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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 inT47D 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.

1. 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 (Barnes, 2010; Hwang et al., 2006; Setchell, 1998). 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 (Rong et al., 2011; Siow and Mann, 2010; Vitetta et al., 2011). 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 (BfR, 2007; Doerge et al., 2000; Maskarinec, 2013). 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 (Andres et al., 2011; Haines et al., 2012; Rietjens et al., 2013; Steinberg et al., 2011). 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 (Gu et al., 2006; Hosoda et al., 2010; Sesink et al., 2001). Glucuronides (Fig. 1) are the predominant metabolites reported in blood plasma (around 75%, depending on the species studied), followed by sulfates (24%) and aglycones (<1%) (Gu et al., 2006; Hosoda et al., 2010; Sesink et al., 2001). This means that estrogen sensitive tissues will predominantly be exposed to conjugated metabolites via 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. (2010) concluded

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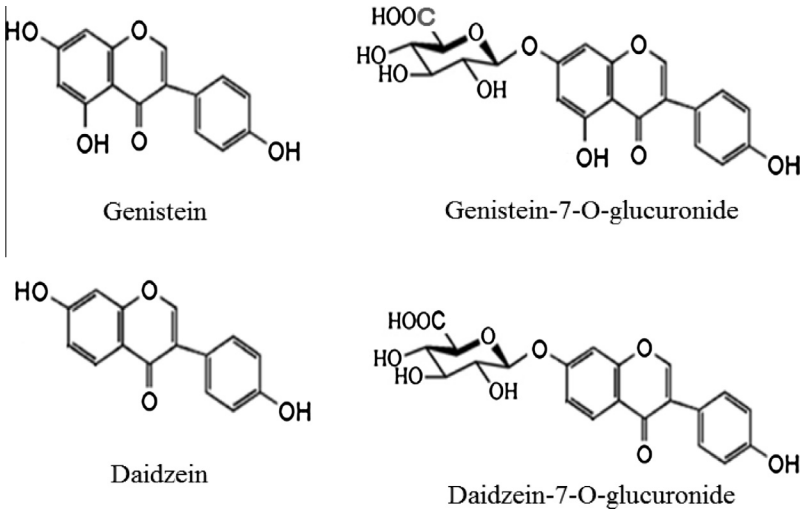

Fig. 1. Molecular structure of genistein, daidzein and their corresponding 7-O-glucuronides, the commercially available and predominant metabolites in the systemic circulation.

from a human intervention study that after consumption of dietary supplements rich is soy isoflavone (SIF), 98% of SIF reached the breast tissue as glucuronide, whereas Yuan et al. (2012) 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 (Hosoda et al., 2010; Terao et al., 2011; Zhang et al., 1999). 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 (Evers et al., 2013; Kuiper et al., 1997; Pearce and Jordan, 2004; Pfaffl et al., 2001). In certain types of breast cancer, the ERα/ERβ ratio is significantly increased compared to healthy tissue (Bardin et al., 2004a; Leygue et al., 1998), mainly due to a decrease of the ERβ levels (Lazennec et al., 2001; Rutherford et al., 2000). 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 (Bardin et al., 2004b; Sotoca et al., 2008; Ström et al., 2004). 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 (Sotoca et al., 2008; ter Veld et al., 2006). 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. (2013) 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 (Gu et al., 2006; Hosoda et al., 2010). The production of SIF aglycones in the cell culture experiments was determined as well.

2. Materials and methods

2.1. 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 Cedex, 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.2H2O; Titriplex), magnesium sulphate ($MgSO_4{\cdot}7H_2O$), 37% HCl, and KCl were purchased from Merck (Darmstadt, Germany). Magnesium carbonate ($(MgCO_3)4Mg(OH)_2{\cdot}5H_2O$), 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).

2.2. 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 (ter Veld et al., 2006). 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 (200 μg/ml) and hygromycin (50 μg/ml), while U2OS-ERβ growth medium was supplemented only with geneticin (200 μg/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. (2004) and used as described before (Sotoca et al., 2008). 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. (2013). 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 (Sotoca et al., 2008). 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.

2.3. 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 (Quaedackers et al., 2001). As a result, the amount of luciferase produced is a direct indication of the amount of estrogen-mediated gene expression via ERs (ter Veld et al., 2006; van der Woude et al., 2005). These two cell lines are ideal models for measuring ER-isotype specific transcriptional activity. Reporter gene assays were performed using the method described before (Sotoca et al., 2008; ter Veld et al., 2006). 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 10^5 cells/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 24 h of incubation. At 48 h, 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 24 h 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 (10 mM Tris–HCl pH 7.8 containing 2 mM DTT and 2 mM CDTA). To lyse the cells the plate was then placed on ice for 10 min to allow swelling and subsequently frozen at −80 °C for at least 30 min. 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 (ter Veld et al., 2006).

