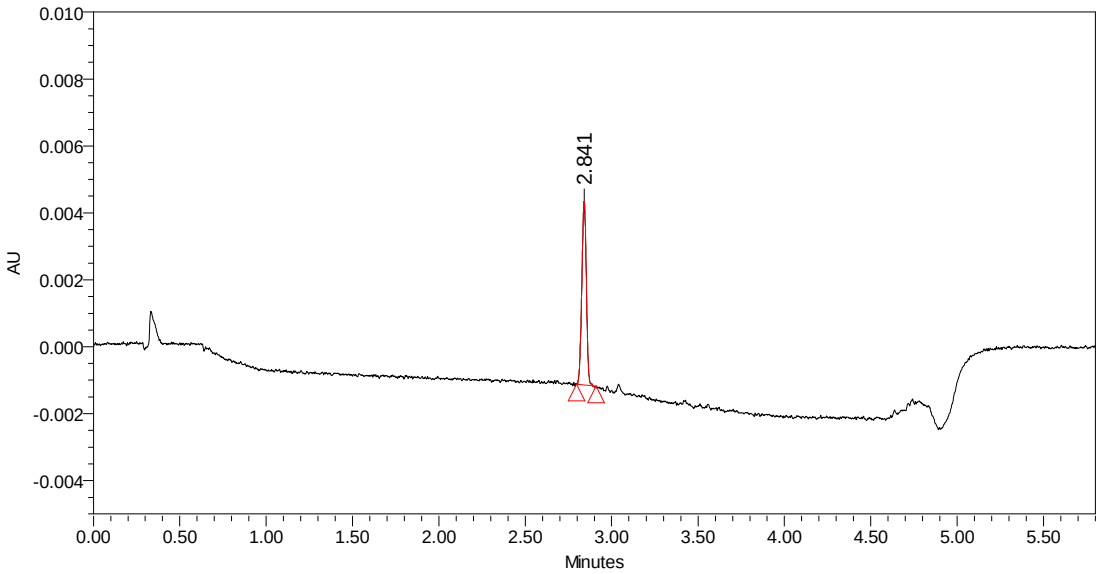
	Name	Retention Time	Area	% Area	Height
1	GG	2.841	2569	100.00	1387

GG (1.0 µM)



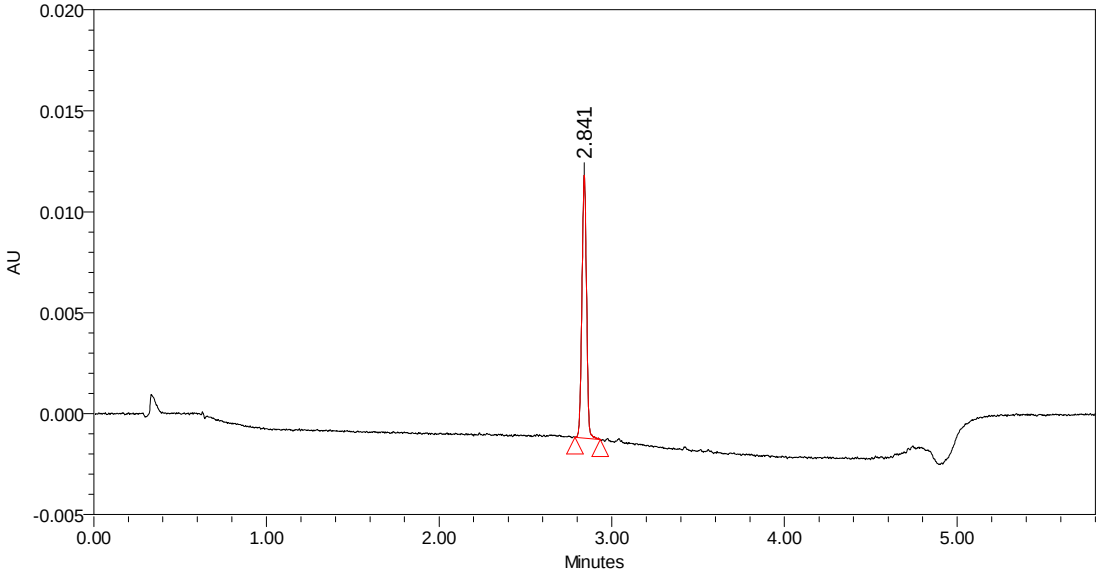
	Name	Retention Time	Area	% Area	Height
1	GG	2.840	5394	100.00	2900

GG (2.0 µM)



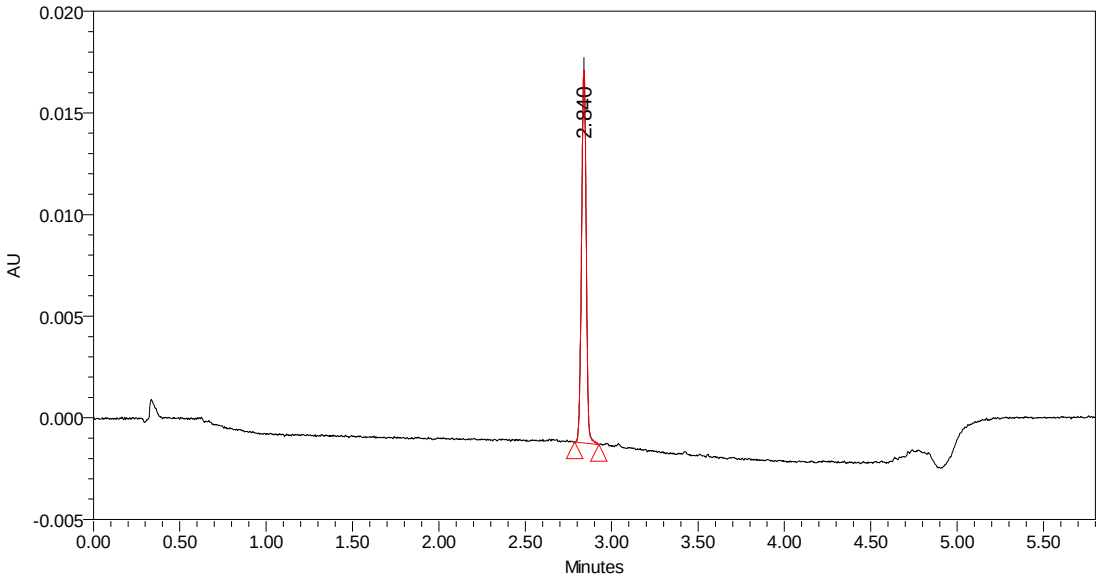
	Name	Retention Time	Area	% Area	Height
1	GG	2.841	10013	100.00	5491

GG (4.0 µM)



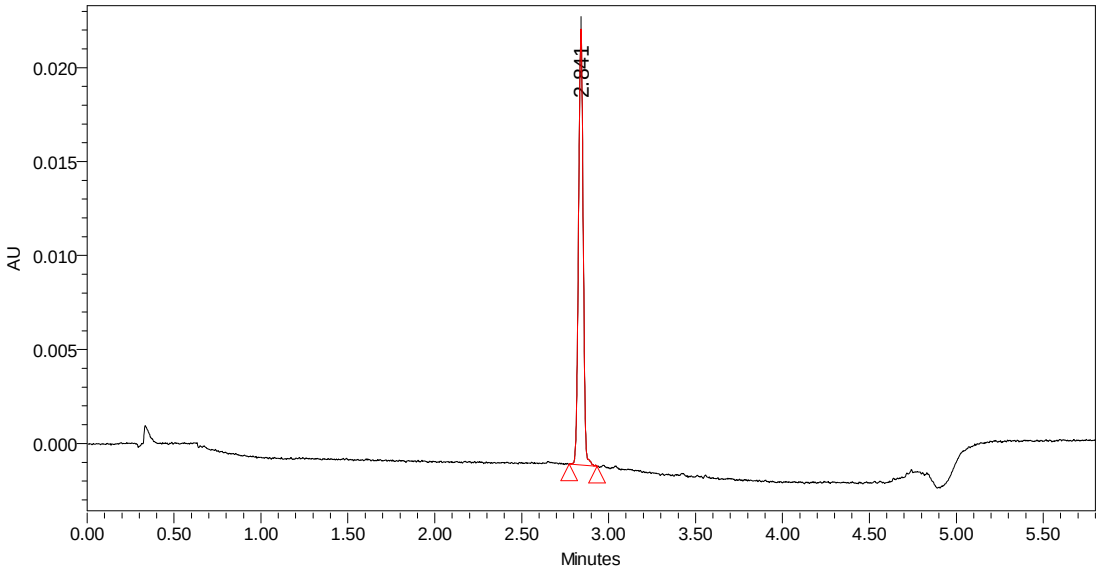
	Name	Retention Time	Area	% Area	Height
1	GG	2.841	24210	100.00	13022

GG (6.0 µM)



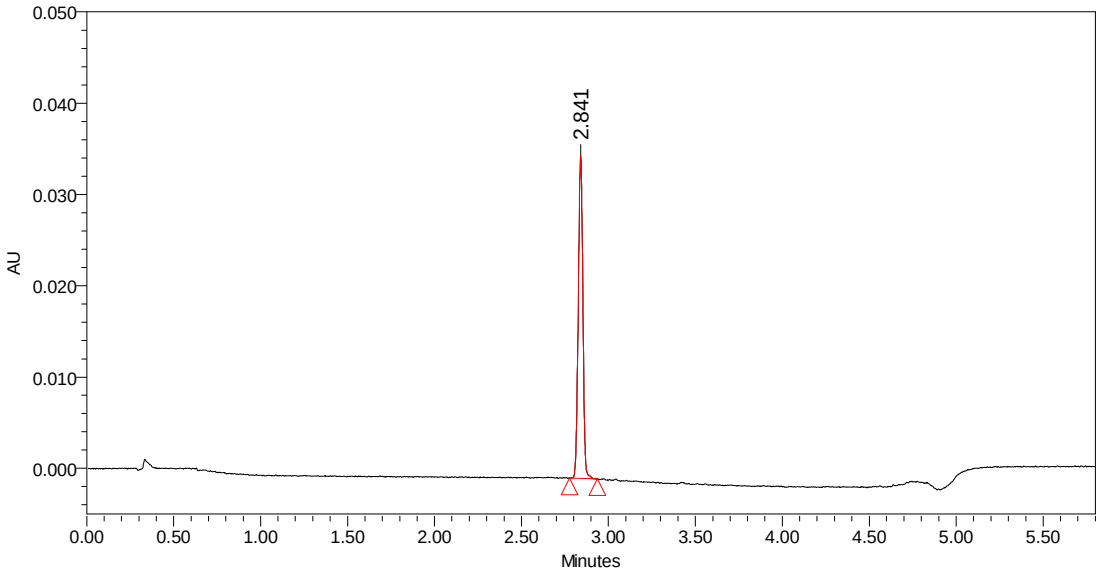
	Name	Retention Time	Area	% Area	Height
1	GG	2.840	34281	100.00	18357

GG (8.0 µM)



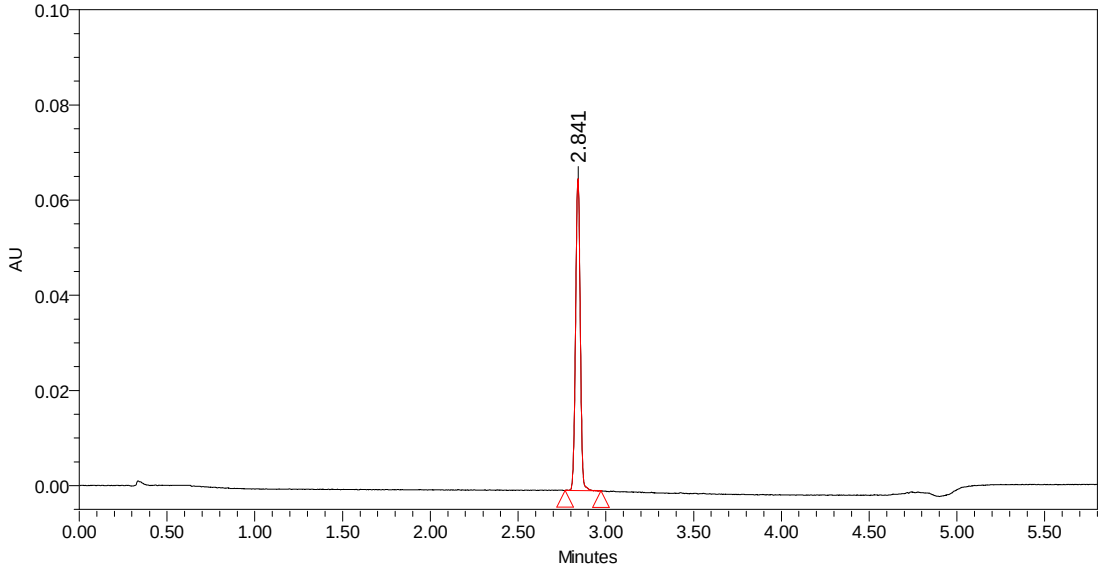
	Name	Retention Time	Area	% Area	Height
1	GG	2.841	43097	100.00	23206

GG (10.0 µM)



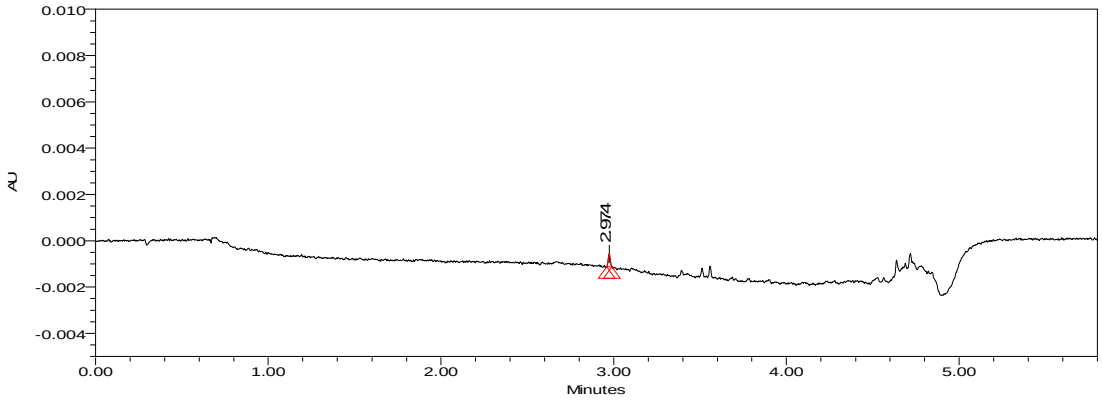
	Name	Retention Time	Area	% Area	Height
1		2.841	65122	100.00	35262

GG (20.0 µM)

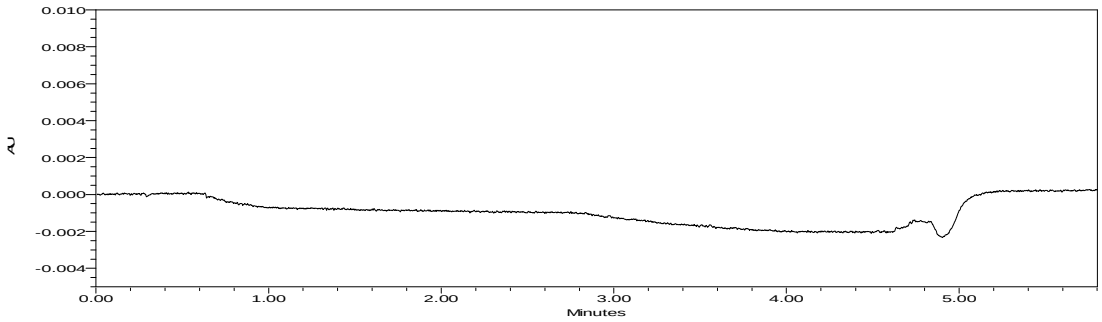


	Name	Retention Time	Area	% Area	Height
1		2.841	121242	100.00	65544

N-pure (in the beginning)



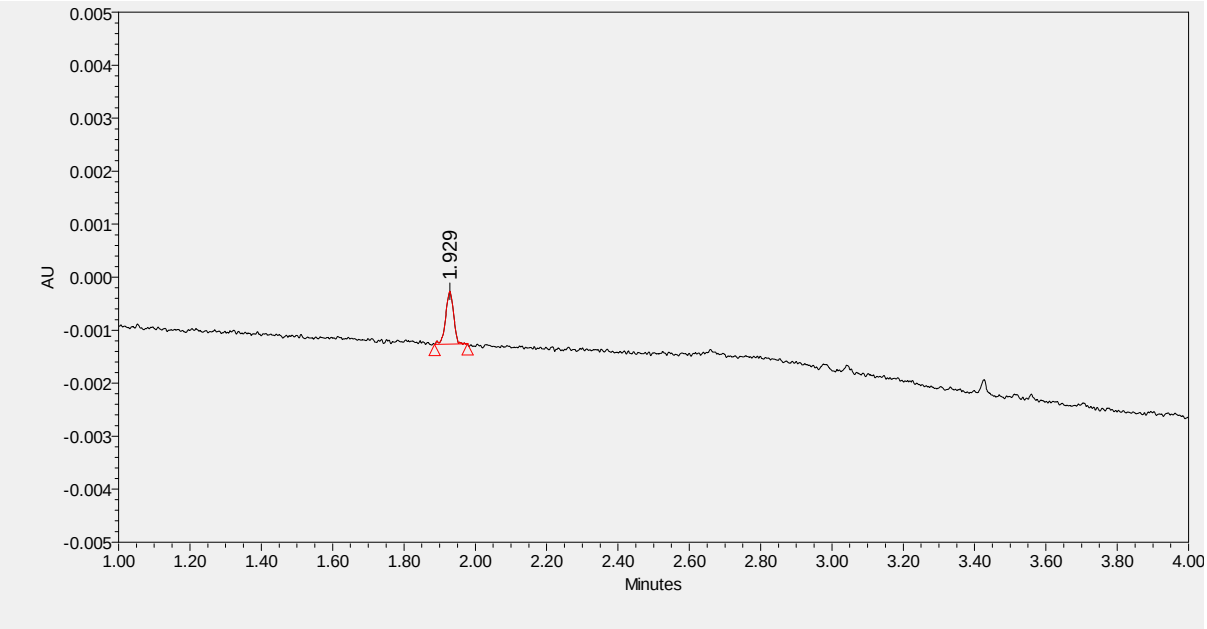
N-pure (in the beginning)



Supplementary document 3

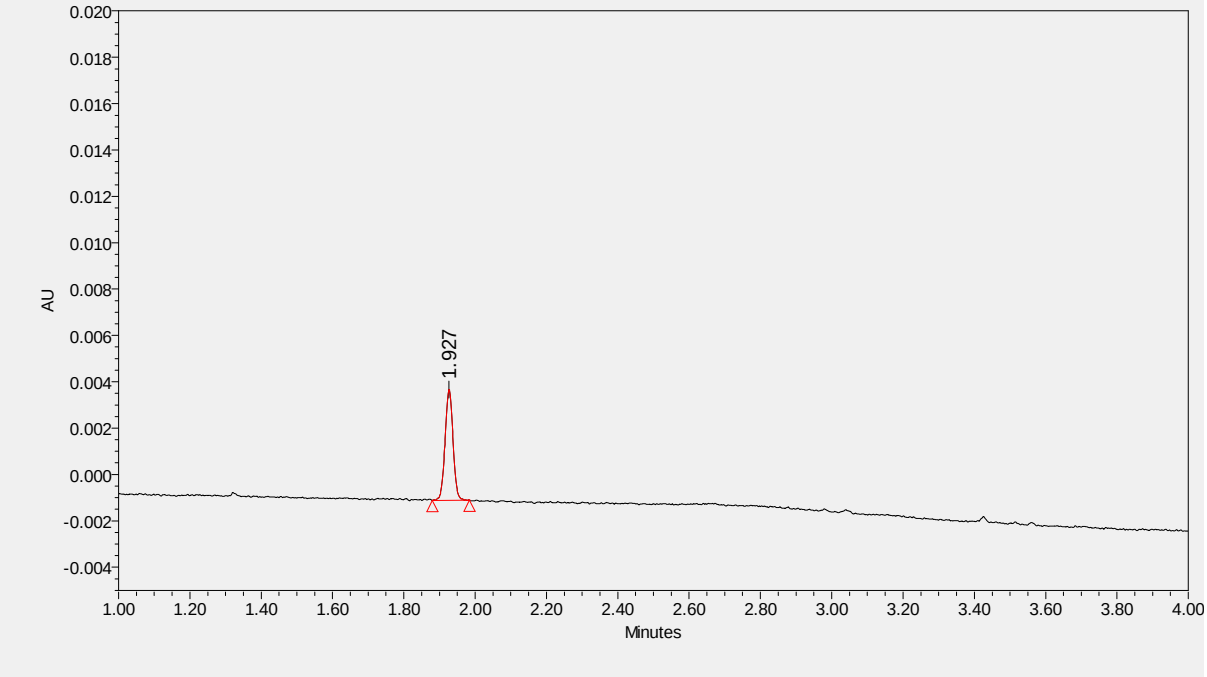
Standard of Daidzein 7-O-Glucuronides (DG)

DG (0.02 µM)



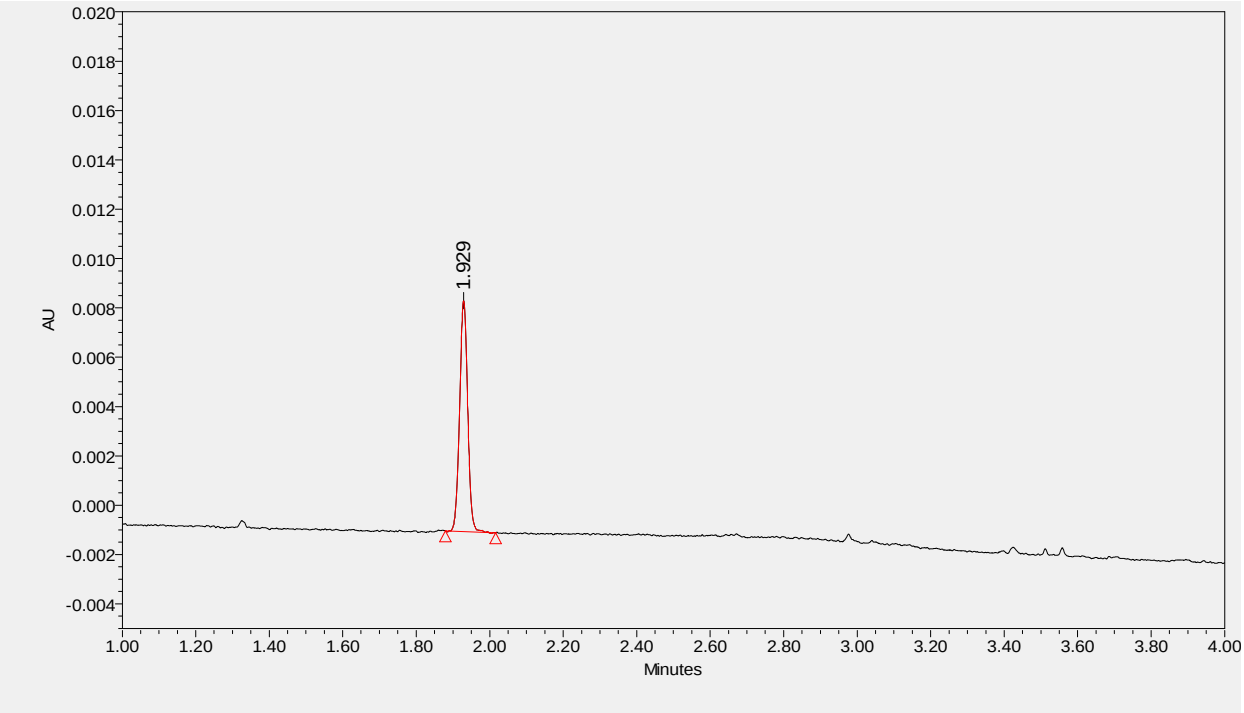
	Name	Retention Time	Area	% Area
1	DG	1.929	1532	100.00

DG (1.0 µM)



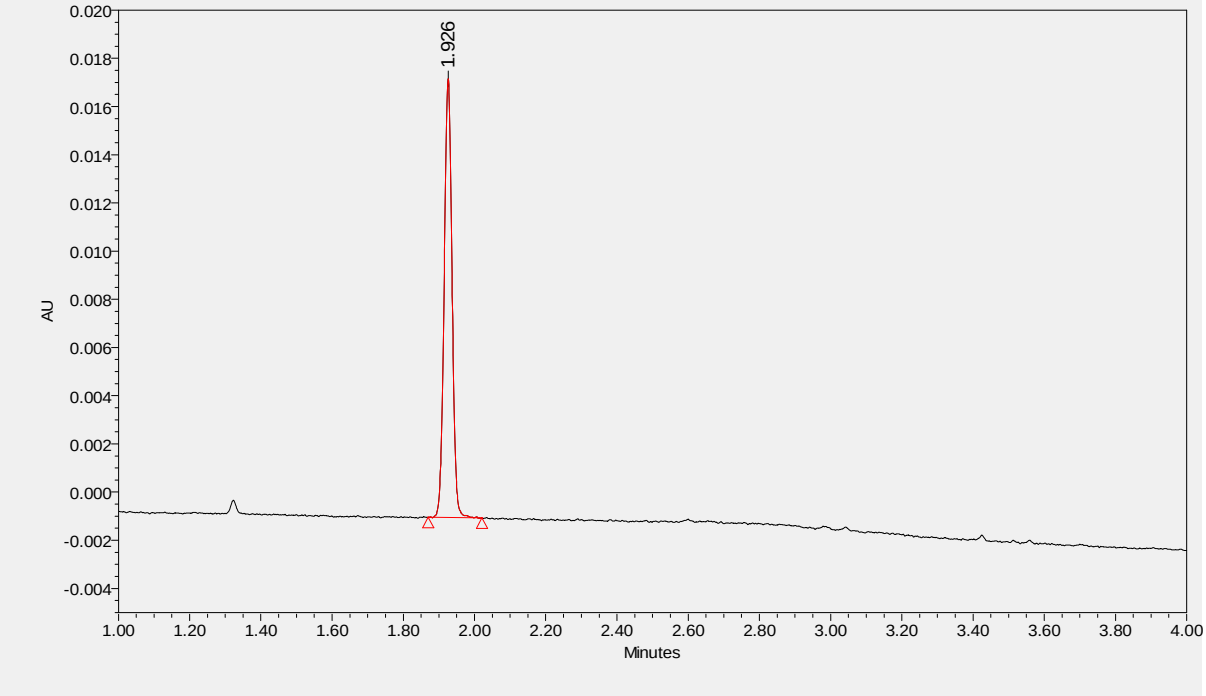
	Name	Retention Time	Area	% Area
1	DG	1.927	7410	100.00

DG (2.0 µM)



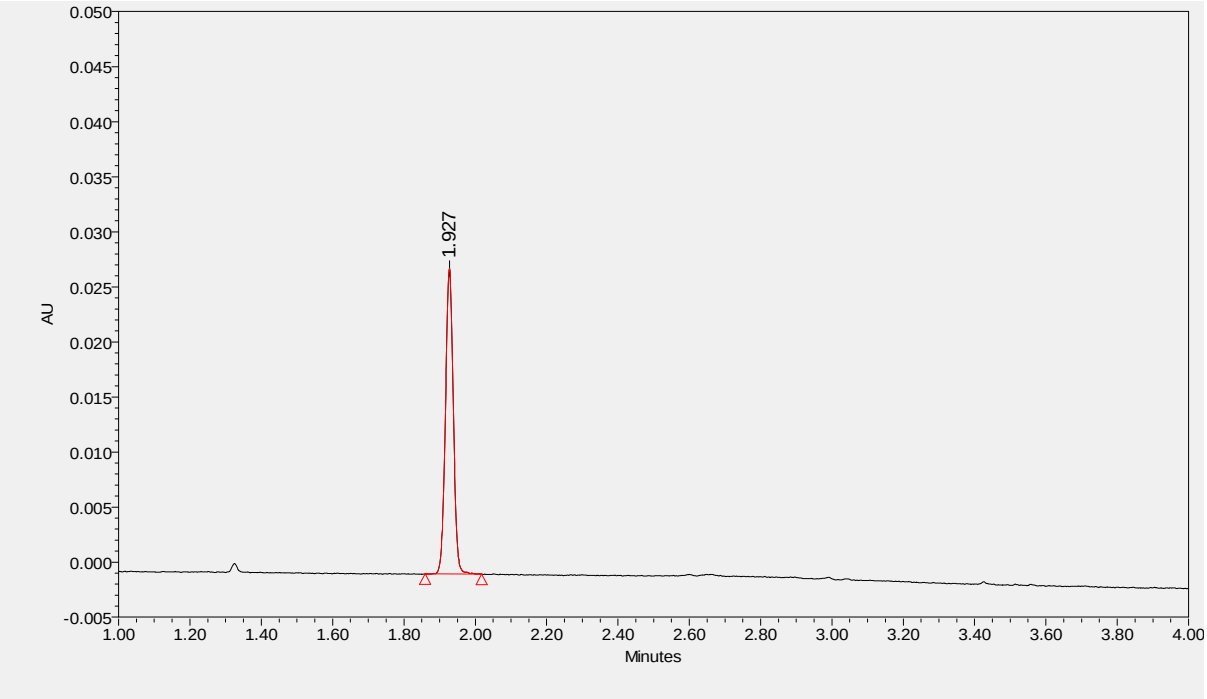
	Name	Retention Time	Area	% Area
1	DG	1.929	14352	100.00

DG (4.0 µM)



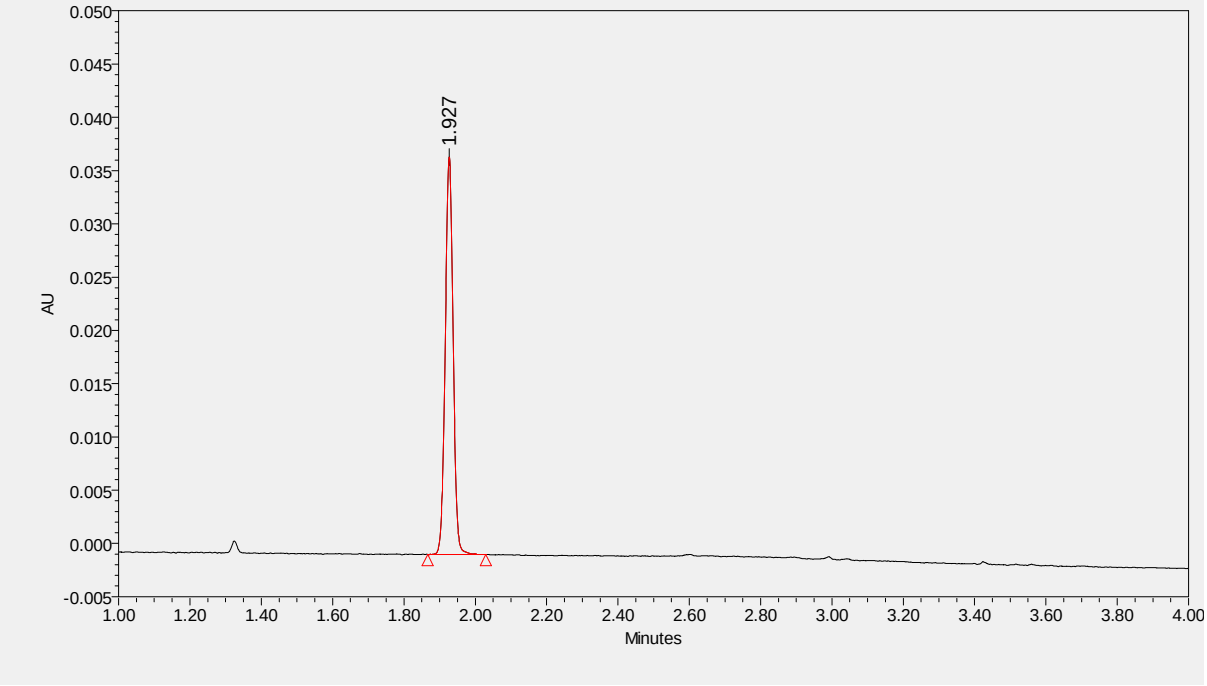
	Name	Retention Time	Area	% Area
1	DG	1.926	28013	100.00

DG (6.0 µM)



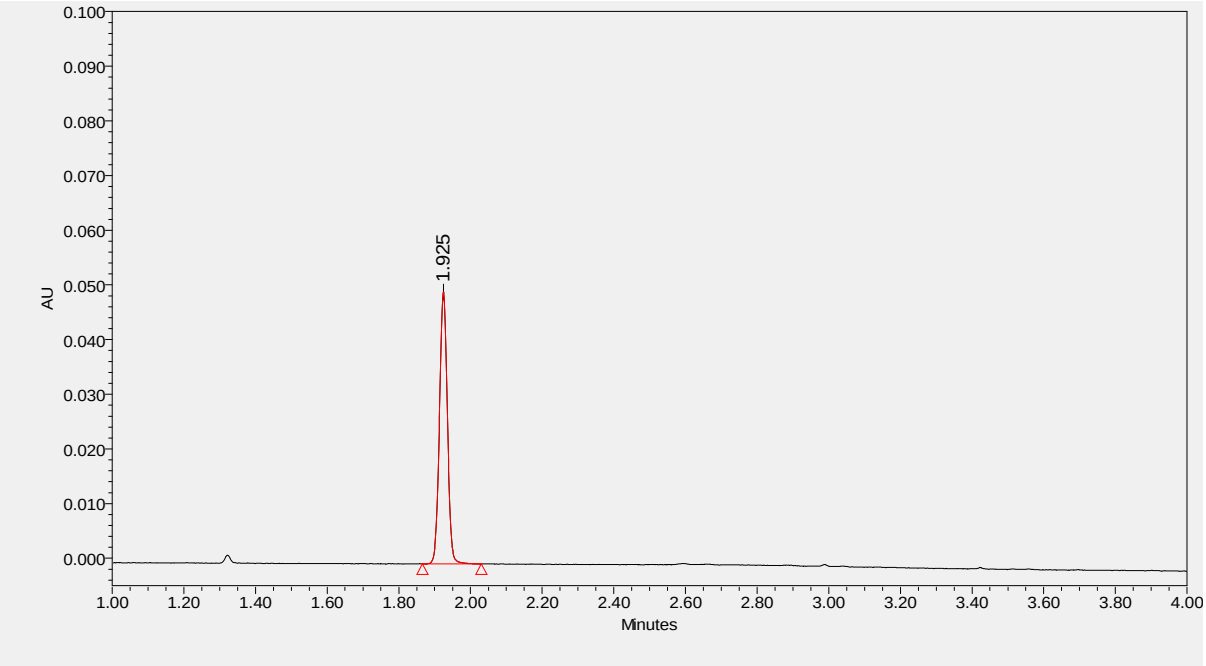
	Name	Retention Time	Area	% Area
1	DG	1.927	42659	100.00

DG (8.0 µM)



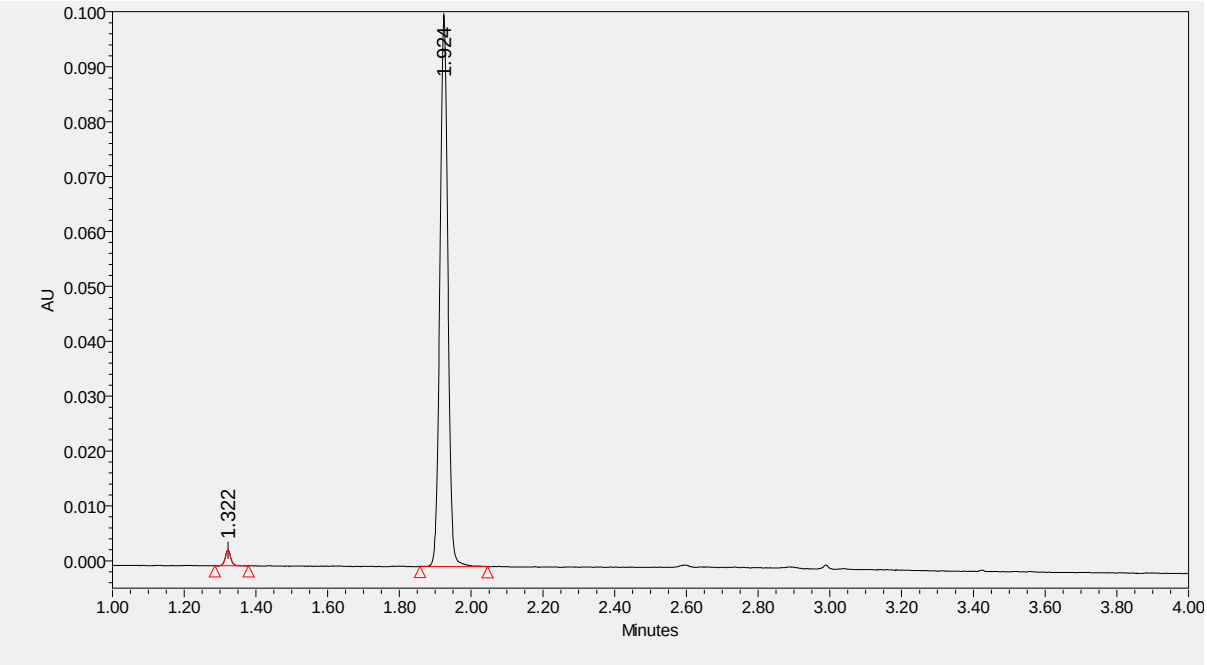
	Name	Retention Time	Area	% Area
1	DG	1.927	57541	100.00

DG (10.0 µM)



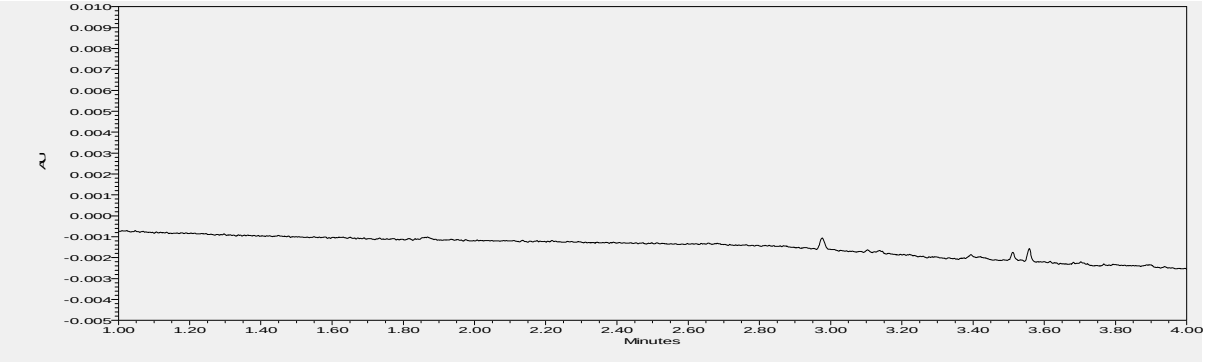
	Name	Retention Time	Area	% Area
1	DG	1.925	76631	100.00

DG (20.0 µM)

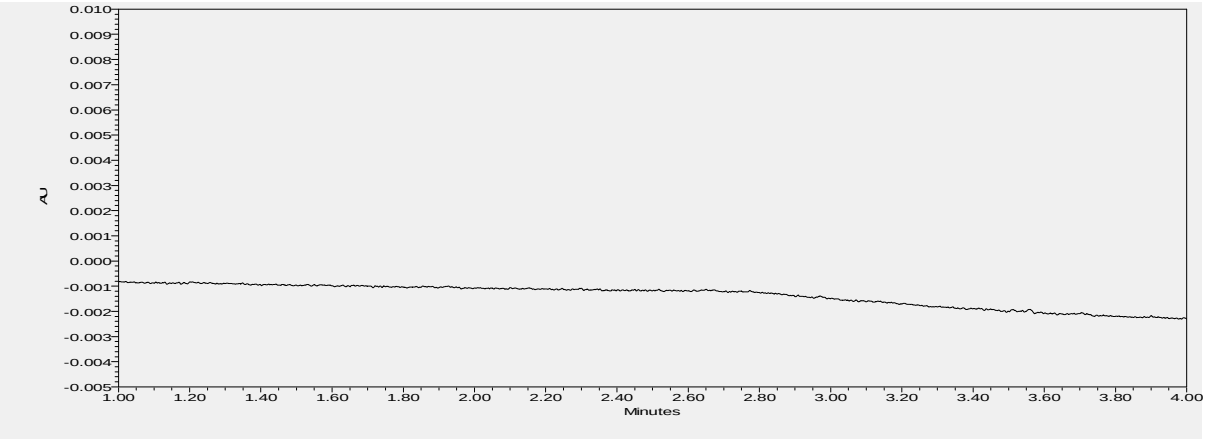


	Name	Retention Time	Area	% Area
1		1.322	3013	1.92
2	DG	1.924	154311	98.08

N-pure beginning



N-pure End



4

Plasma bioavailability and changes in PBMC gene expression after treatment of ovariectomized rats with a commercial soy supplement

Mohammed A. Islam, Guido J.E.J. Hooiveld, Johannes H.J. van den Berg, Mark V. Boekschoten, Vera van der Velpen, Albertinka J. Murk, Ivonne M. C. M. Rietjens and F. X. Rolaf van Leeuwen

Toxicology Reports 2 (2015) 308-321

Abstract

The health effects of soy supplementation in (post)menopausal women are still a controversial issue. The aim of the present study was to establish the effect of the soy isoflavones (SIF) present in a commercially available supplement on ovariectomized rats and to investigate whether these rats would provide an adequate model to predict effects of SIF in (post)menopausal women. Two dose levels (i.e. 2 and 20 mg/kg bw) were used to characterise plasma bioavailability, urinary and faecal concentrations of SIF and changes in gene expression in peripheral blood mononuclear cells (PBMC). Animals were dosed at 0 and 48 hr and sacrificed 4 hr after the last dose. A clear dose dependent increase of SIF concentrations in plasma, urine and faeces was observed, together with a strong correlation in changes in gene expression between the two dose groups. All estrogen responsive genes and related biological pathways (BPs) that were affected by the SIF treatment were regulated in both dose groups in the same direction and indicate beneficial effects. However, in general no correlation was found between the changes in gene expression in rat PBMC with those in PBMC of (post)menopausal women exposed to a comparable dose of the same supplement. The outcome of this short-term study in rats indicates that the rat might not be a suitable model to predict effects of SIF in humans. Although the relative exposure period in this rat study is comparable with that of the human study, longer repetitive administration of rats to SIF may be required to draw a final conclusion on the suitability of the rat a model to predict effects of SIF in humans.

Introduction

Dietary isoflavones are phenolic plant compounds mostly found in soy [1-3]. They are biologically active and, depending on the concentration, they have estrogen agonist and antagonist effects due to structural similarity with estradiol [4]. Therefore, they are also known as phytoestrogens [4, 5]. Soy isoflavones (SIF) have gained increasing interest in recent years because of their potential health benefits such as improvement of cardiovascular functions, bone mineral density, alleviation of menopausal symptoms and reduction of the occurrence of certain cancers like breast and prostate cancers [6-8]. The use of soy products in the USA increased significantly following their approval by U.S. Food and Drug Administration [9] and the decision [10] to allow food manufacturers to make cardiovascular health claims for soy protein products. Moreover a number of SIF-rich supplements have become available for over the counter sale in Western countries during the last decade [11]. In contrast, adverse health effects also have been reported [12-14], and recently the European Food Safety Authority (EFSA) rejected some of the major health claims of SIF, such as maintenance of bone mineral density and reduction of vasomotor symptoms associated with menopause [15]. According to EFSA [15], the data are not sufficient to establish a cause-effect relationship for these endpoints. Therefore, concern remains about the potentially harmful effects from self-administered dosages of these SIF rich products, which are freely available in pharmacies, health food stores and via online shopping on internet [6, 11, 12].

SIF are present in soy based foods and food supplements, predominantly in their glucoside forms namely genistin, daidzin and glycitin. Their corresponding primary metabolites are genistein, daidzein and glycitein. These metabolites are produced initially mainly by β-glucosidase present in the duodenum and upper proximal jejunum [16, 17] and further down in the intestinal tract by gut microflora [11, 18]. These primary metabolites are known as aglycones and considered to be the biologically active forms of SIF [13, 19]. However in the systemic circulation, glucuronides (i.e. the secondary metabolites) are found to be the predominant metabolites [20, 21]. Therefore, enzymatic hydrolysis is commonly used to measure the total aglycone content in plasma [21-23]. Daidzin is one of the major soy glucosides present in the soy supplements. Approximately 20-30 % of the Western population is able to metabolize the aglycone form of daidzin (daidzein), into the more potent metabolite equol, which is more bioavailable and has a higher binding affinity for the estrogen receptors [24-26]. This means that especially in (post)menopausal equol-producing women SIF supplementation will result in a considerable exposure related contribution to endogenous estrogen levels [27-29].

The effects of SIF are thought to be induced via estrogen receptors (ERs) [30, 31] of which an alpha and beta form exist. SIF can regulate transcription of ER target genes via the estrogen-responsive elements (EREs) present within gene promoter regions in target tissues such as breast and uterus. It is

known that activation of ERα stimulates cell proliferation, whereas ERβ is involved in inhibition of proliferation and stimulation of apoptosis [20, 32]. Estradiol (E2) is ERα selective, whereas SIF are ERβ selective [15,32]. Because (post)menopausal women produce low levels of endogenous estradiol, it is expected that the intake of SIF by equol-producing (post)menopausal women will result in beneficial health effects of SIF supplementation [3, 6, 19, 33].

During the past decade, the application of microarray technology has opened up new opportunities to study the effects of food and food supplements in the control of cellular processes. Understanding of the bioavailability, metabolism and induction of gene expression related to different biological processes is key to understand the health effects of SIF supplementation. There is, however, a lack of information whether data obtained from in vivo studies in experimental animals are suitable to predict human risks or benefits. Hence the aim of the present study was to establish the effect of SIF on plasma bioavailability and gene expression changes in ovariectomized rats and to investigate whether these rats would be an adequate model to predict effects of SIF supplementation in (post)menopausal women. The ovariectomized rats, mimicking the hormonal condition of (post)menopausal women, were dosed with a commercial SIF containing supplement. The bioavailability and the excretion of SIF were studied by measuring the concentration of SIF and the main metabolite equol in plasma, urine and faeces. Changes in gene expression of peripheral blood mononuclear cells (PBMC) were characterized, because this could be a basis for rat to human comparison, since PBMC are easy to collect as marker tissue in studies with human volunteers, and are often used to predict changes in target tissue [34]. In addition, the effects on gene expression in rat PBMC were compared with the effect on gene expression in PBMC of (post)menopausal equol-producing women, who received the same commercial SIF supplement in a parallel study [23].

Materials and methods

Chemicals

Pure SIF standards (both glucosides and aglycones) and equol were purchased from LC laboratories (Woburn, MA, USA). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium), and enterodiol, β-glucuronidase H-5 (*H. Pomatia* contain 60620 unit/g solid), Optiprep™ and Tricine from Sigma-Aldrich (Steinheim, Germany). NaCl was from VWR International (Darmstadt, Germany) and acetonitrile and methanol from Biosolve BV (Valkenswaard, the Netherlands). Oasis HLB 1cc, solid phase extraction cartridges from Waters (Milford, MA, USA).

Animals

Fifteen ovariectomized F344 inbred rats, 9-10 weeks of age, were purchased from Harlan, (Horst, the Netherlands). The ovariectomy was performed at the age of 7-8 weeks by the supplier. After 2 weeks of acclimatization the animals were delivered at the animal facility, the Centre for Laboratory Animals

(CKP), Wageningen University (Wageningen, the Netherlands). F344 rats were chosen for two reasons. Firstly, according to US EPA [35], the inbred isogenic strain F344 rats are sensitive to estrogenic compounds and thus particularly suitable to study the effects of SIF. Secondly, the use of an inbred strain will minimize the background noise in micro-array analysis.

Housing and nutrition

After arrival at the animal facility, rats were housed in groups (3 animals/cage) and fed an SIF free RMH-B standard diet (ABDiets, Woerden, the Netherlands) (Table 4.1) ad libitum. Standard housing, day-light hours and humidity were maintained during the acclimatization period of 12 days. The animals were 11-12 weeks of age during the experiment, and had a bodyweight of 162-196 g. Animals were placed individually in metabolic cages, 24 h before receiving the first gavage dose.

Table 4.1: Composition of isoflavone-free RMH-B diet

Analysis	**%**	**Amino acids**	**g/kg**
Crude protein	23.5	Lysine	10.5
Crude fat	5.0	Methionine	3.6
Crude fibre	4.3	Methionine + Cystine	6.6
Starch	38.3	Cystine	3.0
Sugar	4.0	Threonine	7.4
Linoleic acid	1.5	Tryptophan	2.0
Ash	5.5	Isoleucine	7.5
Dry matter	89.8	Arginine	14.7
		Phenylalanine	8.7
Energy(kJ/kg)	16100	Histidine	4.6
		Leucine	11.2
Minerals	**%**	Tyrosine	4.1
Ca	0.92	Valine	10.3
P	0.63		
K	0.90	**Vitamins**	**IU/kg**
Mg	0.12	Vit. A	20500
Na	0.42	Vit. D3	2000
Cl	0.74		**mg/kg**
		Choline	1000.0
		Vit. E	60.0
Trace elements	**mg/kg**	Vit. K3	2.4
Fe	105.0	Vit. B1 thiamine	13.0
Mn	70.0	Vit. B2 riboflavin	10.7
Zn	55.0	Vit. B3 niacin	32.0
Cu	17.5	Vit. B5 pentothenic acid	11.9
Co	0.2	Vit. B6 pyridoxine	12.5
I	0.4	Vit. B9 pyridoxine	3.8
		Vit. B12 pyridoxine	0.1
		Betaine	127.0
		Biotine	200.0
		Vit.C	95.0

Source: ABDIETS (www.abdiets.com)

Animal experiment

All aspects of the experimental protocol were reviewed and approved by the Animal Welfare Committee of Wageningen University (Wageningen, the Netherlands). Animals (5 rats per dose group) received one gavage dose for kinetic part, followed by a second gavage dose after 48 h for microarray analysis. The animals were sacrificed 4 h after the second dose and blood was collected for isolation of PBMC. Based on the life duration of rat and human (i.e. two years and seventy years, respectively) the relative exposure time for rats (i.e. two doses in 2 days, resulting in exposure during about 0.27% of their lifespan) is roughly comparable with that of the human volunteers in the parallel study (i.e. 56 days, resulting in exposure during about 0.22% of their lifespan). In addition, this study design allowed us to collect samples for both kinetic and gene expression studies using same animals, which is in accordance with the principles of the Three Rs (Replacement, Reduction, and Refinement). Different dose groups were 0 (solvent control), 2 or 20 mg SIF/kg bw, where the SIF was administered in the form of a commercial soy supplement (See below: Preparation of test solutions). The low dose (LD) of 2 mg SIF/kg bw is comparable with the maximum daily human consumption (i.e. >100 mg/day; [3]) although the usual dose taken by women using soy supplementation lies between 20-80 mg/day [36]. However, to get a clear effect of SIF in this rat study, a 10 times higher dose (HD) (i.e. 20 mg SIF/kg bw) was used as well.

Preparation of test solutions

The commercial soy supplement, Phytosoya, was obtained from Arkopharma (Carros, France). The total content of the SIF present in the supplement and their composition were determined by HPLC and previously reported [17]. The supplement had a total SIF content of 70.3 mg/gram supplement. The content of the three glucosides; genistin, daidzin and glycitin was 7.54, 40.03 and 22.72 mg/g supplement, respectively. The SIF content in this supplement was in accordance with the content indicated by the supplier on the label. On the basis of an average bodyweight of rats of 200 gram, an oral gavage dose of 2 mg/kg bw corresponds with 0.4 mg SIF per rat. Therefore a dose of 5.69 mg supplement per rat was applied. Similarly, a 10 times higher amount was used for the 20 mg/kg dose group. For the administration of a bodyweight dependent gavage dose (i.e. maximum of 1 mL gavage/200g bw), a 10 mL stock solution (i.e. 0.5 mL DMSO and 9.5 mL nanopure water) containing 56.9 mg supplement was prepared for the low dose group. Similarly, a stock solution with a 10 times higher concentration was prepared for the high dose groups. The stock solutions were freshly prepared and were kept at room temperature overnight. They were shaken thoroughly before use and placed on a multi-axle-rotating-mixture machine (CAT RM-40, Slaufen, Germany) during the period of gavage dosing. The control rats received a gavage dose of nanopure water containing 5 % DMSO.

Sample collection

Blood samples (~250 µl) were taken from the tail vein at 0, 10, 30 minutes and 1, 2, 8, 24, 48 h after dosing and collected in Microvette CB-300 tubes (Sarstedt AG & Co, Nümbrecht, Germany) containing lithium heparin. Directly after collecting the blood samples, plasma samples were prepared by centrifuging the blood samples for 5 minutes at 10,000 rpm (Biofuge centrifuge, Heraeus Sepatech, UK). Plasma samples were collected in Safe-lock 0.5 mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany) which were placed on ice to preserve degradation of the collected samples during the experiment. Urine samples (24 h) were collected in 50 mL graduated sterile polypropylene tubes and finally stored in 14 mL round bottom tubes; the tubes were purchased from Greiner bio-one (Frickenhausen, Germany). Faeces samples (24 h) were collected in 30 mL polystyrene tubes from Greiner bio-one (Gloucestershire, UK). All the urine and faeces samples were stored at -80 ^{0}C until further analysis. After collection of the last blood sample (48 h after dosing), a second dose was administered to the animals to study the effects on gene expression. Then 4 h later the animals were sacrificed after anaesthesia with a mixture of isoflurane and oxygen followed by removal of blood from the dorsal aorta. The collected blood was immediately transferred into 10 mL tubes and kept on a multi-axle-rotating-mixture machine (CAT RM-40, Staufen, Germany) to avoid clotting. This blood was used for the isolation of PBMC. For the isolation procedure see below.

Extraction of SIF from plasma, urine and faeces

Extraction of SIF from plasma was performed with 1 cc Waters Oasis HLB cartridges (Milford, MA, USA). The plasma extraction method included enzymatic hydrolysis and was performed according to Saracino et al. [37] and modified because of the small volume of the plasma samples. In short, 50 µL of plasma sample was mixed with 17 µg (17 µl of a stock solution of 1 mg/mL) of enterodiol (internal standard) and 275 µg (22 µl of a stock solution of 12.5 mg/mL) of *H. Pomatia* mixture. The final volume was made 170 µl by adding sodium-acetate (pH 5, 0.5 M). The samples were then incubated for 18 h at 37 °C. After incubation, 830 µl sodium-acetate was added, followed by centrifugation for 10 minutes at 10,000 × g, at 4 ^{0}C. The collected supernatants were used for solid phase extraction followed by evaporation of the extraction medium. Finally, the dried extracts were re-dissolved in 75 µl eluent B (i.e. 25 mM sodium phosphate, pH 2.4) and injected in the HPLC system.

Extraction of SIF from urine samples was carried out following enzymatic hydrolysis. In short, 70 µL of urine sample was mixed with 375 µg (30 µl from stock 12.5 mg/mL) of *H. Pomatia* mixture. The samples were then incubated for 2 h at 37 °C. After incubation, 200 µl pure acetonitrile and 100 µl of 20 % H_3PO_4 were added. The mixture was vortexed and centrifuged for 10 minutes at 10,000 g at 4 ^{0}C, and the supernatants (100 µl) were collected in HPLC vials.

Before extraction, faeces samples were lyophilised and powdered using pestle and mortar. Duplicate samples of 0.1 g were taken from each faeces sample and mixed with 500 µl absolute ethanol followed by homogenization using Precellys 24 (Bertin) at 6500 rpm for 30 second with a short interval after 15 seconds. Samples were centrifuged for 10 minutes at 13,500 × g at 4 ^{0}C. Finally, 200 µl of the supernatants were collected in HPLC vials for analysis. No enzymatic hydrolysis was applied because from other reports it is known that faeces only contains aglycons, as the gut microbiota are able to completely deconjugate all conjugated SIF, i.e. glucosides, glucuronides or sulphates [18, 38].