2.4. Cell proliferation assays

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 (Evers et al., 2013). The cell proliferation assay was performed using the method described by Sotoca et al. (2010). 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 (1.8×10^5 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 24 h of incubation. At 48 h, 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 48 h 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.

2.5. 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 exposure was limited to 20 μM and 400 μM GG or DG for 24 h. After the exposure, the medium was collected and cells were washed with PBS. Then cells were harvested and lysed in cold 65% aqueous methanol (St Gelais et al., 2012). All samples were stored at −20 °C until analysis.

2.6. 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 ± 0.2 g 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 °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 9000*g* for 15 min at 4 °C. After removing the fat layer from the top, the supernatant was collected as subcellular S9 fractions and stored at −80 °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).

2.7. 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 24 h at 37 °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 25 μl ice-cold acetonitrile. Samples were analyzed immediately.

2.8. UPLC analysis of SIF glucuronides and aglycones

All samples of the deconjugation experiments were analysed using a Waters Acquity™ 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 °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).

2.9. 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 E2at 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 (EPA, 2011). 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.

3. Results

3.1. Estrogenic potency of aglycones and glucuronides

Fig. 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 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 (Kwack et al., 2009; Sotoca et al., 2008; ter Veld et al., 2006). The estradiol equivalency factor (EEF) values for PC10 and PC50 obtained with both cell lines (Table 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 1), which in both ER-transfected cells was up to >200% of the maximum induction by E2.

3.2. Proliferation effects of soy isoflavones (aglycones and glucuronides)

Fig. 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 2).

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.

Fig. 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 100 nM tetracycline (Evers et al., 2013). 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 (Fig. 4 and Table 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

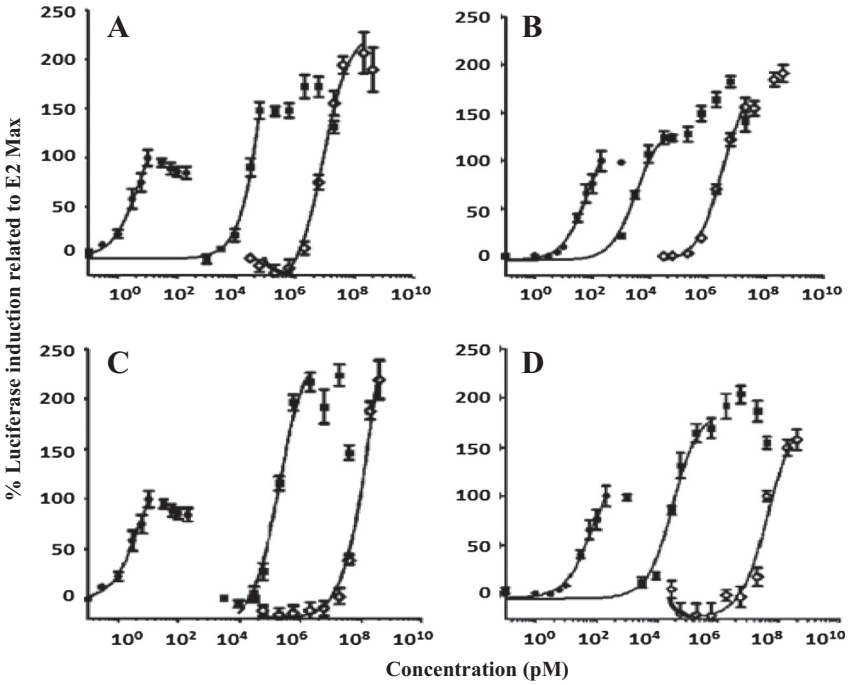

Fig. 2. Induction of ERE-mediated Luc activity in the U2OS-ERα (A and C) and U2OS-ERβ (B and D) cells upon exposure to increasing concentrations of E2 (●), Ge (■) and GG (◇) (A and B) and of E2 (●), Da (■) and DG (◇) (C and D). Induction was expressed relative to the maximum E2 response set at 100%. Data points represent the mean of triplicate determinations ± standard deviation.