HPLC analysis

The SIF content of the supplements was determined according to the method of Penalvo *et al.*[39] by HPLC analysis with electrochemical detection. HPLC analyses were carried out on Lachrom Hitachi equipment (Varian, Sugarland, TX, USA) with a L2100 masterpump, a L2100 auto-sampler and a CoulArray electrochemical detector equipped with a high sensitivity analytical cell (model 6210, 4-sensor cell, ESA Inc., Chelmsford, MA, USA). A data collection system Varian Star 6.2 software was used for controlling the instrument and collecting the data from the electrochemical detector. A Symmetry Shield C18 column (150 mm × 4.6 mm, 5 µm) from Waters with a Brownlee New guard pre-column (7 µm) (Shelton, USA) was used for the analysis of the samples. The SIF were measured on 4 channels; 300, 500, 550, and 600 mV and quantified at 500 mV. The limit of detection for all SIF was 0.01µg/mL, whereas the limit of quantification was 0.02 µg/mL.Solutions of 10 %, 55 % and 30 % acetonitrile in 25 mM sodium phosphate (pH 2.4) were used as eluent A, B and C, respectively. The injection volume for HPLC analysis was 10 µl and the flow rate was 1 mL/minutes. The total running time was 27 minutes. Elution was started with 30 % of solvent B followed by a change of solvent B as 30, 100, 100, 30 and 30 % in 0.0-0.1, 0.1-17, 17-19, 19-20 and 20-27 minutes, respectively. The retention times for genistein, daidzein, glycitein, equol and enterodiol were 17.5 ± 0.2, 12.8 ± 0.1, 12.1 ± 0.1, 16.3 ± 01 and 10.2 ± 0.2 minutes, respectively. The differences in SIF concentrations between the dose groups and the control were analysed by student t-tests using Microsoft Excel.

Isolation of PBMC, mRNA and running microarray analysis

PBMC were isolated immediately after blood collection using OptiPrep^{TM} (60 % w/v iodixanol) as a density gradient medium following the online protocol by Axis-Shield (www.axis-shield-density-gradient-media.com> methodology > cells > C43). After isolation, all samples were dissolved in RLT buffer of Qiagen (Venlo, the Netherlands) for lysis of cells to isolate RNA, and stored at -20 ^{0}C until further analysis. RNA extraction was carried out by using Qiagen RNeasy Mini Kit (Qiagen, Venlo, the Netherlands). The extracted amount of RNA was quantified using a Nanodrop ND 1000 spectrophotometer (Nanodrop Technologies) and integrity (i.e. quality) was measured (ng/µl) using an Agilent 2100 Bioanalyzer with RNA 6000 Nanochips (Agilent Technologies, Amstelveen, the Netherlands). Finally, samples were selected for gene expression analysis on an AffymetrixGeneChip

Rat Gene 1.1 ST plate (Affymetrix, Santa Clara, CA). One hundred nanogram of total RNA was used for whole transcript cDNA synthesis with the Ambion WT expression kit [catalog number 4411974] (Applied Biosystems/Life Technologies, Nieuwekerka/dIJssel, the Netherlands). Hybridization, washing and scanning of the Rat Gene 1.1 ST peg arrays was performed on a GeneTitan Instrument (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations.

Packages from the Bioconductor project [40], integrated in an online pipeline [41], were used to analyse the array data. Various advanced-quality metrics, diagnostic plots, pseudo images, and classification methods were used as described by Haber and Sick [42], to determine the quality of the arrays before statistical analysis. Three controls and one HD sample did not pass the quality control criteria, and were removed from the dataset. The final dataset thus obtained consisted of 11 arrays. The probes on the Rat Gene 1.1 ST array were redefined using current genome information [43]. In this study, probes were reorganized on the basis of the gene definitions available in the National Center for Biotechnology Information (NCBI) Rattus norvegicus Entrez Gene database based on the rat genome build 5.1 (custom CDF v17). Normalized gene expression estimates were calculated with the Robust Multichip Average method [44]. Subsequently, the dataset was filtered to only include probesets (genes) that were active (i.e. expressed) in at least 4 samples using the universal expression code (UPC) approach (UPC score > 0.50) [45]. This resulted in the inclusion of 7,650 of the 19,311 (40 %) probe sets present on the array. Differentially expressed probe sets were identified by using linear models, applying moderated t-statistics that implemented intensity-based empirical Bayes regularization of standard errors [46, 47]. Probe sets that satisfied the criterion of $p < 0.05$, were considered to be significantly regulated. Changes in gene expression were related to functional changes using gene set enrichment analysis (GSEA) performed according to Subramanian et al. [48].GSEA focuses on groups of genes that share a common biological, biochemical or metabolic function. GSEA has the advantage that it is unbiased, because no gene selection step is used. Only gene sets consisting of more than 15 and less than 500 genes were taken into account. Gene sets were derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [49]. Effects of the low and high dose of SIF versus control were compared using ranked lists based on significance (t-values), using methods described by Plaisier et al. [50].

Results

Kinetics

The commercial supplement used in the present study contains predominantly daidzin (~57%), and smaller amounts of glycitin (~32%) and genistin (~11%). See also the “Preparation of test solution” section above. Figure 4.1 shows the plasma concentrations of daidzein and equol following a single oral administration of a low dose of 2 mg/kg bw (LD) and a high dose of 20 mg/kg bw (HD). As is shown in figure 4.1, daidzein was rapidly absorbed with peak plasma concentrations within 1 hour

after dosing. The corresponding pharmacokinetic data are presented in table 4.2. The maximum observed highest plasma concentration (Cmax) for daidzein was 3.2 fold higher and the area under the curve (AUC) was 6.6 fold larger in the HD group compared to the LD group. For equol there was an apparent lag time of about 1 h in both dose groups before its appearance in plasma. Both the Cmax and AUC for equol was 4.2 fold higher in the HD group than in the LD group. Furthermore it can be seen from figure 4.1 that equol is not completely cleared from the plasma within a period of 48 h after dosing.

Figure 4.1

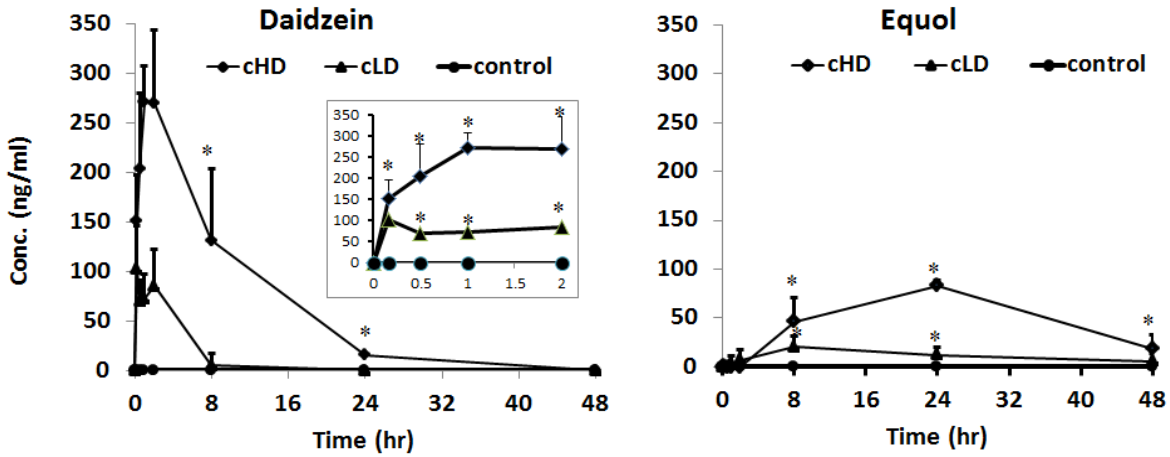


Figure 4.1: Plasma curves of isoflavones in F344 ovariectomized rats after a single oral gavage dose (2 and 20 mg/kg bw) of a commercial soy supplement. Only daidzein and equol could be detected (the limit of detection for all SIF was 0.01µg/mL, and the limit of quantification was 0.02 µg/mL). Data are expressed as mean ± SD. Sample size was 5 rats/group. LD (▲) and HD (♦) stand for low and high dose group, respectively. A significant effect in LD and HD at specific time points is denoted by * ($p < 0.01$). The inserted graph shows the plasma curve of daidzein during the first 2 h.

Table 4.2: Plasma pharmacokinetic parameters of daidzein and equol in female ovariectomized F344 rats that received a single oral gavage dose (2 or 20 mg/kg bw) of SIF from a commercial SIF supplement.

Isoflavone dose (mg/kg b. w.)	Daidzein				Equol		
	C_{max} (ng/mL)	T_{max} (h)	t ½ (h)	AUC (µg.h/mL)	C_{max} (ng/mL)	T_{max} (h)	AUC (µg.h/mL)
2	85±37	0.17	2.5	0.46	20±11	8	0.56
20	272±36	1.0	6	3.02	83±6	24	2.39

Figure 4.2 shows the urinary and faecal excretion of SIF and of equol, in samples collected over a 24 h period. In urine of the LD group, the highest concentration was found for daidzein, followed by equol and genistein, while glycitein could not be quantified because of an interfering peak in the chromatogram. In the HD group the highest concentration was found for daidzein, followed by glycitein, equol and genistein (see figure 4.2a and table 4.3). The concentrations of SIF in different dose groups were significantly different compared to the control group and also between the different dose groups. In faeces highest concentrations were found for equol, followed by daidzein and genistein (see Fig. 4.2b and table 4.3) and we did not find any conjugated SIF (i.e. no glucosides, glucuronides or sulphates). Again due to interference of an unidentified peak in the chromatogram at the same retention time, it was not possible to quantify glycitein in the faeces of both LD- and HD-group rats. To estimate the relative amount (%) recovered in urine and faeces, the administrated dose of the respective SIF as glucoside, was converted in 'aglycon equivalents' using their corresponding aglycone/glucoside molecular weight ratio [1]. For equol, the amount in faeces and urine was first converted into 'daidzein equivalent' based on the respective molecular weights and then compared with the administrated dose of daidzin expressed as 'daidzein equivalents'.

The relative amount of daidzein recovered in urine plus faeces collected over a 24 h period was 36% and 33% of the administered dose of daidzin (expressed as aglycone equivalents) for the LD and HD group, respectively (Table 4.3). When also equol was taken into account a total of 65% (36% + 29%) and 47% (33% + 14%) of the administrated dose of daidzin was recovered in the LD and HD group, respectively. For genistein the relative amount recovered in urine plus faeces was 22% for both the LD and the HD group. Glycitin could not be detected in the urine and faces of LD rats, and only in the faeces of HD rats, at a relative amount of 22% of the administered glycitin dose (Table 4.3).

Table 4.3: Amount of SIF in urine and faeces (µg/rat) after 24 h in F344 ovariectomized rats (n=2 for control and 5 for treatment groups). LD stands for low (i.e. 2 mg/kg bw) and HD for high (i.e. 20 mg/kg bw) dose. Significant differences with the control are denoted by $*$ ($p < 0.05$) or $**$ ($p < 0.01$).

Group	Daidzein			Equol			Glycitein			Genistein		
	Urine	Faeces	[1](%)	Urine	Faeces	[2](%)	Urine	Faeces	[1](%)	Urine	Faeces	[1](%)
LD	48 ± 23**	2.1 ± 1*	36	20 ± 6**	19 ± 4**	29	ND	ND	-	5.5 ± 3.0**	0.32 ± 0.1**	22
HD	434 ± 73**	19 ± 17	33	114 ± 38**	74 ± 30**	14	178 ± 56**	ND	22	56.0 ± 12.7**	1.40 ± 0.7*	22

[1] The relative amounts of the various isoflavones (sum in urine and faeces), expressed as percentage of the administrated dose (%), was calculated based on "aglycone equivalents" by correcting for the difference in molecular weight between the respective glucoside and its aglycone.

[2] For equol, the amount was first converted into daidzein (aglycone) equivalents and then expressed as percentage of the administrated dose of daidzin based on "aglycone equivalents".

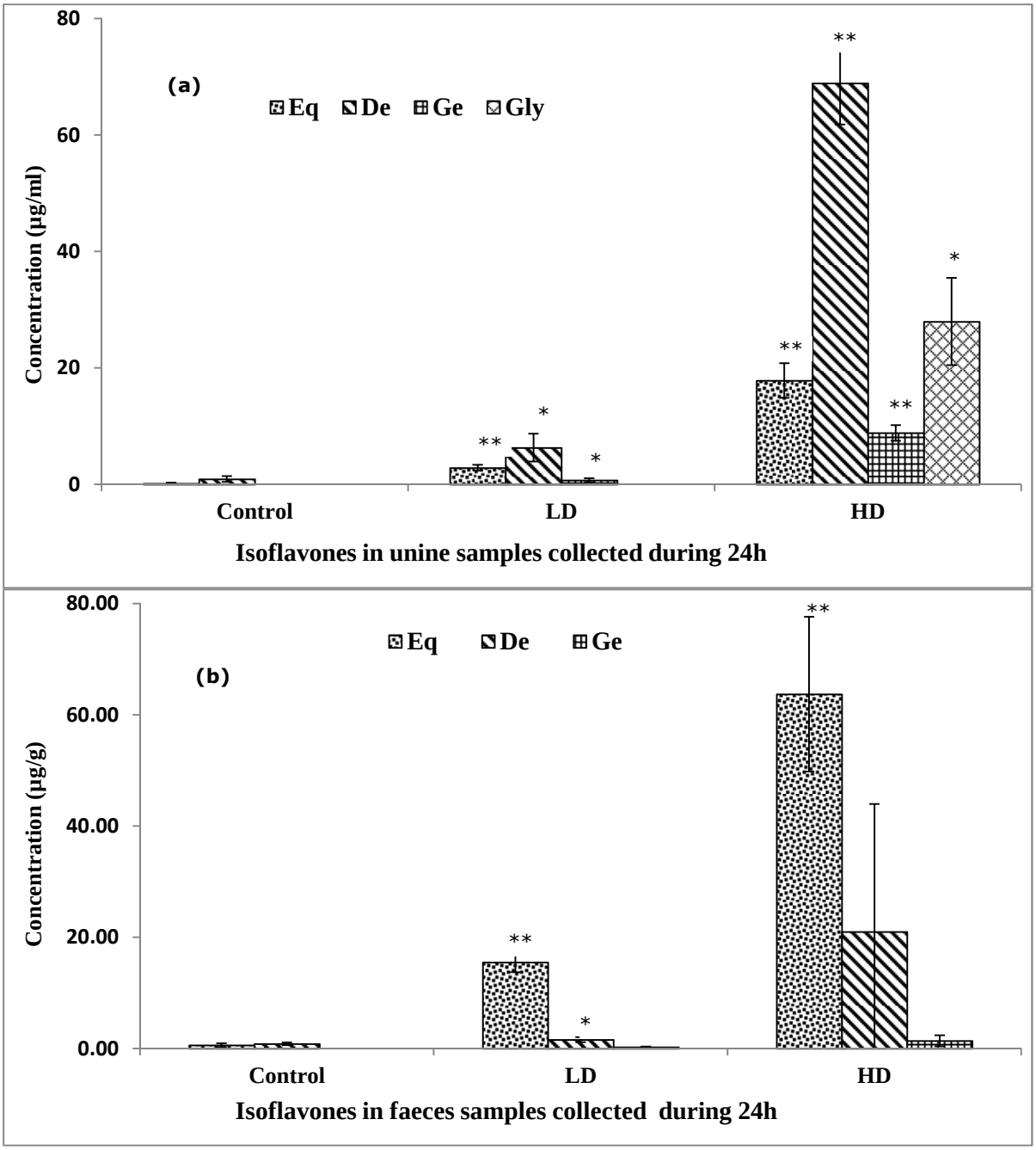


Figure 4.2: Amount of isoflavones in urine (a) and faeces (b) of F344 female ovariectomized rats over a 24 h time period after administration of a single oral dose (2 and 20 mg/kg bw SIF) of a commercial soy supplement. Data are expressed as mean ± SD. Sample size was 5 rats/group. LD and HD stand for low and high dose, respectively. Significant effects between LD and HD groups compared to control at specific time points are denoted by $*$ ($p< 0.01$) and $**$ ($p< 0.001$).

Microarrays

Figure 4.3 shows an overview of the microarray analysis. Out of 7650 filtered genes a total of 3861 and 4785 genes were significantly changed ($p < 0.05$) in the LD and the HD group compared to control, respectively. Of those genes 1338 were up regulated in the LD group and 1527 in the HD group, and 2523 genes were down regulated in the LD group and 3258 in the HD group. Figure 4.4 shows a multidimensional scaling (MDS) plot that was created using the top 500 most affected genes. Separation of the samples on the first dimension (i.e. X-axis), explaining most variation, suggests a dose response. In the 2nd dimension (i.e. Y-axis) a smaller variation and a separation of the LD group from the HD and control groups is observed.

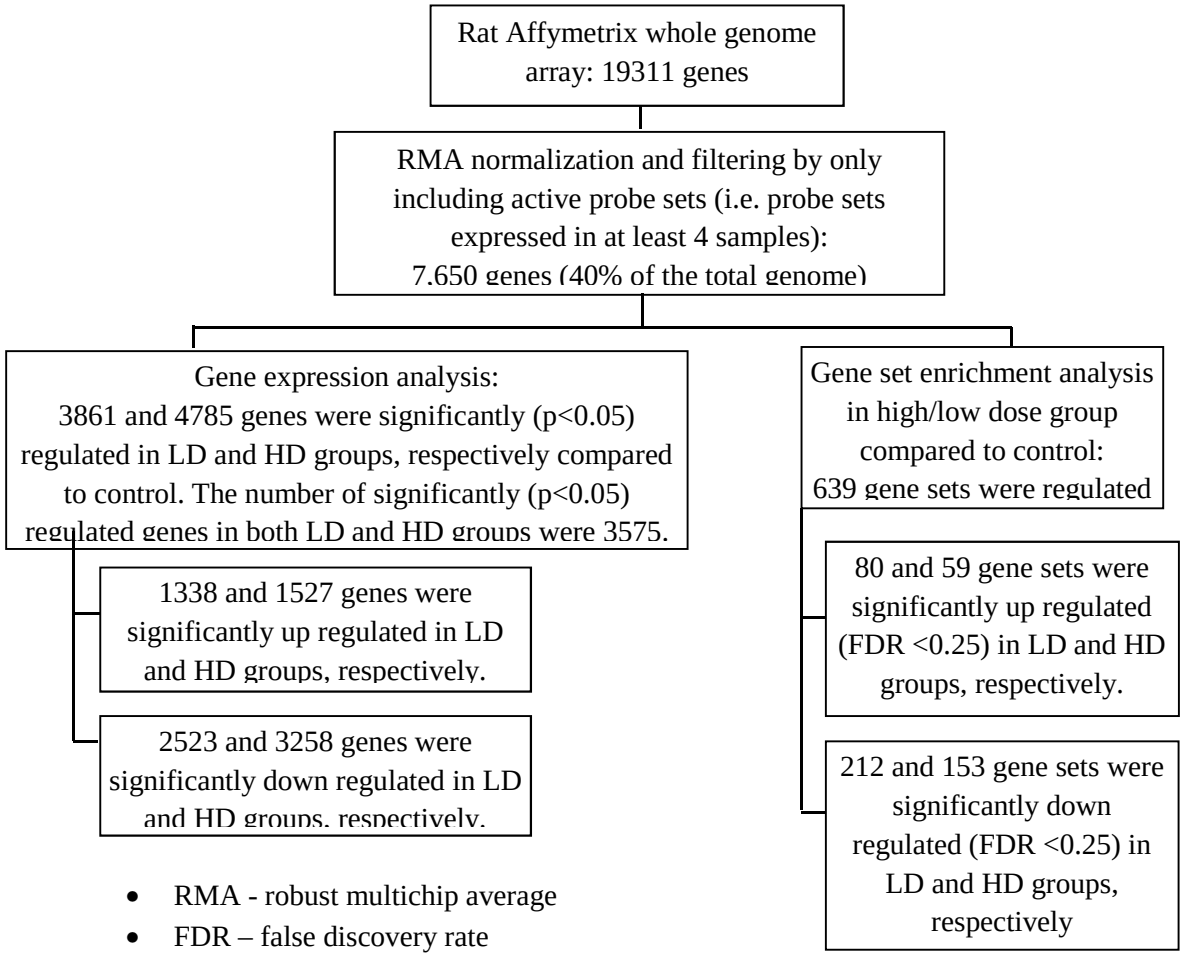


Figure 4.3: Flow chart of microarray analysis.

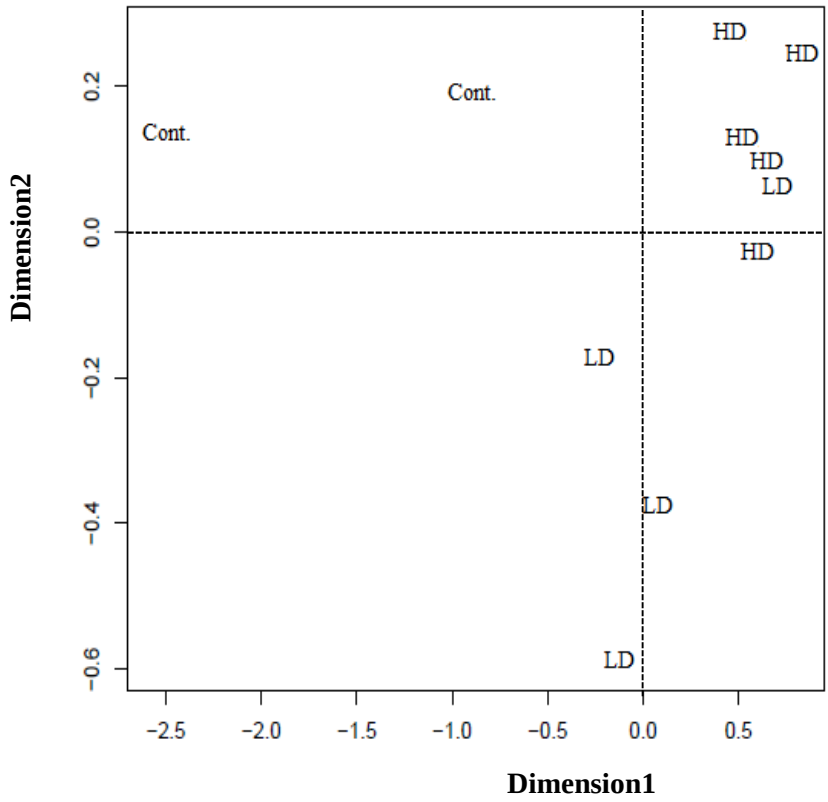


Figure 4.4: Multidimensional scaling (MDS) plot of all arrays. The plot was generated using the top 500 most affected genes. The distances in both dimensions are arbitrary units where LD, HD and cont. stands for low dose, high dose and control rats, respectively.

In figure 4.5 the log (base 2) fold change plot of all the 7650 filtered genes, without using any cut off value, in the two different treatment groups compared to the control is presented. The upper right quadrant shows up regulation of genes in both dose groups and the lower left quadrant shows down regulation of genes in both groups. It can be seen that administration of the SIF supplement produced a maximum 4-fold change in gene expression where more genes were positively regulated. Moreover, there is a positive correlation (r^2 = 0.96) in effects on gene expression between the LD and the HD group.

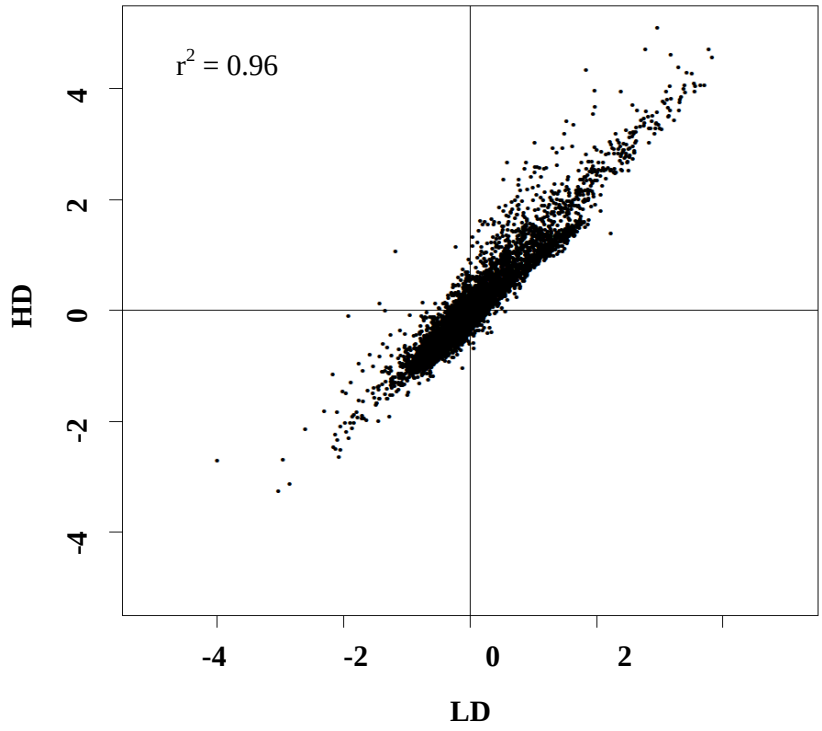


Figure 4.5: Log (base 2) fold change (FC) plot of the LD vs. HD group compared to controls. The log2 FC plots were prepared using data of all 7650 genes.

As the effect of SIF are expected to be induced via estrogen receptors (ERs), we have selected all the estrogen-responsive genes (ERGs) for the rat available in Dragon estrogen-responsive database (http://datam.i2r.a-star.edu.sg/ergdbV2/) which is based on the publication of Tang et al. [51]. Table 4.4 shows the significantly ($p < 0.05$) up and down regulated estrogen-responsive genes (ERGs) in the LD and HD groups. Out of 87 available rat ERGs 16 ERGs were found to be significantly regulated in the LD group, of which 7 genes were up-regulated and 9 down-regulated. In the HD group, 19 ERGs were significantly regulated, of which 8 were up-regulated and 11 down-regulated. A number of 15 ERGs were induced in the same direction in the LD and the HD group, meaning that genes that were up-regulated in the LD group were also up-regulated in the HD group, and those that were down-regulated in the LD group were also down-regulated in the HD group.

Table 4.4: List of significantly ($p < 0.05$) up- and down regulated estrogen-responsive genes (ERG) in PBMC of ovariectomized F344 rats treated with a low (LD, 2 mg/kg bw) or high (HD, 20 mg/kg bw) dose of SIF from a commercial supplement, compared to the control. Up and down regulation are indicated with up and down arrows. A list of ERG in the rat can be found in the Dragon ERG database (http://datam.i2r.a-star.edu.sg/ergdbV2/).

Gene	Description	LD	HD
ANGPT1	Angiopoietin 1	↑	↑
ANXA4	Annexin A4	ns	↑
BCL2	B-cell CLL/lymphoma 2	↓	↓
CCND2	Cyclin D2	ns	↓
CDKN1B	Cyclin-dependent kinase inhibitor 1B	↓	↓
CEACAM1	Carcinoembryonic antigen-related cell adhesion molecule 1 (biliary glycoprotein)	↑	↑
HK1	Hexokinase 1	↑	↑
HPCAL1	Hippocalcin-like 1	↓	↓
IL6R	Interleukin 6 receptor	↓	ns
ITGA6	Integrin, alpha 6	↑	↑
MARK2	MAP/microtubule affinity-regulating kinase 2	ns	↓
NCOA1	Nuclear receptor coactivator 1	↓	↓
NDRG2	NDRG family member 2	↓	↓
PAM	Peptidylglycine alpha-amidatingmonooxygenase	↑	↑
PHF5A	PHD finger protein 5A	↓	↓
PSMA7	Proteasome (prosome, macropain) subunit, alpha type 7	↓	↓
RB1	Retinoblastoma 1	↑	↑
SCP2	Sterol carrier protein 2	ns	↓
SFRP4	Secreted frizzled-related protein 4	↑	↑
SLC25A4	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 4	↓	↓

ns = not significant.

As next step GSEA was performed to elucidate which differentially regulated gene sets related to biological processes could be affected by treatment with SIF. Data revealed that a total of 639 gene sets were changed in the two treatment groups, of which 80 and 59 gene sets were up-regulated and 212 and 153 were down-regulated in LD and HD group respectively, compared to the control and

based on a False Discovery Rate (FDR) < 0.25 (Figure 4.3). Based on the KEGG database, Table 4.5 shows biological pathways (BPs) which are significantly up (↑) or down (↓) regulated. In total 54 KEGG based BPs were significantly changed out of which 21 and 27 gene sets were up regulated and 16 and 21 gene sets were down regulated in the LD and HD group, respectively. About half of these BPs were affected in the same direction in the LD and the HD group (i.e. either significantly up regulated or down regulated in both dose groups). The data show that all the BPs related to genetic information processing such as RNA transport and degradation and DNA replication were down regulated. Some of the BPs related to human diseases such as pathways related to colorectal and pancreatic cancer are down regulated; others such as hypertrophic and dilated cardiomyopathy were up-regulated. Also BPs related to environmental information processing, such as extracellular matrix (ECM)-receptor interactions which serves an important role in tissue and organ morphogenesis, and control of cellular processes such as endocytosis, phagocytosis, proliferation and apoptosis, were significantly up regulated. For a number of BPs a significant effect was found in one dose group only, whereas the effect in the other group was non-significant. This is for instance the case for the citrate cycle, oxidative phosphorylation, regulation of the actin cytoskeleton and others (see table 4.5).

Table 4.5: Gene set enrichment analysis (GSEA) of KEGG based significantly regulated (FDR <0.25) biological pathways (BPs) in the PBMC of ovariectomized F344 rats treated with a commercial supplement compared with control. LD and HD stands for low and high dose group, while up and down regulated enrichments are indicated by up and down arrows. The names of the different pathways are literally quoted from KEGG together with the number of genes involved in the specific pathways.

BPs related to	BP	No of Genes	cLD_c	cHD_c
Metabolism	Citrate cycle (TCA cycle)	24	↓	Ns
	Oxidative phosphorylation	82	↓	Ns
	Glutathione metabolism	30	↑	↑
	N-glycan biosynthesis	36	↓	↓
	Porphyrin and chlorophyll metabolism	17	↑	↑
Genetic information processing	Basal transcription factors	22	↓	Ns
	Spliceosome	94	↓	↓
	Aminoacyl-tRNA biosynthesis	25	↓	Ns
	Ribosome biogenesis in eukaryotes	56	↓	↓
	Ribosome	35	↓	↓
	RNA transport	105	↓	↓
	mRNA surveillance pathway	52	↓	↓
	RNA degradation	49	↓	↓
	Proteasome	40	↓	Ns

Table 4.5 continued

BPs related to	BP	No of Genes	cLD_c	cHD_c
Genetic information processing	Protein export	18	↓	↓
	Protein processing in endoplasmic reticulum	129	↓	Ns
	DNA replication	28	↓	↓
	Base excision repair	18	↓	↓
	Mismatch repair	19	ns	↓
Environmental information processing	ABC transporters	15	↑	Ns
	Neuroactive ligand-receptor interaction	33	↑	Ns
	ECM-receptor interaction	19	↑	↑
Cellular processes	Lysosome	92	ns	↑
	Endocytosis	126	↑	↑
	Phagosome	90	↑	↑
	Regulation of actin cytoskeleton	104	↑	Ns
	Focal adhesion	81	↑	↑
	Gap junction	37	↑	↑
	Complement and coagulation cascades	16	↑	↑
	Toll-like receptor signaling pathway	54	↓	Ns
	Hematopoietic cell lineage	45	↑	↑
	Natural killer cell mediated cytotoxicity	63	ns	↓
	T cell receptor signaling pathway	77	↓	↓
	Intestinal immune network for IGA production	20	ns	↓
	PPAR signaling pathway	26	↑	↑
	Cardiac muscle contraction	29	ns	↑
	Vascular smooth muscle contraction	50	↑	Ns
	Pancreatic secretion	37	↑	Ns
	Aldosterone-regulated sodium reabsorption	18	↓	Ns
	Long-term depression	31	↑	Ns
	Circadian rhythm - mammal	15	↓	↓
	Pathways in cancer	142	↓	↓
	Chagas disease (American trypanosomiasis)	59	↓	↓
	Malaria	23	↑	↑
	Colorectal cancer	46	↓	↓
	Pancreatic cancer	41	ns	↓
	Primary immunodeficiency	26	ns	↓
	Parkinson's disease	81	↓	ns
	Huntington's disease	109	↓	ns
	Prion diseases	18	↓	ns
	Hypertrophic cardiomyopathy (hcm)	23	↑	↑
	Arrhythmogenic right ventricular cardiomyopathy (arvc)	20	↑	ns
	Dilated cardiomyopathy	25	↑	↑
	Staphylococcus aureus infection	18	↑	↑

Figure 4.6(a) presents the rank-rank scattered plot based on the t-value of the significantly changed and common PBMC genes in the two dose groups of rats. Out of 7650 filtered genes (see figure 4.3) 3575 genes were significantly changed in both LD and HD groups, and these were used to prepare the scatted plot. The size of quadrants in figure 4.6(a) is not the same, because the number of significantly down regulated genes was higher than number of significantly up regulated genes (see figure 4.3). A strong correlation in gene expression profile between both treatment groups (LD and HD) relative to the controls of the rat study is observed.

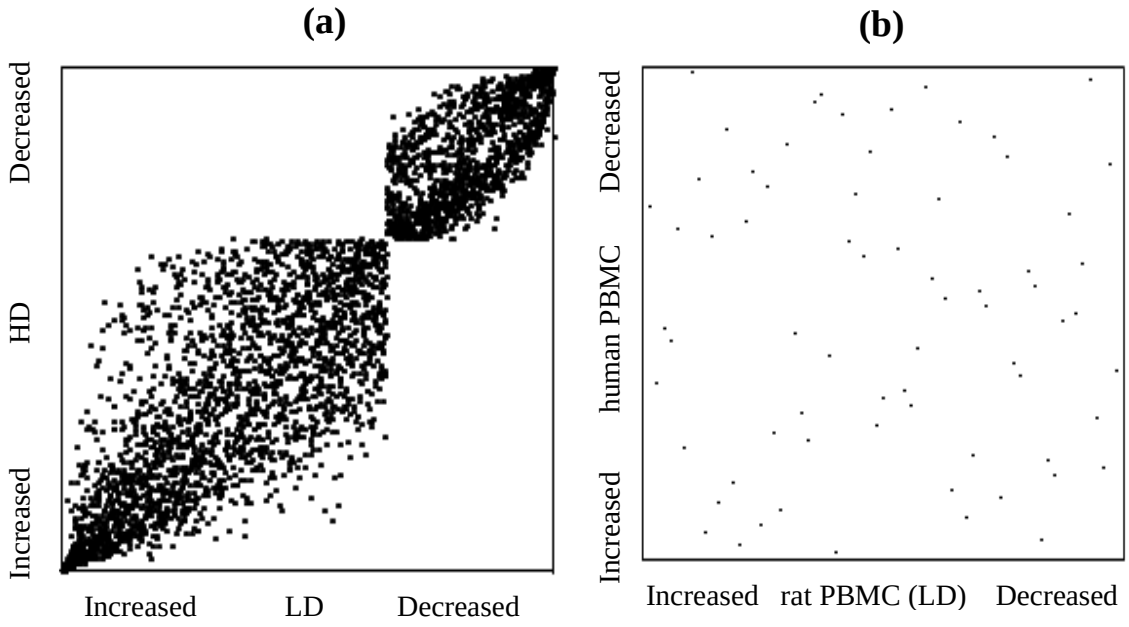


Figure 4.6: Rank-rank scattered plot for significantly changes in gene expression among the LD and the HD group relative to the controls (6a), and for the LD group compared to (post)menopausal women (6b). A similar picture was found for HD rats vs. (post)menopausal women (data not shown). Out of 7650 filtered genes (see figure 4.3) 3575 genes were significantly changed ($p < 0.05$) in both LD and HD groups; while out of 7650 filtered genes, only 69 genes were significantly changed ($p < 0.05$) in both rat and homologous human PBMC. These 3575 and 69 genes were used to build the scattered plot. Each dot represents the t-value of a single gene. Corresponding human homologous genes were retrieved from the study with (post)menopausal women taking the same supplement, in a similar dose as the LD rats [23]. Genes significantly changed in the same direction in both treatments are in Cartesian quadrants I and III, while genes significantly changed in opposite directions are in quadrants II and IV. The density of the dots visualise similar trends in regulation among the two different groups.

Discussion

In this study we evaluated the use of ovariectomized rat model to predict (post)menopausal health risk and/or benefit of SIF supplementation by comparing transcriptomics data obtained in a parallel human intervention study in which a similar SIF supplement were given to (post)menopausal women. The plasma concentration of our study shows a dose dependent increase of SIF after administration of different doses of a commercial supplement. This is also reflected by the differences in Cmax and AUC (Figure 4.1 and Table 4.2). Only daidzein and its metabolite equol could be detected in plasma, because the commercial supplement contained a higher amount of daidzin than of the other SIF. The uptake of daidzein is very fast, peaking at 10 min (see insert of Figure 4.1). In the LD group, which is the relevant dose group to be compared with the experiment in human volunteers, the levels decrease, and then rise again, with a peak at 2 h. This biphasic pattern in the plasma concentration of daidzein, suggesting enterohepatic circulation, is also observed in human intervention studies [52-54]. Franke *et al*. [55] mentioned that this biphasic plasma bioavailability might occur due to the location of uptake of SIF, where the first early peak represent the uptake from the small intestine and the second peak represents the uptake from the large intestine. However, in the HD group, with a 10 times higher dose than the LD group, this biphasic peak did not appear. It can be suggested that due to the higher amount of SIF in the small intestine, compared to the LD group, a longer period is needed to deconjugate the administered dose of SIF glucosides by the intestinal enzymes, leading to a longer absorption time in the small intestine, thereby preventing the occurrence of a biphasic absorption behaviour. Sepehr *et al.* [1] also did not observe any biphasic behaviour in the plasma profile of SIF in Sprague Dawley rats receiving an oral gavage of 20 mg/kg bw of a commercial soy supplement.

In faeces no conjugated SIFs were detected. This is in line with other authors [38, 56] reporting that SIFs are completely deconjugated by gut microbiota. In both the LD and HD group the equol concentration was significantly higher compared to daidzein, indicating an efficient conversion of daidzein into equol by rat colonic microbiota [57, 58]. The later appearance of equol in plasma compared to daidzein is also consistent with its production in the large intestine [1, 59]. All rats are equol producers and several colonic bacteria are involved in the production of equol in rats [59]. However, only 25-30% of the adult Western population is able to produce equol due to lack of the specific colonic microbiota. This percentage is significantly lower than in Eastern Asian populations where more than 50% of all adults can produce equol [25, 48, 59]. Sepehr et al. [1] reported that dosing Sprague-Dawley rats (both male and female) with a mixture of all three SIF glucosides or a commercial SIF supplement increases the production of equol compared to dosing with aglycone alone. In vitro studies with human colonic bacteria suggested that a high carbohydrate environment stimulates the colonic fermentation and increases the rate of conversion of daidzein into equol [16, 17].

From the transcriptomic analysis it appears that out of the 19311 gene probes on the array chip about 20-25% genes were significantly changed in the different dose groups compared to the control (figure 4.3). To the best of our knowledge there is no literature available on the effect of soy supplementation on gene expression in rat PBMC, therefore, it was not possible to directly compare our PBMC data with published data. However in the parallel study of Van der Velpen [23] PBMC of (post)menopausal equol-producing human volunteers, exposed to the same SIF supplement, only 2% of the total number of genes was significantly changed. This suggests that rats might be more susceptible to effects of SIF than humans. It is unclear, however, to what extent the inbred nature of the rats could have contributed to this difference. In both studies the transcriptomic data were produced and analysed in the same laboratory, thus preventing intra laboratory variation between the two studies. It is generally known that rats have a higher metabolic potency compared to humans which is also true for SIF [17]. Therefore a higher enzymatic activity in rats might probably results in a higher number of affected genes [60]. In the rat study also a strong dose dependent correlation was shown by the rank-rank scattered plot (figure 4.6a) and the log (base 2) analysis (figure 4.5). We also found that about 20% of the total rat ERGs was significantly changed: 16 and 19 ERGs were significantly regulated in LD and HD group, respectively out of 87 ERGs. Some of the affected ERGs are involved in important biological endpoints that might be related to beneficial health effects like lower risks for breast cancer as observed in epidemiological studies [13, 61]. For example, the RB1 (retinoblastoma1) gene, which is up regulated in the present study after SIF treatment, is a tumor suppressor gene, and a negative regulator of the cell cycle [62]. Hanahan and Winberg [64] stated that down regulation of the retinoblastoma protein pathway by the RB1 gene is a “hallmark of cancer”. Down regulation of RB1 is positively correlated with tumor formation and in our present experiment this RB1 gene was up regulated, indicating a possible beneficial effect of SIF supplementation on tumor formation.

To investigate whether the observed effects in rats are predictive for effects in humans, the gene expression data from the present rat study were compared with the data of van der Velpen et al. [23] who performed a parallel human intervention study. They found that of the 1069 human ERGs mentioned in the Estrogen Responsive Gene Database [51] only 17 human ERGs were significantly changed, of which 7 were up regulated and 10 were down regulated. We have investigated whether these 17 human ERGs are also significantly regulated in the present rat study. Table 4.6 shows the significantly expressed ERGs in PBMC of human volunteers reported by Van der Velpen et al. [23] and their expression in PBMC of rats of the current study. It is shown that 10 out of these 17 genes were common in both rat and human PBMC. Seven of these 10 genes were significantly changed, but only 3 were changed in the same direction in rats and humans: CACYBP and NME2 were up-regulated and STXBP1 was down-regulated. It is interesting to note that more than half of the number of human ERGs is also found in rat PBMC and that most of them are significantly affected by SIF treatment. However, most of the common genes are regulated in opposite directions in rats and

humans. This means that if they are up regulated in human PBMC they are down regulated in rat PBMC or if they are down regulated in human PBMC they are up regulated in rat PBMC. Therefore rat PBMC do not seem to be a suitable model to predict effects of SIF treatment on ERGs in human PBMC. It should, however, be noted that these human genes identified as ERGs by the Estrogen Responsive Gene Database [51], and also foundin rat PBMC, are not identified as rat ERGs by the Dragon Database. Further information is needed to conclude whether these genes are also estrogen responsive in rats.

Table 4.6: List of the significantly expressed human estrogen responsive genes (ERGs) in PBMC of human volunteers reported by van der Velpen et al. [23] and their expression in PBMC genes in the present rat study. LD and HD stands for low and high dose groups while up and down regulated gene expressions are indicated by up and down arrow marks.

Genes	Description	Regulation		
		Human	Rat	
			LD	HD
BCL2L1	BCL2-like 1	↑	↓	↓
CACYBP	Calcyclin binding protein Y	↓	↓	↓
EDEM1	Endoplasmatic reticulum degradation enhancer, mannosidase alpha-like 1	↑	↓ns	↓ns
ERBB2	v-erb-b2 erythroblastic leukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog (avian)	↑	a	a
FKBP5	FK506 binding protein 5	↑	↓	↓
FOXP1	Forkhead box P1	↑	↓	↓
HSPA1A	Heat shock 70 kDa protein 1A Y	↓	a	a
MYB	v-mybmyeloblastosis viral oncogene homolog (avian)	↑	a	a
NME2	NME/NM23 nucleoside diphosphate kinase 2 Y	↓	↓	↓
NRP1	Neuropilin 1 Y	↓	↑ ns	↑ ns
PSMD8	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 Y	↓	↑	↑
PTPRO	Protein tyrosine phosphatase, receptor type, O Y	↓	↑ns	↑
SLC25A5	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	↓	a	a
SPRED1	Sprouty-related, EVH1 domain containing 1 Y	↓	a	a
STAB1	Stabilin 1 Y	↓	a	a
STXBP1	Syntaxin binding protein 1	↑	↑	↑
TIMELESS	Timeless homolog (Drosophila)	↓	a	a

a - absent; ns - not significant

When the significantly changed and common PBMC genes in the LD group of the rat study and the human intervention study by van der Velpen et al. [23] were compared, it was found that only 69 common genes were significantly changed in both rat and human PBMC. However, in the rank-rank scattered plot (figure 4.6b) no overall correlation for the changes in PBMC of rats and humans was observed. Also for the HD group of the rat study no correlation with the results and the human

intervention study was found (data not shown). This lack of correlation is in line with the observation that of the common affected ERGs in rat and human PBMC. Only three were changed in the same direction. Another important example of a gene that is affected in a different direction in rat and human is BCL2 which is up regulated in human indicating induction of apoptosis [33] but down regulated in rat.

In the present study we have observed beneficial effect of SIF on ovariectomized rat but also significant species differences in the changes in gene expression in PBMC of rats and humans following comparable exposure to a commercial SIF supplement. Setchell et al. [63] also found significant species differences of the circulating concentrations of aglycones among rodent and human. Based on differences in the proportion of unconjugated SIF in plasma of humans in rodents, in particularly for certain strains of mice, they also questioned the value of these rodent models for the assessment of effects of SIF in humans. However, in the article of Setchell et al. [63] different isoflavone sources and administration protocols were used to treat animals and human volunteers. Animals were treated with much higher dose levels compared to humans. In addition the adult human data span wide range of ages (i.e. from 21-65 years). Therefore, the higher aglycone concentrations of IF in the plasma of rats compared to that of humans may also be due to the higher dose levels used in the rat studies and enzymatic variation among different age group of human individuals. In our present study we have overcome these difficulties by using the same source and dose level to compare plasma bioavailability and PBMC gene expression of ovariectomized rat with equol producing menopausal women. To further increase the comparability of rat data with human data, only equol producing menopausal women were included in the parallel human study because all rats are equol producers, while only 25-30 % of the Western populations are equol producers [59].

However, the comparison of the results of the current rat study with those of the parallel study with human volunteers [23] indicates that the rat might not be a suitable model to predict effects of SIF in humans. It should, however, be recognized that, although the relative exposure period in rats and the human volunteers (i.e. 0.27% for rat and 0.22% for humans, see above, Materials and methods/Animal experiment) in relation to their respective life span is comparable, longer term repetitive administration of rats to SIF may be needed to draw a final conclusion on the suitability of the rat model, because longer exposure might lead to a difference in gene expression [62].

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5

Soy supplementation: impact on gene expression in different tissues of ovariectomized rats and evaluation of the rat model to predict (post)menopausal health effects.

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List of abbreviations:

AT- Adipose Tissue, BT- Breast Tissue, UT- Uterus Tissue, LT – Liver Tissue, ST – Sternum Tissue.

Abstract

A toxicogenomics approach was used to compare gene expression changes in different target (i.e. breast (BT), uterus (UT) and sternum (ST)) and non-target (i.e. peripheral blood mononuclear cells (PBMC), adipose (AT) and liver (LT)) tissues of ovariectomized F344 rats exposed to a commercially available soy supplement for eight weeks. The supplement had a total soy isoflavones (SIF) content of 70.3 mg /g supplement and the dose level used provides the equivalent dose (i.e. 2 mg SIF/kg body weight per day) for an adult consuming this soy based supplement. Gene expression changes were analyzed using whole-genome microarray analysis. Rank-rank scattered plots did not show any correlation in gene expression changes among different tissues. Out of 87 estrogen responsive genes (ERG), only 19 were found significantly regulated ($p<0.05$) in different tissues. The significantly regulated ERG were found mostly in LT, AT and UT. Surprisingly, no ERG were significantly regulated in BT and ST although these are considered to be important estrogen sensitive target organs. The changes in gene expression in PBMC of the present study were compared with those of a previously reported short- term rat study where the same type of supplement, and similar dose and rat strain were used, but no correlation was found between PBMC data of the two studies. In addition, we compared the changes in gene expression in PBMC and adipose tissue of our present study with results of parallel human intervention studies, in which the similar dose, supplement and exposure duration was used to evaluate the applicability of the ovariectomized rat model to predict (post)menopausal health risk and/or benefit of soy isoflavone (SIF) supplementation. The overall changes in gene expression indicated that using rat transcriptomic data as a model for human risk or benefit analysis of SIF supplementation is challenging and probably not suitable.

Introduction

The advances in health care and the public initiative towards healthy living have increased the number of women in the menopausal age group worldwide. The menopause transition results in the declining of ovarian function and leads to significant hormonal changes in the female body, in particular in the reduction of the female hormone estrogen. As a result women in this age group face several physical and mental difficulties including hot flashes, night sweats, vaginal atrophy, and an increased risk of developing osteoporosis. Menopause is inevitable and many women during their menopausal period take hormone replacement therapy (HRT) using soy based dietary supplements. These soy based dietary supplements contain phenolic plant compounds called isoflavones [1, 2] which have structural similarities with estradiol [3]. Despite inconsistencies among the available data, there is growing evidence supporting the notion that soy isoflavones (SIF) are effective in the reduction of menopausal symptoms [4, 5]. Therefore, the popularity and availability of soy supplements are increasing significantly. However, concern regarding the safety of long-term exposure to self-administrated high levels of SIF, which may exceed the levels that can be obtained from the diet, is increasing within the governmental and public health related organizations [6, 7].

The effects of SIF are thought to be induced via estrogen receptors (ERs) of which an alpha (ERα) and beta (ERβ) form exist. SIF may regulate transcription of ER target genes via the estrogen-responsive elements (EREs) present within gene promoter regions in target tissues. It has been shown that ERα activation stimulates cell proliferation, whereas ERβ activation leads to inhibition of proliferation and stimulation of apoptosis [8-10]. It is known that the relative occurrence of ERα and ERβ is tissue dependent. For example, ERα is predominant in the mammary gland, epididymis, testis, uterus, kidney and pituitary gland whereas more ERβ is present in the prostate, bladder and lungs [11-14]. When postmenopausal women produce small amounts of endogenous estradiol, it is possible that binding of SIF from food supplements to ERs may produce estrogen-responsive effects especially in ERβ-sensitive target tissues, because SIF have been shown to be particularly ERβ-active [10-11]

During the past decades, the application of microarray technology has opened up new opportunities to study the effects of food and food supplements in the control of cellular processes and related health effects upon exposure to different type of compounds [15-17]. By using transcriptomic techniques it is possible to directly compare changes in gene expression not only in different tissues of the same species but also in similar tissues among different species. We hypothesized that using a transcriptomic approach it might be possible to answer the question whether the ovariectomized rat would be an adequate model to predict (post)menopausal health risks and/or benefits of soy isoflavone (SIF) supplementation. Therefore, in the rat study reported here we have investigated the changes in gene expression in different target (i.e. BT, UT and ST) and non-target (PBMC, AT and LT) tissues of ovariectomized F344 rats, as a model for (post)menopausal women who were exposed to a

commercially available soy supplement in a physiological relevant dose for eight weeks. In addition, the changes in gene expression in PBMC in the current study were compared with those of the previously reported short-term rat study [18]. Finally a cross-species comparison of changes in gene expression in PBMC and adipose tissues among ovariectomized rats and (post)menopausal women was carried out to compare the effects of SIF supplementation in rats with those observed in (post)menopausal human volunteers. The rats were exposed to the same commercial SIF containing supplement in a similar dose as the women in the parallel human intervention studies [4, 19].The overall aim of the present study was to investigate whether such a toxicogenomics approach could be used to predict possible health effects in humans, and would improve the health risk/benefit assessment of SIF.

Materials and methods

Chemicals

Pure isoflavone standards (both glucosides and aglycones) and equol were purchased from LC laboratories (Woburn, MA, USA). Dimethyl sulfoxide (DMSO) was obtained from Acros Organics (Geel, Belgium), and enterodiol, β-glucuronidase H-5 (*H. Pomatia* contain 60620 unit/g solid), Optiprep^{TM}, and Tricine from Sigma-Aldrich (Steinheim, Germany). NaCl was obtained from VWR International (Darmstadt, Germany), and acetonitrile and methanol from Biosolve BV (Valkenswaard, the Netherlands). Oasis HLB 1cc solid phase extraction cartridges were obtained from Waters (Milford, MA, USA).

Animals

Twelve female ovariectomized F344 inbred 8 weeks old rats were purchased from Harlan, (Horst, the Netherlands). The ovariectomy of these rats was done at the age of 6 weeks by the supplier and after 2 weeks of acclimatization they were delivered at the animal facility, the Centre for Laboratory Animals (CKP), Wageningen University, Wageningen, the Netherlands. F344 rats were chosen for several reasons. Firstly, according to US EPA (Environmental Protection Agency) [20], the inbred isogenic strain F344 rats are sensitive to estrogenic compounds and thus particularly suitable to study the effects of SIF. Secondly, the use of an inbred strain will minimize the background noise in micro array analysis. Thirdly, for reasons of comparison, we used the same strain of rats as for the short-term study and measured PBMC gene expression; this enabled comparison of PBMC gene expression after long term exposure (present study) and short term exposure [18].

Housing and diet

After arrival at the animal facility (CKP), the rats were housed in groups (2 animal/cage) and fed an isoflavone free RMH-B standard diet (ABDiets, Woerden, the Netherlands) (Supplementary Table 1) ad libitum. Standard housing, day-light hours and humidity were also maintained during the whole experimental period. The experiment started after a week acclimatization period. After that period animals were about 9 weeks of age with an average body weight of 160.8±9.8 g.

Animal experiment

All aspects of the experimental protocol were reviewed and approved by the Animal Welfare Committee of Wageningen University (Wageningen, the Netherlands) which was in compliance with the Dutch Act on animal experiment (Stb, 1977, 67; Stb 1996, 565), revised February 5, 1997. The animals were dosed a commercial soy containing supplement, corresponding to a dose of 2 mg SIF/kg bw (1 gavage/day) in 1% DMSO solution for seven days per week, during 8 weeks. One day before sacrifice the animals received an extra dose in the evening instead of a dose early next morning. This was done to make the experiment comparable with the time schedule of the two parallel human intervention studies (see below) run by the Division of Human Nutrition, Wageningen University to facilitate direct comparison of both rat and human data after exposure to the same supplement.

Related experiments

The effects on gene expression in PBMC and AT after SIF intake in the present rat study were compared with the gene expression data of a previously reported short term rat study, and of two parallel human intervention studies (see below).

Short term rat study:

A short-term (2 days) rat experiment was performed earlier at the same animal facility centre of Wageningen University, after approval by the ethical committee on animal experimentation of Wageningen University. This short-term study, in which rats from the same strain (i.e. ovariectomized F344) were exposed to a similar SIF supplement, and also PBMC were collected and studied by microarray analysis, was mainly designed for quantification of the bioavailability of SIF [18].

Parallel human intervention studies:

The two parallel human intervention studies were conducted at the Division of Human Nutrition of Wageningen University, and approved by the Medical Ethical Committee of this university [4, 19]. The studies were registered at clinical trials under number NCT01232751 and NCT01556737. Both of these studies were double blind cross-over studies with the exposure time of two eight week intervention periods and an eight week washout period in between. All participants received the same soy supplement used in the rat studies where human volunteer consumed 2 capsules in the morning

and 2 in the evening after the meal in a dose of about 1.5 mg SIF/kg bw and a placebo treatment. PBMC samples were collected from the first human intervention study while AT samples were collected from the second intervention study.

Preparation of test solutions

The commercial soy supplement, Phytosoya, was obtained from Arkopharma (Carros, France). The supplement had a total SIF content of 70.3 mg/gram supplement [21]. The content of the three glucosides; genistin, daidzin and glycitin was 7.54, 40.03 and 22.72 mg/g supplement, respectively. An oral gavage dose of 2 mg SIF/kg bw which corresponds with 0.4 mg SIF/rat was applied per day. The stock solutions were freshly prepared daily and were placed on a multi-axle-rotating-mixer (CAT RM-40, Slaufen, Germany) machine during the period of gavage dosing. The control rats received a gavage dose of nanopure water containing 1% DMSO.