Table 1
The estrogenic potency of SIF expressed as potency concentrations (PC10 and PC50) 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 estradiol equivalency factor (EEF) at PC10 and PC50, 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

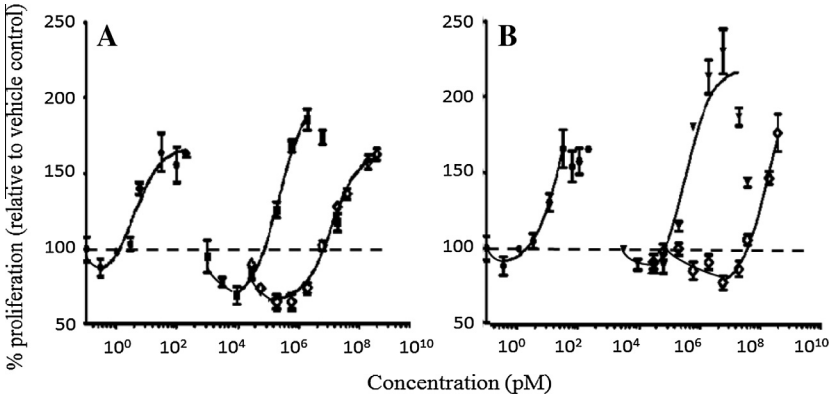

Fig. 3. Proliferation effect of E2 (●), Ge (■) and GG (◇) (A) and E2 (●), Da (▼) and DG (◇) (B) in T47D-wt type cells. Data are presented after normalized with respect to the proliferation of control cells set at 100%. Data points represent the mean of triplicate determinations ± standard deviation.

Table 2
Cell proliferation induced by SIF expressed as proliferation potency concentrations (PC10 and PC50) of genistein (Ge), genistein-7-O-glucuronide (GG), daidzein (Da) and daidzein-7-O-glucuronide (DG) compared to E2 in T47D-wt cells. Also estradiol equivalency factor (EEF) at PC10 and PC50, 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 (%)
T47D-wt	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

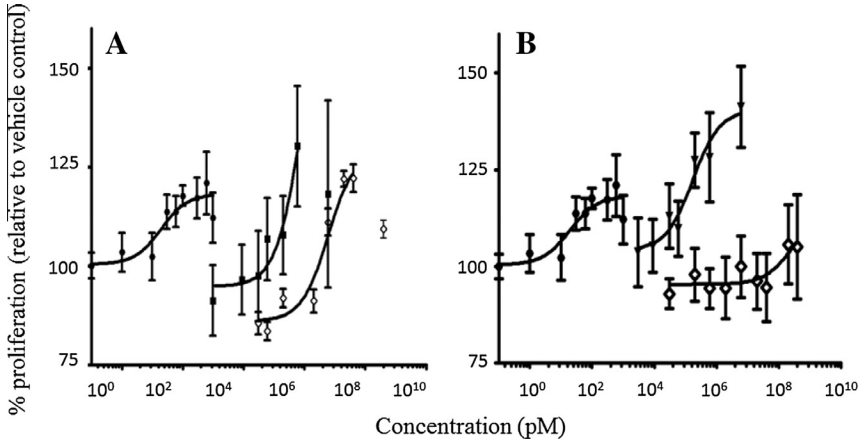

Fig. 4. Proliferation effect of E2 (●), Ge (■) and GG (◇) (A) and E2 (●), Da (▼) and DG (◇) (B), in T47D-ERβ cells when exposure medium contains 100 ng/ml tetracycline to mimic the ERα/ERβ ratio in normal breast tissue. Data are presented after normalized with respect to the proliferation of control cells set at 100%. Data points represent the mean of triplicate determinations ± standard deviation.

Table 3
Cell proliferation induced by SIF expressed as proliferation potency concentrations (PC10 and PC50) of genistein (Ge), genistein-7-O-glucuronide (GG), daidzein (Da) and daidzein-7-O-glucuronide (DG) compared to E2 in T47D ERβ cells. Also the estradiol equivalency factor (EEF) at PC10 and PC50, 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 (%)
T47D-ERβ	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

assay (Table 3). As with the reporter gene assays, the glucuronides were about 100-fold less potent than their respective aglycones (Tables 2 and 3).

3.3. In vitro deconjugation study

3.3.1. Cell lines

The UPLC chromatograms (Fig. 5a–c) show the deconjugation of GG to Ge as detected by analysis of the cell culture medium and the intracellular content after incubation for 24 h 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 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. 0.1–0.7 μM) of glucuronide conjugates was detected in the intracellular extract, where no aglycones were detected (Fig. 5 and Table 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 (0.1 μM) (data not shown).

3.3.2. Tissue S9 model

Fig. 6 shows the fate of GG and DG when incubated for 24 h 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 24 h 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,

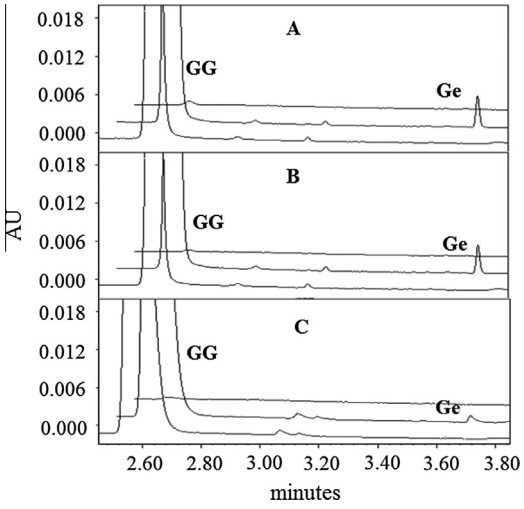

Fig. 5. UPLC chromatograms of culture medium and intracellular content after incubations of GG with (A) U2OS-ERα, (B) U2OS-ERβ and (C) T47D-wt cells. Chromatogram sequences (front to back) are – GG at T0; GG and Ge at T24 h; and intracellular GG at T24 h.