Sample collection and analysis

After the 8 weeks dosing the animals were sacrificed after anaesthesia with a mixture of isoflurane and oxygen followed by removal of blood from the dorsal aorta with a syringe containing 0.1 mL lithium heparin. The collected blood was immediately transferred into 10 mL tubes and kept on a multi-axle-rotating mixer (CAT RM-40, Slaufen, Germany). The average amount of collected blood was 5-6 mL. Collected blood samples were divided into two parts, one part (3.5 mL) for isolating PBMC and another part (about 1-2 mL) for separating plasma. The first part of the collected blood samples was used for micro array analysis and the second part was used for measuring the plasma concentration of SIF. White AT, BT, UT, LT, and ST were also collected. After collection the tissue samples were frozen immediately in liquid nitrogen and stored at -80^{0}C until further analysis. The plasma samples were prepared by centrifuging the blood for 5 min at 10,000 rpm (Biofuge centrifuge, Heraeus Sepatech, UK) and were collected in Safe-lock 0.5 mL Eppendorf tubes (Eppendorf AG, Hamburg, Germany). These samples were placed on ice during the sample collection of the in vivo experiment and stored at -80^{0}C until analysis.

Extraction and analysis of SIF from plasma

The enzymatic hydrolysis, solid phase extraction and HPLC (High Performance Liquid Chromatography) analysis of SIF in plasma samples were performed as described earlier [19, 21]. To avoid inter laboratory variations among the present in vivo rat study and the human intervention studies, analysis of SIF concentrations in plasma and micro-array analysis were performed using similar protocols and performed at the same laboratories. Averages and standard deviations of plasma SIF concentrations of the dose group and the control were calculated using Microsoft Excel.

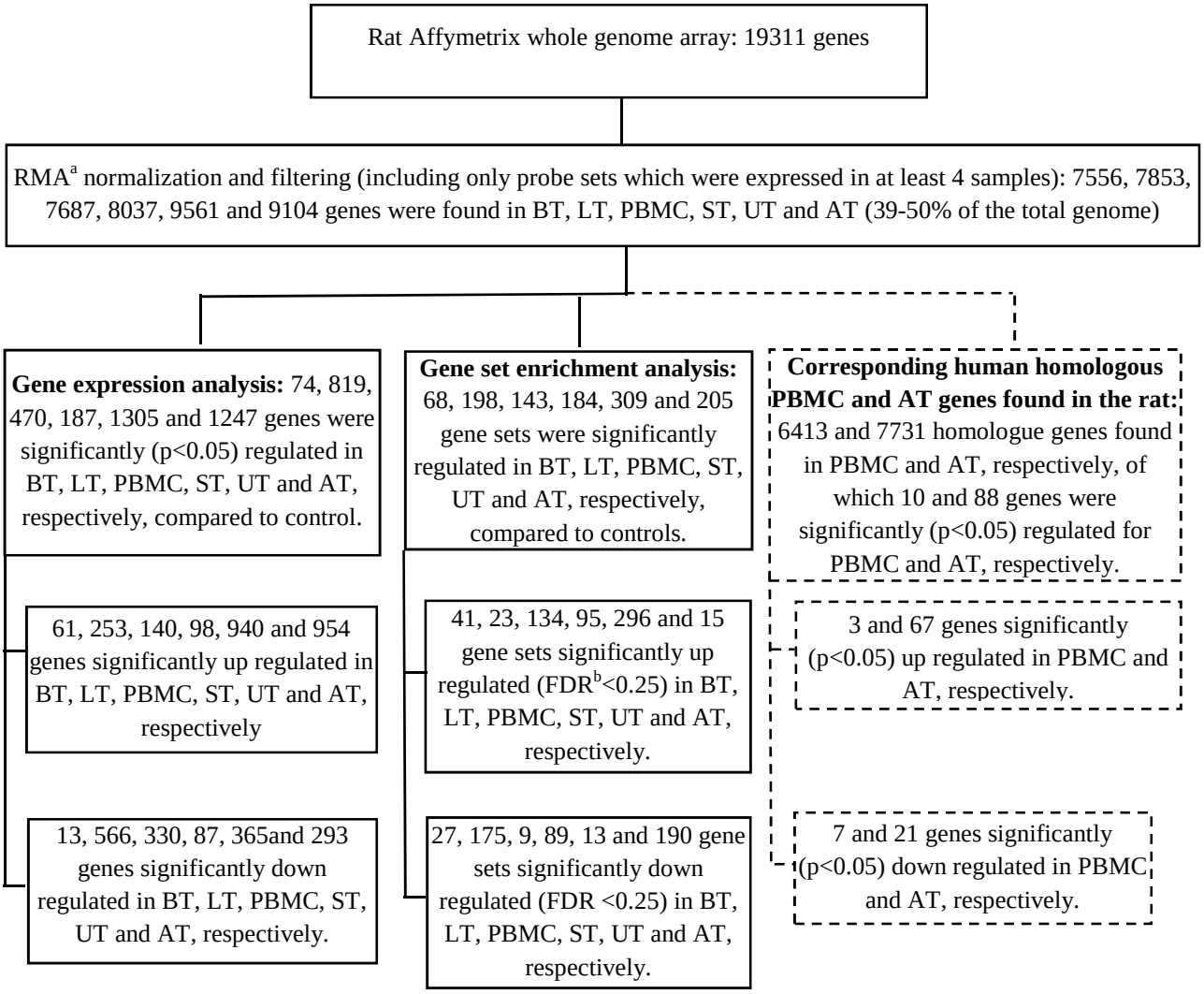
Isolation of PBMC, mRNA and running microarray analysis of different tissues

PBMC were isolated immediately after blood collection using OptiPrep™ (60 % w/v iodixanol) as a density gradient medium following the online protocol provided by Axis-Shield (www.axis-shield-density-gradient-media.com > methodology > cells > C43). After isolation, all the PBMC samples were dissolved in TRIzol reagent (Invitrogen, Sigma, Steinheim, Germany) and stored at -20 ^{0}C until further analysis. The other tissues were stored at -80 ^{0}C and during the day of RNA extraction TRIZol was added immediately after grinding the tissue samples in frozen condition. The different steps of RNA extraction and quality checking were performed as describe earlier [18]. Gene expression analysis was carried out by Affymetrix GeneChip Rat Gene 1.1 ST plate (Affymetrix, Santa Clara, CA). One hundred nanogram of total RNA was used for whole transcript cDNA synthesis with the Ambion WT expression kit [catalog number 4411974] (Applied Biosystems/Life Technologies, Nieuwekerk a/d IJssel, the Netherlands). Hybridization, washing and scanning of the Rat Gene 1.1 ST peg arrays was performed on a GeneTitan Instrument (Affymetrix, Santa Clara, CA) according to the manufacturer's recommendations.

Packages from the Bioconductor project [22], integrated in an online software program [23], were used to analyse the array data. Various advanced-quality metrics, diagnostic plots, pseudo images, and classification methods were used as described by Heber and Sick [24], to determine the quality of the arrays before statistical analysis. The probes on the Rat Gene 1.1 ST array were redefined using current genome information [25]. In this study, probes were reorganized on the basis of the gene definitions available in the National Center for Biotechnology Information (NCBI) *Rattus norvegicus* Entrez Gene database, based on the rat genome build 5.1 (custom CDF v17). Normalized gene expression estimates were calculated with the Robust Multichip Average method [26]. Subsequently, the dataset was filtered to only include probe sets that were active (i.e. expressed) in at least 4 samples, using the universal expression code (UPC) approach (UPC score > 0.50) [27]. Differentially expressed probe sets were identified by using linear models, applying moderated t-statistics that implemented intensity-based empirical Bayes regularization of standard errors [28, 29]. Probe sets with $p < 0.05$ were considered to be significantly regulated. Changes in gene expression were related to functional changes using gene set enrichment analysis (GSEA) performed according to Subramanian et al. [30]. GSEA focuses on groups of genes that share a common biological, biochemical or metabolic function. GSEA has the advantage that it is unbiased, because no gene selection step is used. Only gene sets consisting of more than 15 and less than 500 genes were taken into account. Gene sets were derived from the Kyoto Encyclopedia of Genes and Genomes (KEGG) database [31]. Effects of SIF treatment versus control were compared using ranked lists based on significance (t-values), using methods described by Plaisier et al. [32].

Results

Figure 5.1 shows an overview of the results from the microarray analysis. After normalization and filtering, 39-50% probe sets were retained in different tissues from the total of 19311 probe sets. The percentage of significantly regulated ($p < 0.05$) genes within this data set amounted to only 1-13.6% (i.e. 74 to 1305 genes out of 7556 to 9561 RMA normalized and UPC filtered genes), and was lowest for the BT (i.e. only 1%), with increasing number of genes being regulated in ST, followed by PBMC, LT, AT and UT (i.e. 2.3, 6.1, 10.4, 13.6 and 13.6%, respectively). Figure 1 also shows the number of significantly up and down regulated (false discovery rate, FDR < 0.25) biological pathways. In line with the total number of significantly regulated genes, the lowest and highest number of significantly changed biological pathways was found in BT (i.e. 68 gene sets) and in UT (i.e. 309 gene sets), respectively. In the right column of figure 1 the human homologous genes in PBMC and AT which were also found in the rat study are indicated. These homologous genes were retrieved from the parallel human intervention studies mentioned above [4, 19]. Out of 6413 and 7731 human homologous genes, only 10 and 88 genes were significantly ($p<0.05$) regulated in rat PBMC and AT, respectively.



a) RMA - robust multichip average
b) FDR- false discovery rate

Figure 5.1: Flow chart of the microarray analysis indicating significantly changed genes and gene sets induced in different tissues of ovariectomized F344 rats after oral gavage dosing (2 mg SIF/kg bw per day for 8 weeks) of a commercial soy supplement. The number of corresponding human homologue genes found in rats is also indicated (right hand column).

As the effects of SIF are considered to be mostly mediated via the estrogen receptors (ERs), we investigated the number of differentially regulated ERGs in the six selected rat tissues. These genes were selected based on their occurrence in the Dragon DRGs database (http://datam.i2r.a-star.edu.sg/ergdbV2/). Table 5.1 shows the list of ERGs and the direction of their regulation in the different rat tissues. It is shown that out of 87 ERGs available in the specified website only 19 were found to be significantly regulated in the different tissues. The highest number of ERGs that were significantly regulated was found in LT (7 ERGs) followed by AT (6 ERGs), UT (5 ERGs) and PBMC (3 ERGs). Surprisingly no ERGs were significantly regulated in BT and ST. Moreover, it appeared

that there was no similarity in the pattern of regulation of the various ERGs among the different tissues.

Table 5.1: List of significantly ($p < 0.05$) up and down regulated estrogen-responsive genes (ERGs) in different tissues of ovariectomized rats after 8 weeks oral dosing with the frequency of one gavage dose/day of a commercial supplement (2 mg SIF/kg bw) compared with control. Up and down regulated enrichments were indicated by up and down arrow marks. A list of all ERGs can found in the Dragon ERG database (http://datam.i2r.a-star.edu.sg/ergdbV2/).

Name of the gene	PBMC	AT	BT	UT	ST	LT
AR	↓	↑ns	↓ns	↓ns	↓ns	absent
ANXA4	↓ns	↓ns	↑ns	↑ns	↓ns	↓
DUSP1	↓ns	↓ns	↓ns	↓ns	↓ns	↓
IGF1	absent	↑ns	↑ns	↑ns	↑ns	↓
IL6R	↓ns	↑ns	↑ns	↑	↑ns	↑
IGFBP5	absent	↓**	↑ns	↓ns	↑ns	absent
IGFBP2	absent	↑**	absent	absent	absent	↓ns
KIT	absent	↑**	↑ns	absent	absent	absent
NR2F6	absent	↓**	absent	↑ns	absent	↓ns
PGR	absent	absent	absent	↑	absent	absent
PNOC	absent	↑	absent	↓	absent	absent
PAWR	absent	↑**	↓ns	↓ns	absent	↓ns
RARA	↓*	↓ns	↓ns	↑ns	↓ns	↓
RB1	↑ns	↑ns	↑ns	↑ns	↓ns	↓
SCARB1	↓ns	↑ns	↑ns	↑	↑ns	↑ns
SCP2	↑ns	↑**	↑ns	↓ns	↑ns	↑**
TGFB1	↓ns	↓ns	↑ns	↑	↑ns	↓ns
TEP1	↓*	↓ns	↓ns	↑ns	↑ns	↓ns
TIMP1	absent	↑ns	↑ns	↑	↑ns	↓ns

ns = not significant
* The PBMC genes common in rat and human
** The AT genes common in rat and human

Because most of the ERGs were not or only slightly regulated by SIF dosing, we investigated the genes that were affected most in the three target tissues, namely breast, uterus and sternum, by using the 'fold change (FC)' approach. Only significantly affected genes with an FC value > 2 were selected to investigate their involvement in different biological processes, and the higher the FC value is, the more the gene is affected by SIF dosing. Table 5.2 shows the significantly regulated genes, with an FC>2. In ST, no genes were regulated with an FC value >2. In BT 14 genes with an FC>2 were identified (i.e. 19% of the significantly regulated genes) of which 13 were up regulated, and in UT 11 genes (i.e. 1% of the significantly regulated genes) of which 9 were up regulated. According to the information in the NCBI database the up regulated genes are involved in metabolism such as fatty

acid, lipid and glucose metabolism, in stress responses such as detection of oxidative stress, responses to abiotic stimuli, defence mechanisms and immune responses such as cellular response to tumour necrosis factor, antigen binding, and intercellular signal transduction. Down regulated genes are involved in DNA and protein binding and inflammatory response.

Table 5.2: List of the significantly expressed (fold change >2 and p<0.05) genes in different target tissues of rats (negative numbers indicate down regulation).

Tissue name	Gene symbol	Description	Functions/process	Fold change
Breast tissue	Adipoq	Adiponectin, C1Q and collagen domain containing	Fat cell differentiation, detection of oxidative stress, fatty acid oxidation and glucose metabolism.	**4.9**
	Rnase2	Ribonuclease, RNase A family, 2 (liver, eosinophil-derived neurotoxin)	A protein-coding gene involved in nucleic acid binding.	**4.5**
	Mcpt1l1	Mast cell protease 1-like 1	Involved in proteolysis (protein breakdown).	2.9
	Cpa3	Carboxypeptidase A3, mast cell	A protein-coding gene involved in zinc ion binding	2.9
	Thrsp	Thyroid hormone responsive	Lipid metabolism biosynthesis process.	2.8
	Cd163	CD163 molecule	Receptor mediated endocytosis	2.3
	Tusc5	Tumour suppressor candidate 5	Involved in the response to the biotic stimulus	2.3
	Cdo1	Cysteine dioxygenase type 1	Metabolism of cysteine	2.3
	Fabp4	Fatty acid binding protein 4, adipocyte	Fat cell differentiation, cholesterol homeostasis, fatty acid metabolism and cytokine production	2.2
	Prkar2b	Protein kinase, cAMP dependent regulatory, type II bet	Fatty acid metabolism	2.1
	Mpz	Myelin protein zero	Cell-cell junction maintenance	2.1
	Atf3	Activating transcription factor 3	Involved in DNA and protein binding,	-2.1
Uterus tissue	LOC684146	Ig kappa chain V-II region 26-10-like	No information provided by NCBI database	**10.2**
	Igkv28	Immunoglobulin kappa chain variable 28	No information provided by NCBI database	**8.7**
	LOC500181	Ig kappa chain V-V region K2-like	No information provided by NCBI database	3.4
	Igj	Immunoglobulin joining chain	A protein-coding gene involved in antigen binding	2.9
	Nos2	Nitric Oxide Synthase 2, Inducible	Involved in aging, cellular response to tumour necrosis factor, intracellular signal transduction and nitric acid biosynthetic process	2.8
	Mzb1	Marginal zone B and B1 cell-specific protein	Involved in integrin activation, regulation of B cell proliferation and positive regulation of immunoglobulin biosynthetic process	2.7
	LOC100361706	Lambda-chain C1-region-like	Involved in antigen binding	2.5
	LOC362795	Immunoglobulin G heavy chain	Involved in antigen binding	2.2
	Fgg	Fibrinogen gamma chain	Involved in inflammatory response, platelet activation and aggregation	-2.1
	Rup2	Urinary protein 2	No information provided by NCBI database	**-3.9**

Table 5.3 shows the significantly regulated ($p < 0.05$) biological pathways (BPs) in different tissues of the present rat study, and the related human disease, as indicated in the Kyoto Encyclopaedia of Genes and Genomes (KEGG) data base. The total list of BPs that were significantly up- or down regulated in different tissues can be found in supplementary table 2. A total of 33 diseases related KEGG based BPs were found to be significantly regulated in different tissues in the present rat study. The highest number of up regulated BPs was found in UT (i.e. 24) and then in BT (i.e. 5), whereas 3 BPs were up regulated in sternum and PBMC. The highest number of down regulated BPs was found in LT (i.e. 19) followed by AT (10), PBMC (7), and UT (2). Although only 5 and 3 BPs were significantly regulated in BT and ST, respectively, they all were up regulated, whereas in liver and adipose all the BPs were

down regulated. Interestingly, when a disease related pathway was significantly up regulated in the surrogate tissues, PBMC or AT, it was mostly down regulated in one or more target tissues, and vice versa. No direct correlation was found between effects in the surrogate tissues and the target tissues. Also in none of the gene set enrichment scatter graphs we made to compare up and down regulated BPs in different surrogate and target tissues (data not shown), any correlation was observed.

Table 5.3: Significantly (normalized p value <0.05) regulated biological pathways (BP) after 8 weeks oral administration of a commercial soy supplement in ovariectomized rats. Only KEGG based BPs were included that were related to a distinct human disease. Up and down regulation is indicated by up and down arrows.

KEGG BASES BPs	PBMC	AT	LT	BT	ST	UT
Alzheimer's disease	↑	↓	ns	Ns	ns	ns
Parkinson's disease	↑	↓	ns	Ns	ns	↓
Huntington's disease	↑	↓	ns	Ns	ns	ns
Prion diseases	ns	↓	↓	Ns	ns	ns
Bacterial invasion of epithelial cells	ns	↓	↓	↑	ns	↑
Leishmaniasis	↓	↓	↓	Ns	ns	↑
Chagas disease (americantrypanosomiasis)	ns	ns	ns	Ns	↑	↑
African trypanosomiasis	absent	absent	ns	Ns	ns	↑
Malaria	ns	ns	↓	↑	↑	↑
Toxoplasmosis	ns	ns	↓	Ns	ns	↑
Amoebiasis	ns	ns	↓	Ns	↑	↑
Staphylococcus aureus infection	↓	ns	↓	↑	ns	↑
Hepatitis c	ns	ns	ns	Ns	ns	↑
Pathways in cancer	ns	ns	↓	Ns	ns	↑
Pancreatic cancer	ns	ns	↓	Ns	ns	↑
Glioma	ns	ns	↓	Ns	ns	↑
Thyroid cancer	ns	ns	ns	Ns	ns	↑
Melanoma	ns	ns	↓	Ns	ns	ns
Bladder cancer	ns	ns	↓	Ns	ns	ns
Chronic myeloid leukemia	ns	ns	ns	Ns	ns	↑
Acute myeloid leukemia	↓	ns	ns	Ns	ns	↑
Small cell lung cancer	ns	ns	↓	Ns	ns	↑
Non-small cell lung cancer	ns	ns	ns	Ns	ns	↑
Autoimmune thyroid disease	↓	ns	ns	Ns	ns	↑
Systemic lupus erythematosus	ns	ns	↓	↑	ns	↑
Rheumatoid arthritis	ns	↓	↓	↑	ns	↑
Allograft rejection	↓	absent	ns	Ns	ns	↑
Graft-versus-host disease	↓	absent	ns	Ns	ns	↑
Primary immunodeficiency	↓	absent	ns	Ns	ns	↑
Hypertrophic cardiomyopathy (hcm)	ns	↓	↓	Ns	ns	↓
Arrhythmogenic right ventricular cardiomyopathy (ARVC)	ns	↓	↓	Ns	ns	ns
Dilated cardiomyopathy	ns	↓	↓	Ns	ns	ns
Viral myocarditis	ns	↓	↓	ns	ns	↑

ns = not significant

In Figure 5.2 the gene expression patterns in PBMC of the present rat study were compared to those obtained in the short-term rat study [18]. Only 145 genes were significantly regulated in both data sets (data not shown) of which fifteen marker genes were identified to be highly correlated (deviation value 0 to ±10) and their gene expression was significantly changed in the same direction (increased or decreased) in both studies. As can be concluded from the information included in supplementary table 3, most of the genes thus identified are involved in biological pathways (BPs) related to increase in immune response and in cell proliferation.

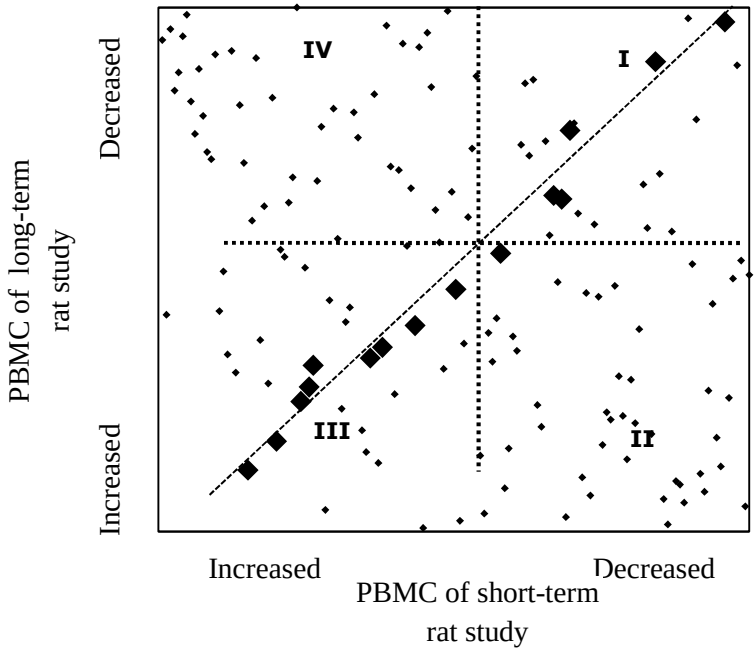


Figure 5.2: Rank-rank scattered plot for significantly changed common PBMC genes in the current long-term study (i.e. 8 week exposure) and from the group exposed to the same dose (i.e. 2 mg SIF/kg bw per day) in the short-term (2 days) study [18]. Each dot represents the t-value of a single gene and the highlighted dots (♦) indicate the highly correlated genes. Genes significantly changed in the same direction in both treatments are in Cartesian quadrants I and III, while genes significantly changed in opposite directions are in quadrants II and IV.

In the parallel human intervention studies mentioned above, Van der Velpen et al. [4, 19] reported some significant regulation of estrogen responsive genes (ERGs) in human PBMC and adipose tissue upon exposure to the SIF supplement. Table 5.4 shows the reported significantly expressed ERGs in PBMC of human volunteers that were also significantly regulated in PBMC in the present rat study. Out of 17 significantly changed human PBMC genes, only 1, gene namely CACYBP, was significantly regulated. Of the 17 genes 5 were insignificantly regulated, and 11 of these 17 genes were not differentially regulated in the PBMC of the present rat study at all. The only one gene that was significantly regulated in both human and rat PBMC (CACYBP) was oppositely regulated. In

summary, no correlation was found between the effects on gene regulation in PBMC from the present rat study and those from the study in human volunteers.

Similarly, table 5.5 shows the reported significantly expressed ERGs in adipose tissue of human volunteers [4] and their expression in the adipose tissue from the present rat study. Out of 82 significantly changed ERGs in human adipose tissue, only 6 genes were also significantly changed in rat AT, 40 genes were non-significantly regulated and 36 genes were not regulated in the rat AT. Out of the 6 genes that were significantly regulated in both human and rat AT, 5 were regulated in the same direction (up or down regulated), and 1 changed in the opposite direction. The down regulated common ERGs in adipose tissue i.e.NR4A1 and IGF2 have a positive relationship with different BPs such as apoptosis, endothelial cell proliferation, RNA and DNA transcription, T-cell proliferation etc. (see supplementary table 4). Thus down regulation of these genes would lead to inhibition of these processes. Although there are some similarities in the response in gene expression in rat and human AT following SIF exposure, there is no clear overall correlation.

Table 5.4: List of the significantly expressed human estrogen responsive genes (ERGs) in PBMC of human volunteers reported by Van der Velpen et al. [19] and their expression in rat PBMC in the present long-term rat study. Up and down regulated gene expressions are indicated by up and down arrows.

Genes	Description	Regulation	
		Human	Rat
BCL2L1	BCL2-like 1	↑	a
CACYBP	Calcyclin binding protein Y	↓	↑
EDEM1	Endoplasmatic reticulum degradation enhancer, mannosidase alpha-like 1	↑	↑ns
ERBB2	V-ERB-B2 erythroblasticleukemia viral oncogene homolog 2 neuro/glioblastoma derived oncogene homolog (avian)	↑	a
FKBP5	FK506 binding protein 5	↑	a
FOXP1	Forkhead box P1	↑	↓ns
HSPA1A	Heat shock 70 kDa protein 1A Y	↓	a
MYB	v-mybmyeloblastosis viral oncogene homolog (avian)	↑	a
NME2	NME/NM23 nucleoside diphosphate kinase 2 Y	↓	↑ ns
NRP1	Neuropilin 1 Y	↓	↓ns
PSMD8	Proteasome (prosome, macropain) 26S subunit, non-ATPase, 8 Y	↓	↑ns
PTPRO	Protein tyrosine phosphatase, receptor type, O Y	↓	a
SLC25A5	Solute carrier family 25 (mitochondrial carrier; adenine nucleotide translocator), member 5	↓	a
SPRED1	Sprouty-related, EVH1 domain containing 1 Y	↓	a
STAB1	Stabilin 1 Y	↓	a
STXBP1	Syntaxin binding protein 1	↑	a
TIMELESS	Timeless homolog (Drosophila)	↓	a

a = absent

ns = not significant

Table 5.5: Significantly expressed human estrogen responsive genes (ERGs) in AT of human volunteers reported by Van der Velpen et al. [4] and their expression in AT in the present long-term rat study. Up and down regulated gene expressions are indicated by up and down arrows.

Sl. No.	Genes	Description	Regulation	
			Human	Rat
1	NR4A1	nuclear receptor subfamily 4, group A, member 1	↓	↓
2	RET	ret proto-oncogene	↓	a
3	TK1	thymidine kinase 1, soluble	↓	a
4	TGFB3	transforming growth factor, beta 3	↓	↑ns
5	NOS3	nitric oxide synthase 3, endothelial cell	↓	↓ns
6	GOT1	glutamic-oxaloacetic transaminase 1, soluble	↓	↓ns
7	TNC	tenascin C	↓	a
8	IGF2	insulin-like growth factor 2	↓	↓
9	PPIF	peptidylprolylisomerase F	↓	↑ns
10	ME1	malic enzyme 1, NADP(+)-dependent, cytosolic	↓	↓ns
11	NME1	NME/NM23 nucleoside diphosphate kinase 1	↓	a
12	TUBG1	tubulin, gamma 1	↓	↑ns
13	THBD	thrombomodulin	↓	a
14	NCAM2	neural cell adhesion molecule 2	↓	a
15	KITLG	KIT ligand	↓	↑
16	ELOVL2	ELOVL fatty acid elongase 2	↓	a
17	CCND1	cyclin D1	↓	↑ns
18	CNKSR3	CNKSR family member 3	↓	a
19	CYCS	cytochrome c, somatic	↓	a
20	DHCR24	24-dehydrocholesterol reductase	↓	↓ns
21	KIR3DL2	killer cell immunoglobulin-like receptor, three domains, long cytoplasmic tail, 2	↓	a
22	PAICS	phosphoribosylaminoimidazole carboxylase, phosphoribosylaminoimidazolesuccinocarboxami	↓	a
23	TFF1	trefoil factor 1	↓	a
24	HSPD1	heat shock protein 1 (chaperonin)	↓	↑ns
25	KPNA2	karyopherin alpha 2 (RAG cohort 1, importin alpha 1))	↓	a
26	ARMCX3	armadillo repeat containing, X-linked 3	↓	↑ns
27	ENO1	enolase 1, (alpha)	↓	↑ns
28	SGCD	sarcoglycan, delta (dystrophin-associated glycoprotein)	↓	↓ns
29	SEMA5B	sema domain, seven thrombospondin repeats (type 1 and type 1-like), transmembranedomai	↓	a
30	G6PD	glucose-6-phosphate dehydrogenase	↓	↑ns
31	RAMP3	receptor (G protein-coupled) activity modifying protein 3	↓	a
32	AURKB	aurora kinase B	↓	a
33	ESR2	estrogen receptor 2 (ER beta)	↓	a
34	C1QBP	complement component 1, q subcomponent binding protein	↓	↑ns
35	CENPA	centromere protein A	↓	a
36	ACO2	aconitase 2, mitochondrial	↓	↑ns
37	RUNX1	runt-related transcription factor 1	↓	↓

Sl. No.	Genes	Description	Regulation	
			Human	Rat
38	MCM4	minichromosome maintenance complex component 4	↓	a
39	NR4A3	nuclear receptor subfamily 4, group A, number 3	↓	a
40	FOXF1	forkbead box F1	↓	a
41	ORMDL2	ORM1-like 2 (S. cerevisiae)	↓	↑ns
42	MARCKS	myristoylated alanine rich protein kinase C substrate	↓	↑ns
43	SLC12A2	solute carrier family 12 (sodium/potassium/chloride transporter), member 2	↓	↑ns
44	SPRY1	sprout homolog 1, antagonist of FGF signaling (Drosophila)	↓	a
45	GARS	glycyl-tRNAsynthetase	↓	↓ns
46	RPA3	replication protein A3	↓	↑ns
47	STMN1	stathmin 1	↓	a
48	IARS	isoleucyl-tRNAsynthetase	↓	↓ns
49	ITGAV	integrin, alpha V	↓	↑ns
50	NUP88	nucleoporin 88	↓	↑ns
51	TXNIP	thioredoxin interacting protein	↑	↑ns
52	NRF1	nuclear respiratory factor 1	↑	↑ns
53	ECE1	endothelin converting enzyme 1	↑	↑ns
54	EFEMP1	EGF-containing fibulin-like extracellular matrix protein 1	↑	↑
55	MPL	Myelo-proliferative leukemia virus oncogene	↑	a
56	GSTO1	glutathione S-transferase omega 1	↑	↑ns
57	HIP1R	huntingtin interacting protein 1 related	↑	a
58	PAX8	paired box 8	↑	a
59	PTPN18	protein tyrosine phosphatase, non-receptor type 18 (brain-derived)	↑	a
60	WSB1	WD repeat and SOCS box-containing 1	↑	↑ns
61	IGF1R	insulin-like growth factor 1 receptor	↑	↑ns
62	GNG7	guanine nucleotide binding protein (G protein), gamma 7	↑	a
63	SFRP1	secreted frizzled-related protein 1	↑	↑ns
64	MCM7	minichromosome maintenance complex component 7	↑	↑ns
65	SATB1	SATB homeobox 1	↑	↓ns
66	INPP4B	inositol polyphosphate-4-phosphatase, type II	↑	↓ns
67	THBS2	thrombospondin 2	↑	↑ns
68	PTGER2	prostaglandin E receptor 2 (subtype EP2), 53kDa	↑	a
69	WISP2	WNT1 inducible signaling pathway protein 2	↑	↑
70	BCL2L11	BCL2-like 11 (apoptosis facilitator)	↑	a
71	ABCB1	ATP-binding cassette, sub-family B (MDR/TAP), member 1	↑	a
72	TSC22D3	TSC22 domain family, member 3	↑	a
73	NFKBIA	nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha	↑	↑ns
74	ANKRD44	ankyrin repeat domain 44	↑	↑ns
75	CTNND1	catenin (cadherin associated protein), delta 1	↑	↑ns
76	BCL2L1	BCL2-like 1	↑	a
77	TNFSF8	tumour necrosis factor (ligand) superfamily, member 8	↑	a
78	CCNG2	cyclin G2	↑	↑ns
79	FKBP8	FK506 binding protein 8	↑	↓ns

80	ZFP36L2	zinc finger protein 36, C3H type-like2	↑	a
Sl. No.	**Genes**	**Description**	**Regulation**	
			Human	**Rat**
81	SLA	Src-like adaptor	↑	↑ns
82	S100P	S100 calcium binding protein P	↑	a

a = absent
ns = not significant

Figure 5.3 shows a rank-rank scattered plot of the significantly regulated genes in PBMC and AT in the rat study and in the parallel human intervention studies. There were 10 human homologous genes significantly regulated in rat PBMC and 88 in rat AT, but no overall correlation was obtained for the 10 significantly regulated PBMC rat genes with the 10 significantly regulated human PBMC genes (figure 5.3a). This implies that the genes which were highest up regulated in rat PBMC were not the same as those in human PBMC. Some genes were even regulated in the opposite direction. A similar observation was found for the 88 common and significantly regulated genes expressed in adipose tissue (figure 5.3b). To identify marker genes, i.e. genes of which the expression was increased or decreased in the same direction in both rat and human PBMC and AT from the rank-rank scattered plots shown in figure 5.3, deviation values were used from the corresponding correlation line. For PBMC (figure 5.3a) a deviation value of 0 to ±
1 was used, because the number of overlapping genes was only 10. For AT (figure 5.3b) a deviation value of 0 to ±4 was used, because the number of overlapping genes was 88. For PBMC (figure 5.3a) three marker genes were identified, which were mainly associated with protein production and regulation of cell growth (see supplementary table 5). Similarly 11 marker genes were identified for AT (figure 5.3b), as can be seen in the supplementary table 6. These marker genes appeared to be involved in biological processes related to immune and inflammatory responses. In addition scattered plots were made using all homologous genes (i.e. 6413 homologous genes for human and rat PBMC and 7731 homologous genes for human and rat AT, see figure 5.1) without applying any significant cut-off value, but no correlation was observed (data not shown).

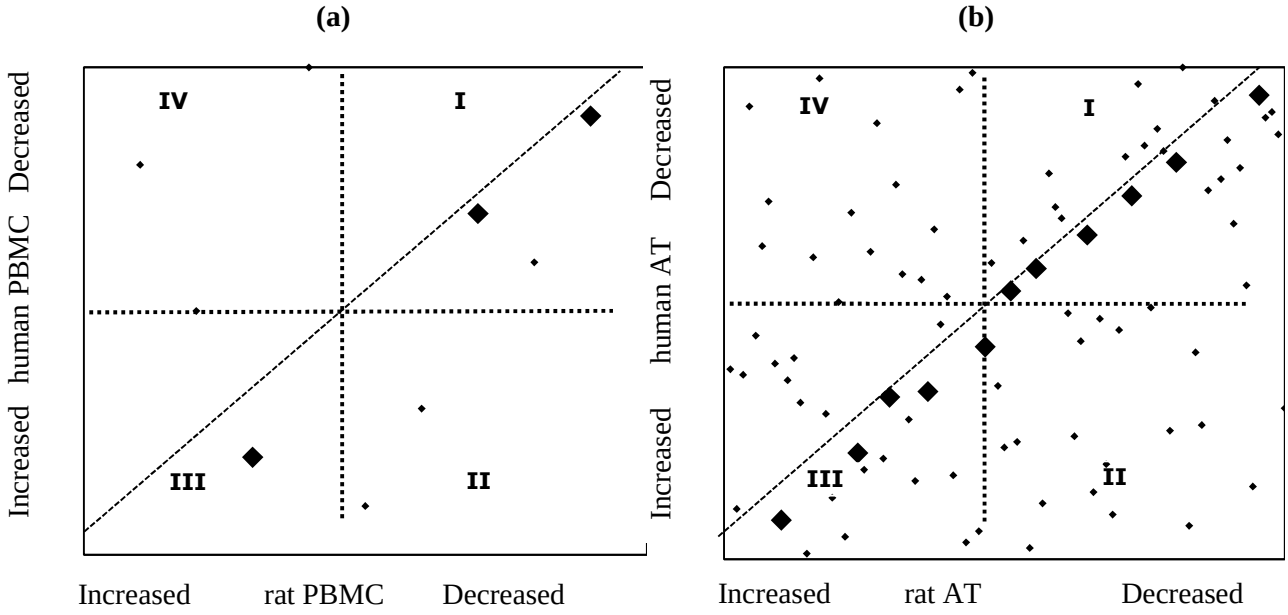


Figure 5.3: Rank-rank scattered plot for significantly changed common PBMC genes in rat and (post)menopausal women (3a), and significantly changed common genes in AT of rats and (post)menopausal women (3b). Each dot represents the t-value of a single gene and the highlighted dots (♦) indicate the correlated genes.Corresponding human homologous genes were retrieved from the study with (post)menopausal women taking the same supplement, in a similar dose (about 1.5 mg SIF/kg bw per day) and time duration (i.e. 8 weeks) [4, 19]. Genes significantly changed in the same direction in both treatments are in Cartesian quadrants I and III, while genes significantly changed in opposite directions are in quadrants II and IV.

Discussion

With this eight weeks study in ovariectomized rats it was shown that the overall gene expression data after SIF supplementation show tissue specific effects of SIF supplementation, for the regulation of estrogen responsive genes (ERGs) (see table 5.1) and even more for genes not known to be ERGs. The different responses in gene expression in different tissues might be explained in part by tissue specific ERα/ERβ ratio occurring in various tissues [11-14]. This may complicate the use of surrogate tissues like PBMC or adipose tissue to predict the effects in target tissues such as breast, uterus or sternum tissue. In addition to differences in gene regulation upon SIF supplementation among the different tissues, there also appeared to be a considerable difference in gene expression in the same tissue of the same species upon different exposure duration, as was evident by the absence of a correlation between the gene expression data in PBMC collected in the short- and the long-term rat study (see figure 2). We also did not observe a correlation between changes in gene expression in similar tissues (PBMC

and AT) from rats and humans upon similar dosing and exposure duration (see figure 5.3; table 5.4 & 5.5).

The present study aimed to predict the effect of SIF supplementation in the human target tissue, such as breast, uterus or sternum tissue, by evaluating the similarities in the gene expression pattern of the surrogate tissues PBMC and adipose in rat and human after the same physiologically relevant dosing to a commercial SIF containing supplement. Because the results of the surrogate tissues (PBMC and AT) in rats differed from those in humans, and because there was no correlation between changes in gene expression between surrogate and target tissues in rats, this prediction of possible effects in humans was not possible. Although transcriptomics techniques are a powerful tool to predict early biological effects, it appeared to be complicated to interpret the early changes in gene expression profile in relation to the adverse or beneficial responses related to different biological process.

The present study revealed that only a few ERGs were significantly regulated and surprisingly not one single ERG was significantly regulated in breast or sternum tissues, which are considered to be estrogen responsive tissues. This observation is in line with the conclusion from Fritz et al. [33] who recently reviewed 131 articles including 40 randomized control trials, and concluded that soy might not have direct estrogenic effects. The authors suggested that SIF may possess anti-estrogenic activity because consumption of 63 mg/day and higher was found to be associated with increased prevalence of hot flashes among premenopausal breast cancer patients.

We have observed before [18] that after an exposure of 2 days of ovariectomized rats to the same supplement as used in the present study, 3861 genes were significantly regulated in the PBMC. This differential gene regulation was significantly less in the present study, where after dosing for a longer period of 8 weeks only 470 genes were found significantly regulated in PBMC. Compared with the total number of probes in the microarray, the number of significantly regulated genes in the short term study was 20% and in the long term study it was 2.4%. Interestingly, in the human intervention study reported by Van der Velpen et al. [19] found that 357 genes were significantly regulated in human PBMC, which was about 1.8% compared to the total probe sets. Possibly long-term exposure might induce homeostasis, resulting in a lower number of regulated genes in the PBMC gene expression profiles, both in rats as well as in humans.

The commercial supplement used in the present study contains predominantly daidzin glucoside (about 57%). After enzymatic hydrolysis the total amount of conjugated and non-conjugated SIF was converted into non-conjugated aglycones, and only daidzein and its metabolite equol were detected in the rat plasma samples. The average plasma concentrations of daidzein and equol were 0.25 and 0.38 µM (see supplementary table 7), respectively. Based on information reported in open literature [34, 35] at best 3% of the total amount of SIF in the systemic circulation could be present as free bioactive

aglycones. Thus for the present experiment it can be estimated that a total plasma concentration of free aglycones in rat plasma of about 19 nM (7.5 nM free daidzein aglycone and 11.4 nM free equol aglycone) could have been reached. Figure 4 shows the estrogenic dose response curves of daidzein in in vitro model systems detecting the induction of luciferase activity in U2OS-ERα and U2OS-ERβ cells and proliferation in T47D-wt and T47D-ERβ as has been previously reported by us [36]. For the estimated total free aglycone concentration of our current rat study (plotted as vertical line in Fig 4). It can be seen this concentration is unlikely to induce an ERα related response, but that it could be able to induce a moderate ERβ related response. This observation might be an explanation for low responses in gene expression, including for ERGs, as observed in the present rat study.

It should be noted, however, that this comparison the total free aglyone concentration of 19 nM with the in vitro estrogenic response of daidzein did not consider differences in estrogenic potency between daidzein and equol. It is know that equol might have a higher estrogenic potency than daidzein, and therefore the use of the value of 19 nM for an expected estrogenic effect might be an underestimation. But unfortunately no formal relative potency factor for the comparison of daidzein and equol is available. However, several authors have addressed the difference in estrogenic potency between daidzein and equol in various test systems. Hwang et al. 2006 [3] used four different cell lines (i.e. human breast cancer cell line MCF-7, human embryonic kidney cells 293, murine calvarial osteoblasts MC3T3E1, and murine monocytic cells RAW 264.7) transfected with the estrogen-responsive reporter gene construct 3×ERE-TATA-Luc and found that the transcriptional potency of equol for the ERα receptor was 1.1 fold higher than that of daidzein and for ERβ receptor 1.2 fold higher.

Kalita and Milligan [37] used Ishikawa and yeast cells, and expressed the relative potencies of different estrogenic compounds based on the EC50 of the compound compared to estradiol (E2). According to their results relative potency of equol is about 2-8 fold higher than that of daidzein. Comparable values, ranging from 1 to 4.7, for the relative estrogenic potency of equol vs daidzein have been reported in a few review papers [38, 39].

In contrast to these studies indicating a relative estrogenic potency for equol compared to daidzein in the range a 1 to 10, a much higher potency factor of 100 was reported by Sathymoorthy and Wang [40] based on proliferation and mRNA expression of the oestrogen-responsive pS2 gene in MCF7 cells. Because the reported relative potencies of equol compared to daizein, based on different test systems, differ considerably, no overall potency factor can be estimated. But assuming a reasonable estimate of a factor of about 10 for the relative potency of equol compared to daidzein, the value of 19 nM used above could be converted in about 120 nM daidzein aglycone equivalents. As can be seen by the dotted lines in Fig. 4 the conclusion that the

plasma levels as observed in the current rat study would not be able to induce an ERα related response, but could induce a moderate ERβ related response, is still valid.

We also calculated the total free aglycone concentrations in human plasma from the parallel human intervention study [19] where Van der Velpen in addition to daidzein and equol, also found genistein and glycitein. After converting the µg/mL concentrations reported by these authors to nM using the molecular weights of the individual aglycones, and using again a value of 3% for the amount of SIF that can be present in the circulation in the free form (see above), the total free aglycone concentration could be estimated to be about 130 nM. This is about 6.8 fold higher than the free aglycones concentration in rat plasma. Considering that the daidzein concentration in human plasma is highest (0.47 µg/mL) followed by equol (0.35 µg/mL), and that lower concentrations were found for genistein (0.18 µg) and glycitein (0.12 µg/mL), one could speculate, that the corresponding free aglycones in human plasma were also not able to induce a significant ERα related response, but that a significant ERβ related response could be possible (figure 5.4).

The observed difference in plasma concentration between rats and humans after dosing the same supplement at a similar dose level (2 mg SIF/kg bw) and for the same duration (i.e. 8 weeks) can be due to species variation in ADME characteristics and/or a difference in the dosing regimen. In the present study the rats were dosed once per day in the morning and in the parallel human intervention study the volunteers took 2 capsules in the morning and 2 in the evening after the meal. Gu et al. [35] also observed considerable species differences in plasma SIF concentrations between female adult Sprague-Dawley rats and premenopausal woman volunteers 4 h after a similar single dose; the concentration of total daidzein was 4 fold higher in human plasma compared to that of the rat. This is in line with the results of the present study.

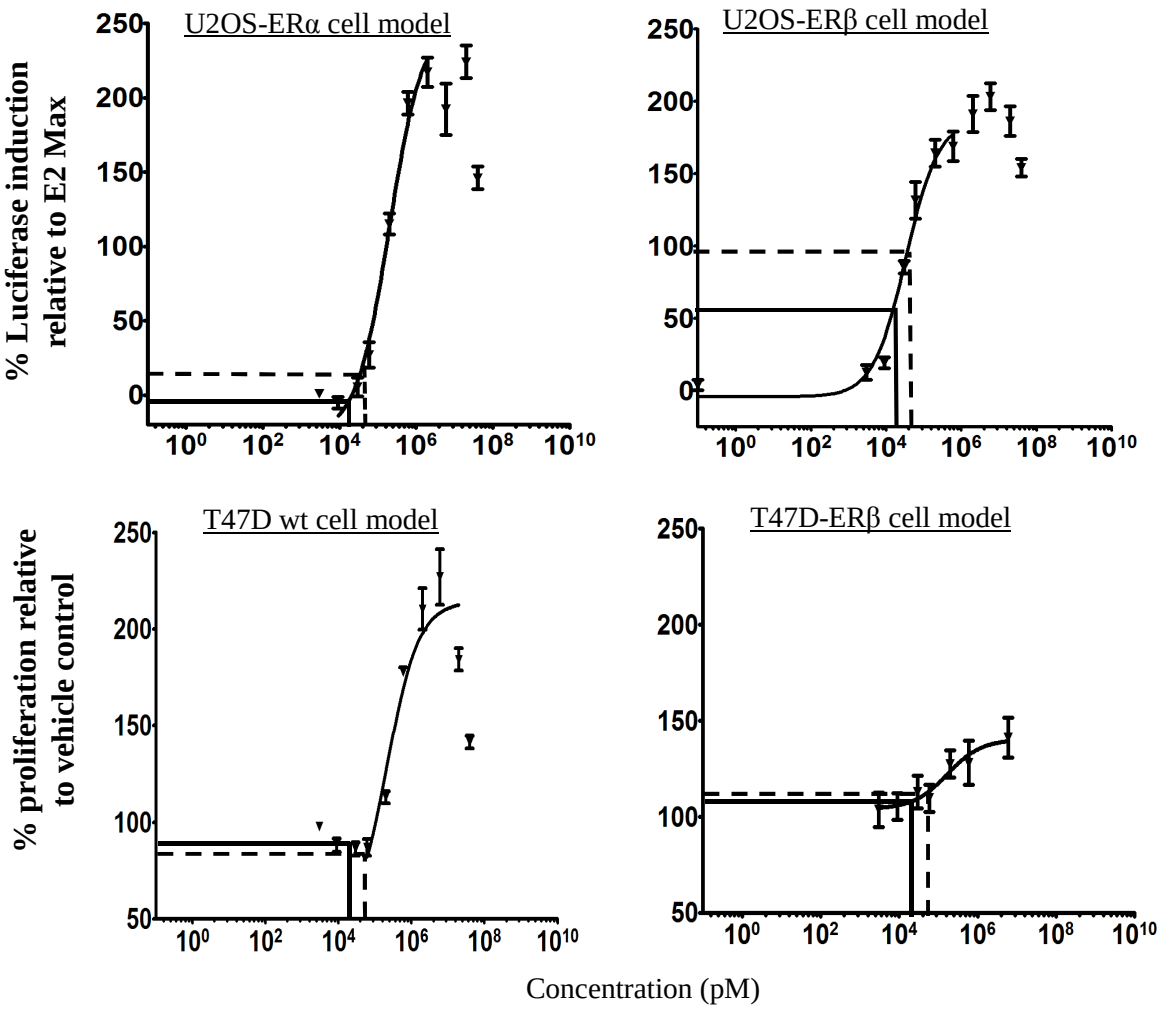


Figure 5.4: Comparison of the estimated in vivo concentration of total free aglycones with the concentration-response curve of daidzein in different in vitro cell models for estrogenicity. (Figure adapted from Islam et al. [36]). The solid lines indicate the expected effect of the total free aglycones present in the rat plasma, the dotted lines taking into account the higher estrogenic potency of equol compared to daidzein.

It has been shown that activation of ERα or ERβ has opposite effects on cell proliferation, i.e. activation of ERα leads to cell proliferation whereas activation of ERβ induces an anti-proliferative effect [8-10]. Therefore, our results, in comparison with the information on the plasma levels of the parallel intervention study, indicate that following SIF supplementation a beneficial health effect might be expected via ERβ, as the concentrations appeared to be too low for an ERα related response. This ERβ selectivity of SIF could also partly explain the beneficial health effects that have been reported in many human intervention studies or randomised control trials. Not only alleviation of menopausal effects, but activation of ERβ is associated with anti-proliferative and anti-carcinogenic effects in hormone sensitive breast cancer patients [33, 41]. Moreover, in the breast, ERβ is wider

distributed in different types of breast cells, such as ductal and lobular epithelial cells and stromal cells, whereas ERα is only found in epithelial cells [42]. Therefore, higher ERβ selectivity and wider distribution of ERβ in the breast tissue might facilitate the inhibition of E2 or ERα promoted cell growth by SIF supplementation.

We also investigated some pathways that might have been affected by SIF. It has been reported that SIF are capable to bind and activate all three isoforms namely, PPARα, PPARβ and PPARγ, of the peroxisome proliferator-activated receptors (PPARs) [43]. These PPARs are a group of transcription factors, which play an essential role in the regulation of cellular differentiation, carbohydrate, lipid and protein metabolism and tumorigenicity [43]. In the present study the PPAR signalling pathway was up regulated in breast uterus and liver tissues (see supplementary table 2) indicating a positive effect of SIF treatment. However this pathway was significantly down regulated in human PBMC after exposure with the same supplement. Other studies have shown that both ERs and PPARs influence each other and thus might lead to different effects of SIF [44-45].

It was found that the p53 pathway was significantly up regulated in PBMC and breast tissue of rats (see supplementary table 2). P-53 is an important and one of the most studied stress response pathways conserving stability of DNA, and protecting cells from DNA damage [46]. Under normal conditions this tumour suppressor gene is constitutively expressed, but it is negatively regulated by the pathway sensor called Mdm2, and then degraded. However, a variety of stress events in the cells, especially those related to DNA damage, activate a series of events that stabilize the p53 protein by inhibiting its degradation. Once activated p53 regulates divergent groups of target genes related to cell cycle arrest [47], DNA repair mechanism [48, 49] and induction of apoptosis [50]. Hence activation of this p53 gene has been reported to be associated with prevention of cancer. And therefore up regulation, as observed in the present study, could be considered to be a beneficial effect of exposure to SIF.

Given the fact that the correlations in gene expression in PBMC and AT between the present rat study and the human intervention study, in which the same SIF supplement was used, were limited, it is of importance to consider the possible limitations of the current rat study, which could have lead to this result. In the rat study the animals were dosed once per day in the morning by gavage, whereas the human volunteers took the dose in two portions per day, consisting of one tablet in the morning and another one in the evening, normally just after having a meal. This difference in dose regimen, together with possible effects of the circadian cycle could have influenced the gene expression. Furthermore, the comparison of the effects of SIF in rats and humans was based on the use of a similar external dose, and no consideration was given to the internal dose. According to Gu at al. [35], and in line with our own data, higher plasma concentrations of SIF were found in humans compared to the rat. In addition, we previously reported that rats can metabolize SIF faster than humans [21]. So providing the same external dose on a kg/bw basis appeared to result in somewhat dissimilar plasma

concentration in rats and humans, and this could have contributed to the differences in gene expression between rats and humans, as observed in the present study. Finally, in our study we have used young ovariectomized rats and considered them as a suitable model for (post)menopausal women. Although it is a well-established model, fully developed, aged female rats may have better physiological similarities with (post)menopausal women and may therefore be a more appropriate animal model.

Conclusion

In the present study it appears that there are considerable differences in gene expression between various surrogate and target tissues and no cross-species early biomarkers for health effects of SIF could be identified. Based on this observation it is concluded that using young ovariectomized rat transcriptomic data as a model for human risk or benefit analysis for SIF supplementation is challenging and probably not suitable. The limited effects on ERGs, may suggests that the positive effects of SIF in alleviating menopause problems may occur via other mechanisms, possibly indirectly related to estrogenic effects.

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The authors have declared no conflict of interest.

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Supplementary Table 1: Composition of isoflavone-free RMH-B diet

Analysis	**%**	**Amino acids**	**g/kg**
Crude protein	23.5	Lysine	10.5
Crude fat	5.0	Methionine	3.6
Crude fibre	4.3	Methionine + Cystine	6.6
Starch	38.3	Cystine	3.0
Sugar	4.0	Threonine	7.4
Linoleic acid	1.5	Tryptofan	2.0
Ash	5.5	Isoleucine	7.5
Dry matter	89.8	Arginine	14.7
		Phenylalanine	8.7
Energy(kJ/kg)	16100	Histidine	4.6
		Leucine	11.2
Minerals	**%**	Tyrosine	4.1
Ca	0.92	Valine	10.3
P	0.63		
K	0.90	**Vitamins**	**IU/kg**
Mg	0.12	Vit. A	20500
Na	0.42	Vit. D3	2000
Cl	0.74		**mg/kg**
		Choline	1000.0
		Vit. E	60.0
Trace elements	**mg/kg**	Vit. K3	2.4
Fe	105.0	Vit. B1 thiamine	13.0
Mn	70.0	Vit. B2 riboflavin	10.7
Zn	55.0	Vit. B3 niacin	32.0
Cu	17.5	Vit. B5 pentothenic acid	11.9
Co	0.2	Vit. B6 pyridoxine	12.5
I	0.4	Vit. B9 pyridoxine	3.8
		Vit. B12 pyridoxine	0.1
		Betaine	127.0
		Biotine	200.0
		Vit.C	95.0

Source: ABDIETS (www.abdiets.com)

Supplementary table 2: List of significantly ($p<0.05$) regulated biological pathways (BPs) after 8 weeks of oral gavage by a commercial soy supplement in ovariectomized rats. Only KEGG based BPs were included. Disease related BPs can be found in table 1.

KEGG BASES BPs	**PBMC**	**AT**	**LT**	**BT**	**ST**	**UT**
Glycolysis / Gluconeogenesis		↓			↑	
Citrate cycle (TCA cycle)	↑				↑	↓
Pentose phosphate pathway		↓			↑	
Pentose and glucuronateinterconversions			↑			
Fructose and mannose metabolism		↓				
Galactose metabolism						↑
Ascorbate and aldarate metabolism			↑			
Fatty acid metabolism			↑		↑	↓
Oxidative phosphorylation	↑	↓				
Glycine, serine and threonine metabolism						↓
Cysteine and methionine metabolism			↑			
Valine, leucine and isoleucine degradation			↑		↑	↓
Arginine and proline metabolism			↑			
Tryptophan metabolism					↑	↓
beta-alanine metabolism			↑			
Glutathione metabolism				↑		
Starch and sucrose metabolism			↑			↑
N-glycan biosynthesis			↓			↑
Other types of o-glycan biosynthesis						↑
Amino sugar and nucleotide sugar metabolism				↑		↑
Glycerolipid metabolism				↑	↑	
Inositol phosphate metabolism					↑	↑
Glycerophospholipid metabolism	↓					
Arachidonic acid metabolism		↑	↓			
Sphingolipid metabolism				↑		↑
Pyruvate metabolism					↑	↓
Propanoate metabolism			↑		↑	↓
Butanoate metabolism			↑			
Porphyrin and chlorophyll metabolism			↑			
Metabolism of xenobiotics by cytochrome p450						↓
Drug metabolism - cytochrome p450		↑			↑	↓
Drug metabolism - other enzymes			↑			
Biosynthesis of unsaturated fatty acids					↑	
Ribosome biogenesis in eukaryotes	↑	↑			↓	
Ribosome	↑			↓		
RNA transport	↑	↑				
RNA degradation					↓	
RNA polymerase				↓		
Basal transcription factors					↓	
DNA replication					↓	

Supplementary table 2: continue

KEGG BASES BPs	PBMC	AT	LT	BT	ST	UT
Spliceosome	↑	↑				
Proteasome	↑	↓			↓	
Protein export	↑			↑	↓	
PPAR signaling pathway			↑	↑	↑	
Nucleotide excision repair	↑				↓	
MAPK signaling pathway		↓	↓			
ERBB signaling pathway	↓					↑
Calcium signaling pathway	↓	↓			↑	
Cytokine-cytokine receptor interaction	↓		↓	↑		↑
Chemokine signaling pathway			↓			↑
Phosphatidylinositol signaling system	↓				↑	↑
Neuroactive ligand-receptor interaction				↑		
p53 signaling pathway	↑			↑		
Ubiquitin mediated proteolysis					↓	
Protein processing in endoplasmic reticulum	↑					↑
Lysosome				↑		↑
Endocytosis						↑
Phagosome				↑		↑
Peroxisome			↑		↑	
Apoptosis			↓			↑
Cardiac muscle contraction	↑	↓				
Vascular smooth muscle contraction					↑	
Notch signaling pathway			↓	↓		
TGF-beta signaling pathway			↓			↓
Axon guidance			↓			
VEGF signaling pathway		↓				
Osteoclast differentiation	↓	↓	↓			↑
Focal adhesion		↓	↓	↑	↑	
ECM-receptor interaction	↑		↓	↑	↑	
Cell adhesion molecules (cams)	↓		↓	↑	↑	↑
Adherens junction			↓			
Tight junction					↑	
Gap junction					↑	
Complement and coagulation cascades			↓	↑		
Antigen processing and presentation				↑		↑
Toll-like receptor signaling pathway						↑
Nod-like receptor signaling pathway			↓			↑
Cytosolic dna-sensing pathway				↓		↑
Jak-stat signaling pathway						↑
Hematopoietic cell lineage			↓	↑		↑
Natural killer cell mediated cytotoxicity	↓		↓			↑

Supplementary table 2 continue

KEGG BASES BPs	PBMC	AT	LT	BT	ST	UT
T cell receptor signaling pathway						↑
B cell receptor signaling pathway	↓	↓				↑
FC epsilon ri signaling pathway						↑
FC gamma r-mediated phagocytosis			↓			↑
Leukocyte transendothelial migration			↓	↑	↑	↑
Intestinal immune network for iga production	↓					↑
Long-term potentiation		↓				
Neurotrophin signaling pathway			↓			↑
Long-term depression					↑	
Regulation of actin cytoskeleton			↓			↑
Insulin signaling pathway		↓			↑	
GNRH signaling pathway		↓	↓		↑	↑
Progesterone-mediated oocyte maturation					↑	
Type II diabetes mellitus		↓				
Type I diabetes mellitus	↓					↑
Aldosterone-regulated sodium reabsorption						↑
Collecting duct acid secretion						↑
Salivary secretion					↑	↑
Gastric acid secretion					↑	
Pancreatic secretion			↓		↑	
Carbohydrate digestion and absorption						↑
Protein digestion and absorption			↓	↑		
Fat digestion and absorption					↑	
Bile secretion		↓			↑	↑

Supplementary table 3: Common, significantly and highly correlated PBMC genes (highlighted in figure 5.2) in two rat experiments with their relation in different biological processed (based on NCBI database).

Name of the gene	Probe ID	Processed involve (according to NCBI data base)
Numb (numb homolog)	29419_at	- Adherens junction organization - Axonogenesis - Cell differentiation - Cell proliferation - Forebrain development - Lateral ventricle development - Lung epithelial cell differentiation - Negative regulation of Notch signalling pathway - Nervous system development - Neuroblast division in subventricular zone - Neuroblast proliferation - Positive regulation of dendrite morphogenesis - Positive regulation of neurogenesis - Positive regulation of polarized epithelial cell differentiation - Regulation of neuron differentiation
Phf2 (PHD finger protein 2)	306814_at	- Histone H3-K9 demethylation - Negative regulation of chromatin silencing at rDNA - Protein demethylation
Trmt10a (tRNAmethyltransferase 10 homolog A)	295496_at	- Magnesium ion homeostasis - Methylation
Zdhhc18 (zinc finger, DHHC-type containing-18)	362613_at	- Cellular protein localization - Metabolic process - Protein palmitoylation
Ngp (neutrophilic granule protein)	301026_at	- Defense response - Negative regulation of endopeptidase activity
Wiz (widely-interspaced zinc finger motifs)	314598_at	- Positive regulation on nuclear cell cycle DNA replication - Protein heterotrimerization - Protein stabilization
Zswim8 (zinc finger, SWIM-type containing 8)	361004_at	- Not found
Ikzf3 (IKAROS family zinc finger 3)	303511_at	- Regulation of B cell differentiation - Regulation of apoptotic process - Regulation of lymphocyte differentiation
Pim3 (Pim-3 proto-oncogene, serine/threonine kinase)	64534_at	- Apoptotic process - Histone phosphorylation - Negative regulation of apoptotic process - Negative regulation of insulin secretion involved in cellular response to glucose stimulus - Protein autophosphorylation - Protein phosphorylation - Regulation of mitotic cell cycle

Supplementary table 3 continue

Name of the gene	Probe ID	Processed involve (according to NCBI data base)
Hspa4 (heat shock protein 4)	266759_at	- Chaperone-mediated protein complex assembly - Kidney development - Negative regulation of DNA binding - Negative regulation of apoptotic process - Negative regulation of cell death - Negative regulation of protein phosphorylation - Positive regulation of angiogenesis - Positive regulation of protein binding - Protein import into mitochondrial outer membrane - Response to heat
Cd83 (CD83 molecule)	361226_at	- Negative regulation of interleukin-4 production - Positive regulation of CD4-positive, alpha-beta T cell differentiation, - Positive regulation of interleukin-10 production - Positive regulation of interleukin-2 production - Response to organic cyclic compound
Kdm6b (lysine (K)-specific demethylase 6B)	363630_at	- Cardiac muscle cell differentiation - Cellular response to hydrogen peroxide - Endothelial cell differentiation - Histone H3-K27 demethylation - Histone demethylation - Mesodermal cell differentiation - Positive regulation of transcription from RNA polymerase II promoter.
Whamm (WAS protein homolog associated with actin, golgi membranes and microtubules)	293057_at	- Not found
Mettl5 (methyltransferase like 5)	502632_at	- Methylation
Glod4 (glyoxalase domain containing 4)	363644_at	- Not found

Supplementary table 4: Common and significantly regulated ERGs in rat and human PBMC and AT with their relation in different biological processed (based on NCBI database)

Name of the common ERGs	Biological processes
CACYBP	- Aging, - Cardiac muscle cell differentiation, - Cellular response to calcium ion, - Heart development, - Negative regulation of cell death, - Positive regulation of DNA replication, - Response to growth hormone
NR4A1	- Apoptotic process, - Cell migration involved in sprouting angiogenesis, - Cell response to fibroblast growth factor stimulus, - Cellular response to organic substance, - Cellular response to vascular endothelial growth factor stimulus, - Endothelial cell chemotaxis, intracellular receptor signaling pathway, - Negative regulation of cysteine-type endopeptidase activity involved in apoptotic process, - Positive regulation of apoptotic process, - Positive regulation of endothelial cell proliferation, - Positive regulation of transcription from RNA polymerase II promoter, - Positive regulation of transcription, DNA template, - Skeletal muscle cell differentiation, - Steroid hormone mediated signaling pathway, - Transcription from RNA polymerase II promoter, - Transcription, DNA-templated.
IGF2	- Cell proliferation - Cellular response to mechanical stimulus, - Exocrine pancreas development, - Female pregnancy - Glucose metabolic process - Insulin receptor signaling pathway via phosphatidylinositol 3-kinas, - Memory - Organ morphogenesis - Osteoblast differentiation, - Positive regulation of MAPK cascade, - Positive regulation of activated T cell proliferation, - Positive regulation of catalytic activity, - Positive regulation of cell division, - Positive regulation of glycogen (starch) synthase activity, - Positive regulation of glycogen biosynthetic process, - Positive regulation of insulin receptor signaling pathway, - Positive regulation of mitosis

Supplementary table 4 continue

Name of the common ERGs	Biological processes
KITLG	- Cell adhesion, - Ectopic germ cell programmed cell death, - Embryonic hemopoiesis, - Extrinsic apoptotic signaling pathway I absence of ligand, - Male gonad development, - Negative regulation of apoptotic process, - Negative regulation of mast cell apoptotic process, - Neural crest cell migration, - Ovarian follicle development, - Positive regulation of DNA replication, - Positive regulation of MAP kinase activity, - Positive regulation of Ras protein signal transduction, - Positive regulation of cell proliferation, - Positive regulation of mast cell proliferation,
Runx1	- Behavioral response to pain, - Cellular response to transforming growth factor beta stimulus, - Central nervous system development, - Definitive hemopoiesis, - Embryonic hemopoiesis, - Hair fallicle morphogenesis, - Hemopoiesis, - In utero embryonic development, - Liver development, - Myeloid cell differentiation, - Myeloid progenitor cell differentiation, - Negative regulation of granulocyte differentiation, - Neuron differentiation
EFEMP1	- Biological process, - Epidermal growth factor receptor signaling pathway, - Negative regulation of chondrocyte differentiation, - Peptidyl-tyrosine phosphorylation, - Regulation of transcription, DNA-templated,
WISP2	- Cell adhesion, - Negative regulation of cell proliferation, - Regulation of cell growth.

Supplementary table 5: Common, significantly and highly correlated PBMC genes (highlighted in figure 5.3a) in rat and human with their relation in different biological processed (based on NCBI database).

Name of the gene	**Probe ID**	**Processed involve (according to NCBI data base)**
Sumo1 (small ubiquitin-like modifier 1)	301442_at	- Negative regulation of DNA binding - Negative regulation of sequence-specific DNA binding transcription factor acvity - Negative regulation of transcription, DNA-templated - Palate development - Positive regulation of proteasomal ubiquitin-dependent protein catabolic process - Positive regulation of protein complex assembly - Protein localization to nuclear pore - Protein sumoylation - Regulation of transcription, DNA-templated.
Il1b (interleukin 1 beta)	24494_at	- Chronic inflammatory response to antigenic stimulus - Cytokine-mediated signalling pathway - Ectopic germ cell programmed cell death - Estrogen metabolic process - Immune response - Inflammatory response - Negative regulation of cell proliferation - Positive regulation of 1-kappaB kinase/NF-kappaB signalling - Positive regulation of cell death - Positive regulation of apoptotic process - Positive regulation of T cell proliferation - Positive regulation of interleukin-2 and interlukin-6 biosynthetic process - Positive regulation of interleukin-8 production
Siglec10 (sialic acid binding Ig-like lectin 10)	292844_at	- Hematopoietic progenitor cell differentiation

Supplementary table 6: Common, significantly and highly correlated AT genes (highlighted in figure 5.3b) in rat and human with their relation in different biological processed (based on NCBI database).

Name of the gene	Probe ID	Processed involve (according to NCBI data base)
Casp8ap2 (caspase 8 associated protein 2)	313128_at	- Fas signalling pathway - Activation of cysteine-type endopeptidase activity in apoptosis process - Biological process - Cellular response to mechanical stimulus - Extrinsic apoptotic signalling pathway via death domain receptors
RGD1306820 (Erythroid differentiation regulatory factor 1)	309069_at	- Not found
Kdm5a (Lysine (K)-specific demethylase 5a)	312678_at	- Positive regulation of transcription, DNA-templated
Lamp2 (Lysosomal associated membrane protein 2)	24944_at	- Protein stabilization
Cpq (Carboxypeptidase Q)	58952_at	- Peptide catabolic process - Proteolysis - Thyroid hormone generation - Tissue generation
Atxn3 (Ataxin 3)	60331_at	- Actin cytoskeleton organization - Cellular response to heat - Cellular response to misfolded protein - Exploration behaviour - Histone H3 deacetylation - Intermediate filament cytoskeleton organization - Microtubule cytoskeleton organization - Misfolded or incompletely synthesized protein catabolic process - Monoubiquitinated protein deubiquitination - Proteasome-mediated ubiquitin-dependent protein catabolic process - Protein K48-linked deubiquitination - Proteolysis - Regulation of cell-substrate adhesion - Regulation of transcription, DNA-templated - Ubiquitin-dependent protein catabolic process
Rell1 (RELT-like 1)	289635_at	Not found
Decr1 (2,4-dienoyl CoA reductase 1, mitochondrial)	117543_at	- Fatty acid beta-oxidaton - Protein homotetramirization
Ndufa4 (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 4)	681024_at	- Hydrogen ion transmembrane transport

Supplementary table 6 continue

Name of the gene	Probe ID	Processed involve (according to NCBI data base)
Ndufa1 (NADH dehydrogenase (ubiquinone) 1 alpha subcomplex, 1)	363441_at	- Not found
S1pr3 (Sphingosine-1-phosphate receptor 3)	306792_at	- Adenylatecyclase-inhibiting –protein coupled receptor signalling pathway - Cytokine production - Inflammatory response - Negative regulation of establishment of endothelial barrier - Regulation of interleukin-1 beta production - Sphingosine-1-phosphate signalling pathway

Supplementary Table 7: Average plasma concentrations of daidzein and equol in female ovariectomized F344 rats that received 8 weeks oral gavage dose (2 mg/kg b.w.) of SIF from a commercial SIF supplement.

Daidzein	Equol
0.25± 0.01	0.38 ± 0.09

6

General Discussion

GENERAL DISCUSSION

Soy based products are gradually getting more popular and easily available in the Western societies. However, despite the long and safe history of soy consumption by the East and South-East Asian population, the benefit and safety of soy have been challenged in recent years and concerns have been raised about possible adverse health effects [1, 2]. These concerns were raised primarily because of the weak estrogenic and proliferative effects of soy isoflavone (SIF) aglycones due to their structural similarities with the natural female hormone estradiol (E2) and secondary because of stimulating effects of SIF aglycones on the proliferation of MCF-7 cells (a human estrogen sensitive breast cancer cell line) implanted into ovariectomized athymic mice [3]. These mice do not have a thymus and therefore do not produce T cells and are thus not able to reject tumour cells or other cells transplanted from other species. As breast cancer is a major cancer type in Western women, concerns have been raised for menopausal and post-menopausal woman who take soy based dietary supplements as a natural alternative for hormone replacement therapy [1, 4]. These concerns formed the basis for the research project of which the results are described in this thesis. It was the aim of this project to evaluate the use of in vitro and in vivo animal and human models to study early biomarks of effect, in order to improve the risk and/or benefit assessment of SIF intake for humans.

This was done by using up-to-date toxicological approaches in combination with the results of two parallel epidemiological studies conducted at the Division of Human Nutrition of Wageningen University, using the same soy based food supplement [5, 6]. We hypothesized that integration of toxicological and epidemiological data after standardization of exposure time and dose could improve the risk-benefit analaysis of SIF. Therefore, together with detailed in vitro toxicological studies, a transcriptomic approach was taken to indentify early biomarkers (Fig. 6.1) which allows direct comparison of animal and human data.

The principle of the so-called parallellogram approach is based on the fact that when comparison of study ourcomes in rat surrogate tissues (PBMC and adipose) would match with those in rat target tissues (breast, uterus, and sternum), an extrapolation of results in available human surrogate tissues to human target tissues, which are experimentally not easily acessible, can be made with confidence. As basis for such an extrapolation rat and human surrogate tissues should show similar responses. In this way pedictions for human target tissues can be made based on study data obtained in the rat. In the present study evidence to support this “tox-epi” approach combining rat and human transcriptomic data to improve the risk-benefit assessment was collected, taking SIF as the model compounds. The endpoints studied were gene expression profiles since these were expected to provide insight in effects on a wide range of possible endpoints, both adverse and beneficial, and also because SIF were expeced to express their beneficial and adverse health effects via expression of estrogen responsive genes (ERGs).

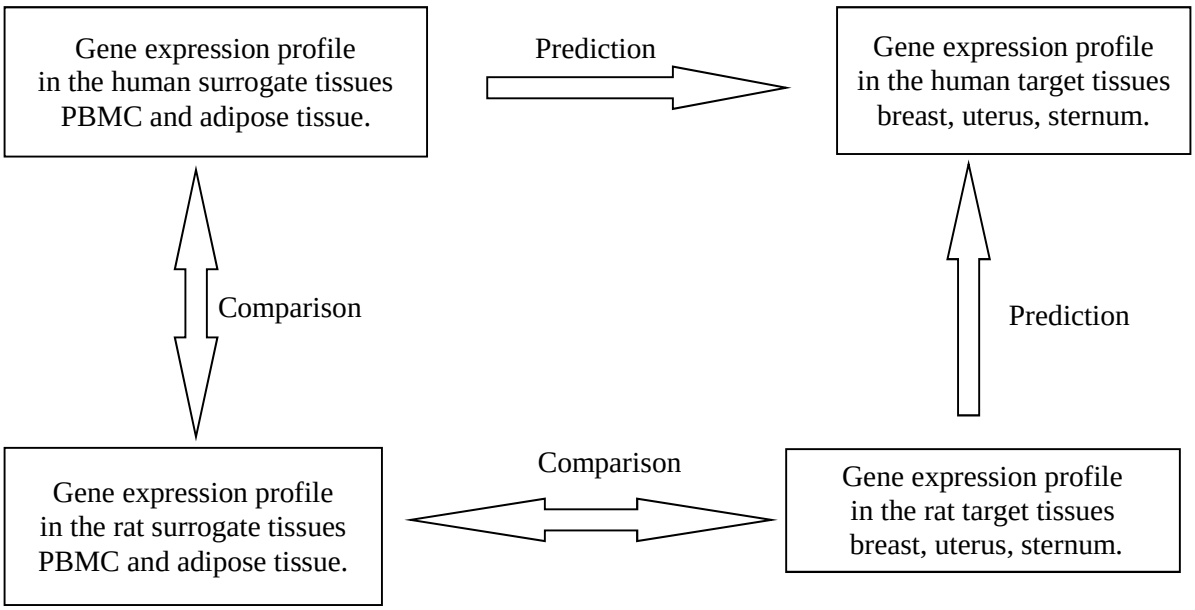

Figure 6.1 Scheme of the integrated "tox-epi" approach combining rat and human transcriptomic data to improve the risk-benefit assessment of soy supplementation for humans. PBMC: peripheral blood mononuclear cell.

Upon analysis of the SIF contents of nine soy supplements acquired commercially (Chapter 2), marked differences were found between the soy contents detected by HPLC and the contents mentioned in the product information sheets. This gives enough space for doubts to be raised about the quality control of the products and definitely warrants further and more stringent quality control of such products before introducing them on the market. Not only the actual SIF contents were considerably lower than the mentioned amounts, there also were significant differences between amounts of SIF present in the different products making it even more difficult to compare the data on possible biological effects resulting from exposure to them.

One of the key physiological events in the digestion of SIF is the breakdown of glucosides and the release of aglycones – which are believed to be the most biologically active forms of the SIF. The chemical hydrolysis of glucosides requires harsh chemical conditions with elevated temperatures (>80°C) and long incubation times, which are physiologically irrelevant. The passage time within the buccal cavity or the stomach is also not long enough to enable this hydrolysis of glucosides. On the other hand, only the aglycones (and not the glucosides) were shown to be transported across an in vitro Caco-2 monolayer (Chapter 2). This indicates the need for biocatalysis, and thus a role for enzymes, making this hydrolysis possible under physiological conditions (37°C). The data described in Chapter 2 of this thesis indicate that part of this hydrolysis may happen in the small intestine and not in the

stomach, and confirm the significant role of enzymes in the hydrolysis of the glucosides and subsequent release of the bioactive aglycones. The role of gut microbial flora can be crucial in providing the enzymes necessary for this hydrolysis [7-9]. Additionally, the lactase phlorizin hydrolase (LPH) enzymes present within the brush borders of the small intestine are also identified to play a role in this hydrolysis [10, 11]. Experiments described in Chapter 2 of this thesis, using cell free small intestine S9 samples, confirmed the role of LPH. To further evaluate the appropriateness of the in vitro enzymatic hydrolysis performed by the cell free S9 samples, the catalytic efficiencies of the hydrolysis, as derived from the in vitro kinetic data, were compared to the in vivo catalytic efficiencies, calculated based on data from intestine perfusion studies reported in literature. This comparison revealed that in vitro S9 hydrolysis as determined by the rat intestinal S9 incubations results in a 3 to 7 fold overestimation of the level of hydrolysis compared to rat intestinal perfusion studies. The presence of cytosolic and membrane-bound enzymes in intestinal S9 fractions might be the cause of this overestimation in the in vitro models, but also the contact between the enzymes and the glucosides in the S9 incubations might be more efficient than in the perfusion studies. In in vivo perfusion studies, especially the extracellular enzymes (LPH) and only part of the intracellular enzymes (i.e. glucocerebrosidase and broad-specificity β-glucosidase (BSβG)) may be able to deconjugate glucosides. In addition, a strong inter-species variation in the enzymes involved in hydrolysis of the glucosides has been reported. For example, in rats a concerted role of lactase phlorizin hydrolase (LPH), glucocerebrosidase and BSβG enzyme systems was recognized, whereas for humans, the hydrolysis was mostly catalysed by BSβG enzymes [12]. Furthermore, the role of enzymes in the deconjugation, but also in the reconjugation in so-called phase II metabolism is important for the ultimate concentration of SIF metabolites, present in the systemic circulation. The data presented in Chapter 2 revealed that the catalytic efficiency of the small intestine in re-conjugation of the SIF by phase II conjugation may be higher than that of the liver with the exception of the catalytic efficiencies for phase II metabolism of daidzin. It is worthwhile to mention that the fact that this reconjugation process shows a larger catalytic efficiency than the deconjugation of the glucosides, may explain why the detection of aglycones in the systemic circulation is unlikely. So high blood levels of the active SIF form are not to be expected. Moreover, the data obtained with the Caco-2 transwell model showed that the transport rates of the aglycones across the intestinal membrane were genistein > daidzein > glycitein.

As follow up to the in vitro studies on the kinetics of the SIF (Chapter 2), Chapter 3 presents investigations carried out to understand some of the toxicodynamics of the SIF. To this end the estrogenic effects of the glucuronidated (conjugated) SIF were compared with the activity of the respective aglycones. It has long been hypothesized that the conjugated (glucuronidated) SIF are less biologically active than the physiologically active aglycones [13-15], and the obtained results substantiated these claims with novel data. The potential of the SIF aglycones and their glucuronide

metabolites to induce cell proliferation was assessed using a T47D-wt cell line with a very high ERα/ERβ ratio, and a T47D-ERβ cell line with an ERα/ERβ ratio comparable to human breast tissue. Interestingly, the aglycones induced a 36-4500 fold higher estrogen-dependent cell proliferation compared to their glucuronidated metabolites. However, the maximal potency exhibited by the aglycone (genistein) was still three orders of magnitude lower than that of the known positive control estradiol (E2). It was also revealed that in the in vitro cellular incubations the glucuronides got hydrolysed to a small extent (0.6-1.6%) into aglycones, resulting in a level of the aglycones that could actually fully account for the mild estrogenic activities exhibited by these glucuronides. In additional studies described in Chapter 3, the estrogenicities of the SIF aglycones and their glucuronide metabolites towards ERα or ERβ receptors were characterised using reporter gene assays. In these studies the aglycones showed 450-1730 times higher efficiencies compared to the glucuronides. These results indicate that the estrogenic effects of SIF are dependent on efficient deconjugation of the glucuronide metabolites generating the biologically active aglycones at the target site. An interesting observation in the in vitro experiments was that exposure of the cells to physiologically relevant SIF concentrations in the exposure medium inhibited the breast cancer cell proliferation. It should be noted however, that the expressions of different ERs in the human breast tissue samples used in the present study as well as the ratio between ERα and ERβ was not quantified and this may be an interesting topic to be taken into account in further studies.

The results in Chapter 3 also pointed towards a significant inter-species variation between rats and humans in the ability to deconjugate the glucuronidated forms to aglycones. For example, at similar experimental conditions the rat S9 fractions of breast tissue deconjugated the glucuronidated forms about 30 times faster than the S9 fraction of human breast tissue. This greater bioactivation potency should be taken into account when extrapolating results from in vivo experiments performed in rats to humans, and should thus be considered when performing a risk assessment for humans based on rat data.

In Chapters 4 and 5 of the thesis, in vivo studies were described that were performed to further elucidate important modes of action underlying biological effects of SIF and to facilitate an interspecies comparison of the effects observed in rats with those observed in the human intervention studies performed in the framework of a parallel PhD project conducted at the Division of Human Nutrition of Wageningen University, after approval from the Medical Ethical Committee (Govt. clinical trials registration number NCT0123275 and NCT01556737. Chapter 4 describes the results of an in vivo experiment performed with inbred Fischer344 rats exposed to a low (2 mg/kg bw) or a high (20 mg/kg bw) SIF dose for 48 hours. Blood levels of SIF in the rats were measured at different time points during this period. The low dose was similar to the supplement dose used in the parallel human intervention study. Transcriptomic analysis of a broad set of 19,311 genes in peripheral blood mononuclear cells (PBMC) of rats was performed to assess the physiological and genetic responses to

this SIF exposure. Interestingly, the bioavailability of the SIF in the rat blood was different for the lower and higher doses. In the low dose group, a biphasic response in blood concentration of SIF with two spikes was observed. In contrast, for the high dose group a more time-dependent effect was seen. This observation of a biphasic response at low doses after 10 min and 2 h could be explained by absorption of the SIF from two distinct sites, the small intestine and the large intestine, as reported by Franke et al. [16]. However, for the high dose, it can be expected that due to the longer passage time spent in the small intestine, most of the absorption occurred from the small intestinal, thus decimating the biphasic response found for the low dose. The absence of SIF in the faeces is in line with other studies, indicating systemic de-conjugation of the SIF [17, 18].

In Chapter 4, the data derived from transcriptomic analysis of rat PBMCs were compared to similar data from a comparable human study from the parallel PhD project. The low dose of 2 mg SIF/kg bw applied in the rat study is comparable with the maximum daily human consumption (i.e. >100 mg/person per day) [19] and with the parallel human intervention study mentioned above (i.e. ~100 mg/person per day) [5]. Moreover, based on an assumed life span of rats and humans of two years and seventy years, respectively, the relative exposure time for rats (i.e. two doses in two days, resulting in exposure during about 0.27% of their lifespan) is roughly comparable with that of the human volunteers in the parallel study (i.e. 56 days, resulting in exposure during about 0.22% of their lifespan). The transcriptomic analysis of both the rat and human PBMCs was carried out in the same laboratory to avoid any experimentation bias. Interestingly, in the rat study 25-30% of the total number of the investigated genes showed significant changes compared to only 2% for the human study. This higher % of affected genes in rats than in humans may be the result of higher bioactivation (deconjugation) of SIF in rats than in humans. Compared to humans, the inbred rats, as expected, showed genetically less intra-species variation and were more comparable to each other. Genetic diversity has been observed in SIF metabolism before [20, 21] and this genetic incongruity between inbred rats and humans can have added to the observed dissimilar responses between rats and humans. Also for estrogen responsive genes (ERGs) a higher response was found in rats than in human volunteers. In rats 16 and 19 out of 87 ERGs were significantly regulated (i.e. both up or down regulated) for respectively the low and the high dose, whereas only 17 out of 1069 ERGs responded in human volunteers as result of SIF exposure. Of the responsive ERGs, only 10 overlapped for rat and human PBMCs. Intriguingly, only three of these ten genes changed in the same direction (increased or decreased) in both rats and humans. This lack of overall correlation in genetic up- or down-regulation between data obtained from rat and human PBMC was observed for both the low and the high dose animal group, and indicates that the rat might not be an appropriate animal model to extrapolate data to humans. However, the dissimilar results may also be due to the difference in exposure period of 48 h for rats compared to human exposure, which lasted for eight weeks. It might well be possible that some genes may be differentially regulated only in the first hours after exposure, after which an

opposite or no response would occur. For example, in a multi-generation study Chalabi et al. [22] observed that the F_1 generation did not express the same genes as the F_0 generation following administration of the same soy isoflavone-containing diet. In spite of the difficulties in the comparison of the SIF induced gene-expression in rats and humans, an interesting observation was the down-regulation of some cancer inducing genes (like retinoblastoma protein pathway RB1) in rat PBMC, which may point towards a beneficial effect of SIF related to the occurrence of cancer.

The results of an additional rat study, in which an exposure period of eight weeks was chosen, similar to that of the parallel human intervention study, to provide more comparable exposure conditions, are described in Chapter 5. It was concluded that the calculated total free aglycone SIF concentration that could be reached in blood of rats after eight weeks of exposure (i.e. about 19 nM) was lower compared to that calculated for the plasma of the human volunteers (about 130 nM). For this comparison we converted the concentrations of the individual SIF expressed as µg/ml reported by van Velpen [5] into nM using the molecular weights of the individual aglycones. One reason for the discrepancy in plasma levels reflected by a difference in the calculated total free aglycone concentration, can be the difference in dosing pattern. While the rats received a single oral dose every day in the morning, human volunteers consumed 2 capsules in the morning and 2 in the evening after the meal. This different dosing scheme may (partly) explain this finding. The significant inter-species variation in the kinetics of the SIF between rats and humans, as also shown in Chapter 2 of the thesis, may also contribute to this 6.8 fold difference in the calculated total free aglycone concentration in rats and humans following exposure to similar daily doses on a bodyweight basis.

In chapter 5 we have also addressed the issue of the relative estrogenic potency of equol, the main metabolite of daidzein, compared to that of its parent compound, to get an impression of the overall estrogenic activity of the estimated amount of the total free aglycones in rat plasma. We found that relative potency factors for equol compared to daidzein as reported in published literature varied widely, based on the test system used, but that most reports indicated a value in the range of 1 to 10. However, a formally accepted potency factor is lacking, which hampers a sound risk/benefit assessment of SIF. The same is true for other SIF like genistein and glycitein. Our own results reported in Chapter 3, showed that the relative estrogenic potency of genistein compared to daidzein also depends on the test system used. The estrogenic potency of genistein was 4.7 and 7.3 fold higher than that of daidzein in U2OS ERα and U2OS ERβ estrogen responsive cell lines, respectively (See Table 3.1), whereas a similar potency of genistein and daidzein was found in T47D-wt cells (See Table 3.2). In T47D ERβ cells, the potency of genistein was about 5.7 fold lower than that of daidzein (See Table 3.3). Also in the open literature different relative potencies for genistein compared to daidzein were reported, but the differences in outcomes of different test systems were less than we found. Kalita and Milligan [23] found that the potency of genistein is about 1.3 fold higher than that of daidzein in estrogen responsive Ishikawa cells and 2 fold higher in yeast cells. Hwang et al. [24] using

four different cell lines (i.e. human breast cancer cell line MCF-7, human embryonic kidney cells 293, murine calvarial osteoblasts MC3T3E1, and murine monocytic cells RAW 264.7) transfected with the estrogen-responsive reporter gene construct 3×ERE-TATA-Luc, reported a slightly lower potency of genistein compared to dadzein (i.e. 1.15 fold lower for the ERα receptor and 1.05 fold lower for the ERβ receptor response). For glycitein only limited information on its relative estrogenic potency is available in the open literature. Song et al. [25] reported that in a competitive binding assay glycitein and daidzein had a similar potency. Based on the different outcomes of the various test systems it is conceivable that the derivation of a single potency factor for the estrogenic activity of each of the individual SIF (i.e. genistein, glycitein and equol) compared to daidzein is difficult, because this may require a conclusion on which of the in vitro test systems is most relevant for the in vivo situation. Of course ER-activation is a principally different endpoint than induction of cell proliferation, which can be related to non ER-mediated mechanisms as well. In vivo experiments such as the uterotrophic assay in juvenile mice, could be helpful to solve the problem of quantification of the estrogenic potency.

It has been reported that while E2 activated ERα (which induces cell proliferation), SIF preferentially activated ERβ mediated gene expression, which would result in inhibition of cell proliferation and induction of apoptosis [26, 27]. This finding has been corroborated by the data presented in Chapter 3 of this thesis and this observation can partially explain the (claimed) beneficial effects of SIF in reducing cancer incidences, especially the incidences of breast cancer [28-30]. The transcriptomic analysis reported in Chapter 5, however, revealed that for the ERGs, surprisingly only non-significant changes were observed in their expression levels in estrogen responsive target organs, like breast and sternum. In addition, comparison of the calculated plasma concentrations of the total free SIF aglycones with the in vitro concentration response curves for the estrogenic effects of daidzein (see Chapter 5), revealed that these concentrations may be too low to activate ERα or to induce substantial ERβ mediated effects. Also, the target organs (prostate, breast and sternum) are structurally and physiologically quite different from the non-target organs (adipose tissue or PBMC) – making it difficult to derive solid comparisons between the different types of tissues, based on the data obtained. Paradoxically, it has been reported that consumption of 104 mg SIF per day can inhibit estrogenic effects as seen by the reduction in prevalence of hot flushes in postmenopausal women taking SIF containing supplements [31-32]. Marmugi et al. [33] stated that this might be due to the down-regulation of estrogen receptors, because the presence of xeno-estrogens increased the total level of estrogenic compounds in the circulation.

Also another important gene system (i.e. peroxisome proliferator-activated receptors/PPAR or tumour suppressor p53) was investigated within the framework of the in vivo experiments. An induction of PPAR signalling pathways was seen in breast and uterine tissues, whereas they were down-regulated in human PBMC. Activation of tumour suppressor p53 genes could also be observed upon SIF exposure which further strengthens the alleged beneficial roles of these SIF in cancer prevention.

Concluding remarks

Although the consumption of SIF as food supplements, particularly by post-menopausal women in Western societies, is increasing over the last decades, the mechanism of the estrogenic action of SIF in vivo, remains to be elucidated. The conventional animal models (e.g. rats) show insufficient capabilities to mimic the physiology of (post)menopausal women. In our in vivo studies we therefore made use of ovariectomized rats to have a better animal model to be compared with the parallel human intervention studies. It was the aim of the reseach presented in this thesis to improve the risk and/or benefit assessment of SIF for humans, by using in vitro and in vivo animal and human models, and gene expression data in various animal and human tissues, as early biomarkers of effects of exposure to SIF. The in vitro studies clearly showed that the aglycones are the bioactive form of SIF, although the glucuronide conjugates are the major fraction of SIF present in blood. The various SIF are capable of stimulating the ERα and ERβ-receptor differently, although the mechanism how the SIF interact with different ERs still remains to be resolved. Changes in gene expression results in surrogate tissues (PBMC and adipose tissue) of ovariectomized rats exposed to a SIF containing supplement did not correlate with changes in the same tissues of human volunteers exposed to the same supplement. There was also no clear correlation between effects on gene expression in surrogate and target tissues (i.e. breast, uterus, sternum) in the rat. This lack of a clear comparison in results in surrogate and target tissues of rats, and in that of surrogate tissues of rats and humans, implies that by using the so-called parallelologram approach, it is unfortunately not possible to make pedictions for effects in non-accessible human target tissues. Thus, the “tox-epi” approach we applied, by combining rat and human transcriptomic data to improve the risk-benefit assessment of isoflavones, indicates that the results should be interpreted with great care since the responses in surrogate tissues may not reflect those in target tissues. In addition, our results also revealed that plasma levels of SIF achieved after intake of a commercial SIF containing supplement may be relatively low compared to concentrations required to have a significant impact on ERGs.

Taking all together we were not able to predict possible effects in human target tissues based on the results obtained in ovariectomized rats. It can be suggested that aged rats might be a more appropriate model than young ovariectomized rats.

Future perspectives

Based on the results obtained and observations made in the thesis, several suggestions for further research can be put forward as future perspectives:

- In most of the in vivo studies performed with SIF, rats were used as animal model. In spite of some similarities in responses between humans and rats after exposure to SIF (e.g. low responses in ERG activation and low levels of aglycones in blood), major inter-species variation generates a mismatch between the results obtained in rats and humans. This discrepancy can be attributed to several factors e.g. the different enzymatic capabilities, differences in dosing schemes and different metabolic rates. In the literature, we did not find experiments where a similar type of transcriptomic approach was used as applied by us for a cross species comparison. Also not for other compounds. For a better understanding and comparison of cross-species gene expression profiles, particularly between experimental animals and humans, the availability of experiments following a similar study protocol for animals and humans would be instrumental.
- Another important aspect to be considered is the use of aged female rats instead of ovariectomized young animals as a more suitable model to mimic post-menopausal women. Also the use of omnivorous mini pigs can be a significant improvement as compared to using rats in such studies, as it has been shown that the biotransformation capacity of these mini pigs is more comparable to humans than that of rats [34-36].
- One of the major drawbacks of the studies reported in open literature is the fact that they have been conducted mostly with aglycones, whereas the aglycones hardly occur in the systemic circulation. Data presented in this thesis strongly suggest that conjugated forms of SIF like glucuronides and sulphates (and not aglycones) exist in the systemic circulation, and that deglucuronidation is needed to convert them into bioactive aglycones. Therefore, future in vivo studies should consider administration of conjugated SIF forms, i.e. glucosides by oral dosing. It is also important to measure the level of glucosides in blood and more emphasis should also be given to understand the pharmacokinetics of SIF in further in vivo studies. It would also be useful to link new in vivo studies with PBK (physiologically-based kinetic) modelling. The physiologically-based kinetic (PBK) models are becoming more advanced and they can give indications as well as explanations on how SIF are metabolised inside the body and what levels of the various metabolites could be expected in vivo in plasma and/or different target tissues.
- As discussed above the relative estrogenic potencies reported for the various SIF in open literature differ based on the test system used to derive them. Information on the relative potency of the different SIF is important in relation to the risk/benefit assessment of food supplements containing different compositions of SIF. Although it might be very challenging to derive a generally agreed potency factor for the different SIF, such potency factors will allow an easy comparison of the different SIF based on their dose, and will facilitate a better prediction of their expected effects. This, however, will greatly depend on the endpoint studied (purely ER-mediated or a more overall effect such as cell proliferation).
- Only few of the ERGs showed a change in expression after administration of SIF to rats and humans. One possible explanation can be that the levels of SIF required in blood to induce a clear

genetic up- or down-regulation were not reached in the described experiments, and that higher dose levels might be required. Another explanation could be that the main effects of SIF are not mediated via ERGs.

- Because SIF have different effects on the ERα and ERβ receptor, and levels of these receptors differ in different tissues, there is a need for better information on the ERα/ERβ ratio in target tissues, in order to better predict effects of SIF (or other estrogenic compounds) in these target tissues.
- Another important point is that studies investigating the effects of soy supplementation did not distinguish between the effects of SIF and of other constituents such as soy proteins, or other non-isoflavone phytochemicals such as phytic acid, sterols, saponins and lignans [37]. It might be possible that these other constituents alone or in combination with SIF, may be involved in the health effects of soy supplements, including anticancer, antioxidative, anti-viral and cardiovascular protective effects [38-40]. Therefore, future toxicological experiments, but also epidemiological studies, should not only focus on the effects of supplements as a whole, but also on individual isoflavones and other soy constituents.

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7

Summary

SUMMARY

This thesis describes the results of a research project that aimed at the improvement of the risk/benefit assessment of soy isoflavones (SIF) by combining toxicological and epidemiological methods. The toxicological studies were carried out at the Department of Toxicology and part of the results were compared with the outcome of human intervention studies, that were carried out in parallel research project at the Division of Human Nutrition. In Chapter 1 it is explained why we considered such an integrated "tox-epi" approach to be useful for the prediction of possible effects of SIF in humans on the basis of animal data. SIF are constituents of soy based supplements, which became more and more popular in Western societies over the last decades, because of their putative beneficial health effects, that were related to the SIF present in these supplements. In spite of the long and safe history of soy consumption by the East and the South-East Asian population, the benefit and safety of soy have been challenged in recent years and concerns have been raised about possible adverse health effects. These concerns focussed primarily on the weak estrogenic and proliferative effects of SIF. Chapter 1 also provides some background information on the individual SIF, their structural similarity with the steroid hormone estradiol (E2) and their interaction with the estrogen receptors ERα and ERβ.

Chapter 2 describes the differences between rats and humans in the conversion of the three major soy isoflavone glucosides, daidzin, genistin and glycitin, and their aglycones in a series of in vitro models. Results of studies in a Caco-2 transwell model confirmed that deconjugation of the isoflavone glucosides is essential for their transport across the intestinal barrier. It was shown that both rat and human intestinal S9 fractions were able to deconjugate the glucosides, and that intestinal enzymes plaid an important role in this deconjugation reaction. It was demonstrated that in the rat lactase phlorizin hydrolase, glucocerebrosidase, and cytosolic broad-specificity β-glucosidase contribute significantly to this deconjugation, and that in humans deconjugation mainly appeared to occur through the activity of broad-specificity β-glucosidase. Species difference in glucuronidation and sulfation were smaller than for the deconjugation reaction, and it was shown that 7-*O*-glucuronides were the major metabolites for all the three isoflavone aglycones. The in vitro results also indicated that glucuronidation in rats might be more efficient than in humans, again pointing towards species differences in the metabolism of isoflavone glycosides between rats and humans. It was also shown that the reconjugation reaction has a larger catalytic efficiency than the deconjugation of the glucosides, which corroborates that the detection of aglycones in the systemic circulation is unlikely.

It has been reported in literature that following administration of SIF to humans or animals, these compounds are mainly (~98%) present in the systemic circulation in their conjugated form (i.e. as glucuronide and sulphate) of which the estrogenic potency is not yet clear. Chapter 3 provides evidence that in an intact cellular model the major SIF glucuronide metabolites in blood, genistein-7-O-glucuronide (GG) and daidzein-7-O-glucuronide (DG), only become estrogenic after deconjugation.

The estrogenic potencies of genistein (Ge), daidzein (Da), GG and DG were determined using stably transfected U2OS-ERα, U2OS-ERβ reporter gene cells and proliferation was tested in T47D-ERβ and in T47D breast cancer cells. In all these assays the estrogenic potency of the aglycones was significantly higher than that of their corresponding glucuronides. UPLC analysis revealed that in the in vitro cell line assays, 0.2-1.6% of the glucuronides were deconjugated to their corresponding aglycones. It was also found that, under similar experimental conditions, rat breast tissue S9 fraction was about 30 times more potent in deconjugating these glucuronides than human breast tissue S9 fraction. The results presented in Chapter 3 confirm that SIF glucuronides are not estrogenic as such when tested in an intact cellular model system, and that the small fraction of aglycones account for the observed estrogenic effects. They also provide evidence for a significant species difference in the metabolism of SIF.

In Chapters 4 and 5 of this thesis, two rat studies are described, that were performed to further elucidate important modes of action underlying biological effects of SIF and to facilitate an interspecies comparison of the effects observed in rats with those observed in human intervention studies. In these studies inbred ovariectomized Fischer344 rats were used, as an animal model for (post)menopausal women. In the first study described in Chapter 4, two dose levels (i.e. 2 and 20 mg/kg bw) were used to characterise plasma bioavailability, urinary and faecal concentrations of SIF and to investigate changes in gene expression in peripheral blood mononuclear cells (PBMC). The low dose was in line with the type of dosing relevant for human supplement use. Animals were dosed at 0 and 48 hr and sacrificed 4 hr after the last dose. A clear dose dependent increase of SIF concentrations in plasma, urine and faeces was observed, together with a strong correlation in changes in gene expression between the two dose groups. In the transcriptomic analysis, all estrogen responsive genes (ERG) and related biological pathways (BPs) that were found to be affected by the SIF treatment were regulated in both dose groups in the same direction, and indicate possible beneficial effects of SIF. However, most of the common genes in PBMC of rats and of (post)menopausal women, exposed to a comparable dose of the same supplement, were regulated in opposite direction. Thus based on these results no correlation was found between the changes in gene expression in rats and humans, leading to the conclusion that rats might not be a suitable model for humans.

In Chapter 5 an animal experiment is described, in which rats received a dose of 2 mg SIF/kg body weight per day for a period of eight weeks. This dosing regimen was similar as that of the parallel human intervention study. Changes in gene expression in different target (i.e. breast (BT), uterus (UT) and sternum (ST)) and non-target (i.e. peripheral blood mononuclear cells (PBMC), adipose (AT) and liver (LT)) tissues were compared. Rank-rank scattered plots did not show any correlation in gene expression changes among different tissues. Out of 87 estrogen responsive genes (ERG), only 19 were found to be significantly regulated ($p<0.05$) in different tissues. The significantly regulated ERG were

mostly found in LT, AT and UT. Surprisingly, no ERG were significantly regulated in BT and ST, although these are considered to be important estrogen sensitive target tissues. No correlation was observed with the changes in gene expression in the PBMC of two rat studies. Correlation was also not seen in the changes of gene expression in PBMC and adipose tissue between rat and humans.

In Chapter 6 the results of the research project described in this thesis are evaluated. It was the aim of thesestudies to contribute to theimprovement ofthe risk and/or benefit assessment of SIF for humans, by using in vitro and in vivo animal and human models, and gene expression data in various animal and human tissues, as early biomarkers of effects of exposure to SIF. Although important information has been gathered on the metabolism and the estrogenic activity of SIF and their aglycones, we were not able to predict possible effects in human target tissues based on the results of changes in gene expression in target tissues obtained in the 8 weeks rat study. Possibly aged rats might be a more appropriate model than young ovariectomized rats.

Appendices

Acknowledgement
Curriculum Vitae
List of publications
Overview Training Activities
List of Abbreviations

Acknowledgement

The journey started on the 12th August 2008 when I arrived at Wageningen University to pursue my MSc degree in Food Safety. It was a dream coming true to study in Wageningen. Today I am not only happy but also proud to be able to successfully complete it. It would not have been possible to come to this stage without the help and support of the kind people around me and the organizations with their financial support. Therefore I would like to take this opportunity to express my sincere gratitude to honour their contributions.

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I am grateful to Dr. Ans, Hans, Bert and Marelle at the Division of Toxicology; Dr. Guido, Dr. Vera, Dr. Mark, Jenny and Shohreh at the Department of the Human Nutrition; Dini and Dr. Peter at RIKILT, and the people in the Experimental Animal Facility for their valuable contribution and technical support. Thanks again to my two paranymphs Hans and Bert for their effective support and help.

I would also like to thank the staff and (former) PhD students at the Division of Toxicology, Dr. Gerrit (my supervisor during MSc research), Prof. Ruud, Irene, Gre, Laura, Sourav (my daily supervisor during MSc research and also the only Bengali speaking person at Toxicology with whom I could discuss different scientific problems), Jaime (my daily supervisor during MSc minor thesis), Henrique, Ana, Ala, Walter, Alicia, Merel, Linda, Jochem, Wasma, Erryana, Nynke, Suzanne, Reiko, Barae, Samantha, Myrthe, Karsten (my nice roommate at Toxicology), Rungnapa, Elise, Alex, Hequn, Sunday, Rozaini, Jonathan, Nacho, Amer, Abdullah and Myrtho. You all were very friendly and helpful; it was a pleasure to work with all of you.

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I am grateful to the very nice and integrated Bangladeshi community at Wageningen. They really make the "out- of-lab-time" (especially the weekends and holidays) very enjoyable with many parties, festival and sports. I am also thankful to our very nice, friendly, active and helpful Muslim community at Wageningen. Finally I am deeply thankful to my parents, my wife, daughters, brothers and sister for their continuous support and encouragement. My father passed away during my PhD and I had to stay away, partly together with my wife and daughters…I am deeply sorry for the time we spent apart.

Curriculum Vitae

Mohammed Ariful Islam was born in May 9th, 1975 in Pabna, Bangladesh. After his secondary education in Dhaka in 1992, he started his undergraduate study in Agriculture at the Bangladesh Agricultural University in Mymensingh. In 2001 he completed his MSc in Agricultural Chemistry from the same University and joined as a lecture in the department of Agricultural Chemistry at the Sher-e-Bangla Agricultural University, Dhaka in 2003. In 2008 he moved to the Netherlands in a Nuffic funded Food Safety MSc program at Wageningen University. After receiving his MSc degree in 2010, he got the opportunity to conduct a PhD research at the Division of Toxicology on the project presented in this thesis funded by RIVM (National Institute for Public Health and the Environment) and in collaboration with a parallel project at the Department of Nutrition and Health. During his PhD study, he followed several postgraduate courses in toxicology which enabled him to register as a European Toxicologist.

List of Publications

Publications (from this PhD research work)

1. **M. A. Islam,** Hooiveld, J. E. J. G., van den Berg, H. J., Boekschoten, V. M., van der Velpen, V., Murk, A. J., Rietjens, I. M. C. M., and van Leeuwen, F. X. R. *Plasma bioavailability and changes in PBMC gene expression after treatment of ovariectomized rats with a commercial soy supplement.* Toxicoloy Reports, 2015. **2**, 308-321.
2. **M.A. Islam**, Bekele, R., van den Berg, J.H.J., Kuswanti, Y., Thapa, O., Soltani, S., van Leeuwen, F. X. R., Rietjens, I. M. C. M., and Murk, A. J., *Deconjugation of soy isoflavone glucuronides needed for estrogenic activity.* Toxicology in Vitro, 2015. **29**(4), 706-715.
3. **M.A. Islam**, Punt A., Spenkelink B., Murk A.J., van Leeuwen F.X.R. and Rietjens I.M.C.M.. *Conversion of major soy isoflvone glucosides and aglycones in in vitro intestinal models.* Molecular Nutrition and Food Research. 2014. **58**, 508-513.

Publications in preparation (from this PhD research work)

1. **M. A. Islam**, Hooiveld, J.E.J.G, van den Berg, H.J., Boekschoten, V.M., van der Velpen, V., Murk, A.J. Rietjens I.M.C.M. and van Leeuwen F.X.R. *Soy supplementaion: impact on gene expression in different tissues of ovariectomized rat and evaluation of the rat model to predict (post)menopausal health effect.*
2. V. van der Velpen, Geelen A, **Islam M.A.**, Braak, C.J.F., van Leeuwen F.X.R., Afman L.A., Hollman, P.C., Schouten, E.V. and van't Veer P. *Quantitative comparison of gene expression profiles of humans and rats after isoflavone supplementation.*

Publications (from other research work)

3. S. Bhattacharjee, Ershov, D., **Islam**, **M.A.,** Kampfer, A.M., Maslowska, K.A., van der Gucht, J., Alink, G.M. Marcelis, A.T.M., Zuilhof, H., and Rietjens, I.M.C.M. *Role of membrane disturbance and oxidative stress in the mode of action underlying the toxicity of differently charged polystyrene nanoparticles.* RSC Advance. 2014. **4**, 19321-19330.
4. M.O. Goni, **Islam, M.A.** and Hasanuzzaman, M. *Identification of arsenic hyperaccumulating plants for the development of phytomitigation technology.* Journal of phytology. 2010. **2**(1), 41-48.
5. **M.A. Islam**, Shiragi, M.H.K., Baque, M.A., Rahman M.S. and Khanam, T. *Arsenic contamination in food chain due to arsenic contaminated groundwater irrigation.* International Journal of Agricultural Technology. 2009. **5**(9), 40-45.

Overview of training activities

Courses

Organ toxicology, Utrecht University, Jan 10-14, 2011

Mutagenesis & carcinogenesis, Leiden University, Feb 7-11, 2012

Toxicogenomics. Maastricht University, Feb 28- March 4, 2012

Pathobiology, Utrecht University, Oct. 15-19, 2012

Molecular toxicology, Utrecht University, March 21-25, 2013

Ecotoxicology, Utrecht University and Wageningen University, Aug 19-30, 2013

Meetings

Nederlands Vereniging voor Toxicologie (NVT annual meeting, June 5-6, 2013, Zeist, Netherlands, oral presentation).

The 49th congress of the European societies of toxicology (EURO TOX, Sep 1-4, 2013, Interlaken, Switzerland, poster presentation).

The 18th congress of the European society for toxicology in vitro (ESTIV, June 10-13, 2014, Egmond aan Zee, Netherlands, oral presentation).

NVT 2014.

General courses

Laboratory animal science (LAS), Utrecht University, Feb. 7-18, 2011

VLAG PhD week, April 11-14, 2011

Risk assessment, Wageningen University, Oct 22-26, 2012

Review a scientific paper, Wageningen University, Nov 8, 2012

Optional activities

PhD excursion, Switzerland and Italy, June, 2011 and United Kingdom, June, 2013

Preparation of PhD research proposal

Attending scientific presentations at Division of Toxicology (2010-2014)

General toxicology (Tox.-20303, MSc course), Wageningen University, 2011

List of abbreviations

ADME - adsorption, distribution, metabolism and excretion,

AFSSA – French agency of food, environment and occupational health and safety

AT – adipose tissue

ATCC – american type culture collection

AUC – area under curve

AUC - area under the curve,

BfR – federal institute for risk assessment

BPs – biological pathways

BrdU – 5-bromo-2'-deoxyurimidine

BSA – bovine serum albumin

BSA - bovine serum albumin,

BS□G - broad specific □-glucosidase,

BT – breast tissue

Caco2 – (human) colorectal adenocarcinoma cell line

CDTA – Trans-1,2-diaminocyclohexane-N,N,N,'N'-tetraacetic acid monohydrate

CE – catalytic efficiency

CNS – central nervous system

COT – committee on toxicology

Da - daidzein

DCC-FCS – dextran-charcoal treated FCS

DFG – German research foundation

DG – daidzein-7-o-glucuronide

DMEM - Dulbecco's modified Eagle's medium,

DMSO - dimethyl sulfoxide,

DNA – deoxyribonucleic acid

DTT – dithiothreitol

E2 – estradiol

EDI – estimated daily intake

EDTA – ethylenedinitrotetraacetic acid

EEF – estradiol equivalency factors

EFSA – European food safety authority

EPA – (U.S.) environmental protection agency

ERα - estrogen receptor alpha

ERβ - estrogen receptor beta

ERGs – estrogen responsive genes

ERs – estrogen receptors

FBS – fetal bovine serum

FBS - fetal bovine serum,

FC – fold change

FDA – (US) food and drug administration

FDR – false discovery rate

Ge – genistein

GG – genistein-7-o-glucuronide

GI – gastrointestinal,

GSEA – gene set enrichment analysis

HRT – hormone replacement therapy

KEGG – Kyoto encyclopedia for genes and genome

Km – Michaelis-Menten constant

LDL – low density lipoprotein

LPH - lactase phlorizin hydrolase,

LT – liver tissue

MEM - minimum essential media,

miRNA- micro ribonucleic acid

MSD – multidimensional scaling

NB-DGJ - N-(nButyl)deoxygalactonojirimycin,

NEAA – non-essential amino acid

PAPS - 3'-phosphoadenosine 5'-phosphosulfate,

PBMC – peripheral mononuclear blood cells

PBS – phosphate-buffered saline

PC – potency concentration

PDA – photodiode array

PDA - photodiode array,

PMSF – phenylmethylsulfonyl fluoride

RIVM - the National Institute for Public Health and the Environment,

RMA – robust multichip average

SD – spraque dawley

SF - scaling factor

SIF – soy isoflavone

SKLM – senate commission on food safety

ST – Sternum tissue

T47D – (a well-known human) breast cancer cell line

T47D-wt – T47D wild type cell line

TEER – trans-epithelial electrical resistance

TEER - trans-epithelial electrical resistance,

TFA – trifluoro acetic acid

U2OS – (human) osteosarcoma cell line

UDPGA - uridine 5′-diphosphoglucuronic acid trisodium salt

UPLC - ultra performance liquid chromatography

USDA – United States department of agricultural

UT – uterus tissue

Vmax – maximum velocity

Propositions

1. Rats are not an adequate model to study human health effects of isoflavone food supplements. (this thesis)

2. It cannot be proven that the health effects of isoflavone food supplements are due to the estrogenicity of the isoflavones present. (this thesis)

3. A car with a small thorium-fuelled nuclear reactor is the dream car for the near future.

4. Think big, think nano.

5. The advantages of dual nationality do not outweigh the disadvantages.

6. The Netherlands is a good model for the socio-economic development of Bangladesh.

Propositions belonging to the thesis, entitled

"Improvement of risk assessment by integrating toxicological and epidemiological approaches: the case of isoflavones"

Mohammed Ariful Islam
Wageningen, 9 October, 2015.