Table 4
Deconjugation of 400 μM genistein-7-O-glucuronide (GG) into genistein (Ge) and daidzein-7-O-glucuronide (DG) into daidzein (Da) after 24 h incubation with U2OS-ERα, U2OS-ERβ and T47D wild type cells (concentration and % of the total amount of compound). Also the intracellular concentration of the glucuronides were determined.

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

respectively (Table 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 5).

4. 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 (Gu et al., 2006; Yuan et al., 2012; van der Velpen et al., 2013). 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 (Evers et al., 2013). 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 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 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. (1999), 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 (Montano et al., 2010; Sotoca et al., 2010).

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 (Evers et al., 2013). 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 (Tables 2 and 3) as in the reporter gene assays (i.e. E2 > Ge > Da > GG > DG) (Table 1). Again, the aglycones showed much higher (36–>4500 fold) potencies than their corresponding glucuronides (Table 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 (Tables 2 and 3 and Figs. 3 and 4). This result is in accordance with the earlier reported decrease in proliferation with increasing ERβ levels (Sotoca et al., 2008). 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 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 (Gu et al., 2006; Yuan et al., 2012; van der Velpen et al., 2013). In this concentration the percentage of aglycones could be 1–4%, which corresponds to 20–80 nM. Our results (Figs. 3 and 4 and Table 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 6, also the glucuronide concentrations found in blood inhibited cell proliferation in our in vitro experiments. Thus based on

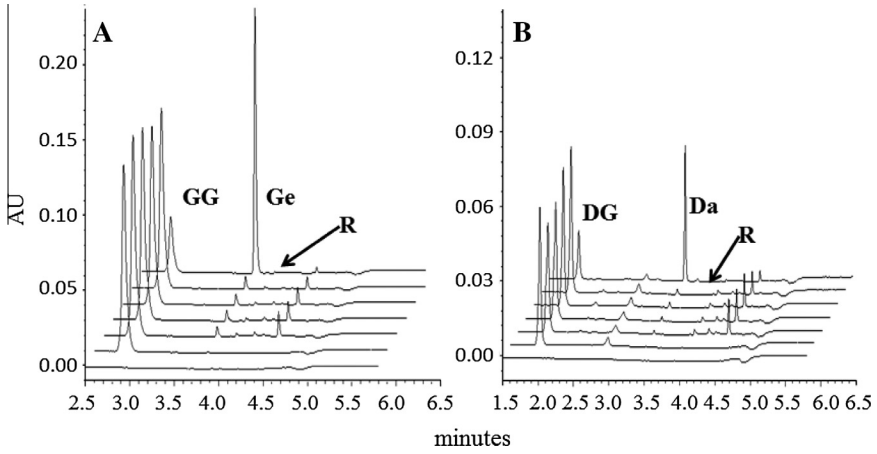

Fig. 6. UPLC chromatograms showing deconjugation of SIF glucuronides (i.e. formation of aglycones) by S9 fractions prepared from four normal human breast tissue samples and 1 pooled rat sample (indicated with R) after 24 h incubation with glucuronide GG (A) and DG (B). In all the incubation, protein concentration in the S9 fractions were 0.1 mg/ml. The chromatograms were extracted at 260 nm 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 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 24 h using a substrate concentration of 40 μM, and analysed with UPLC.

Substrate	Breast tissue S9 sample	% Deconjugation after 24 h	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	0.01109

Table 6
Concentrations of SIF genistein (Ge), genistein-7-O-glucuronide (GG), daidzein (Da) and daidzein-7-O-glucuronide (DG) in which inhibition of proliferation observed by 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	0–20,000	6000	24
T47D-ERβ	Ge	0–9	1	10
	GG	0–2000	60	16
	Da	ND	ND	0
	DG	0–40,000	200	6

ND = not detected.

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 (Bairey Merz et al., 2006; Legette et al., 2011; Novotny et al., 1975), might contribute to the beneficial instead of adverse effects of soy supplementation observed in epidemiological and clinical trials.

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 24 h. 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 (Tables 1–3). 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 4). These aglycone concentrations are higher than their PC50 values observed in the reporter gene assay (Table 1) and high enough to induce maximum cell proliferation (Table 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. (2012) 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 (Islam et al., 2014). 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 (Islam et al., 2014), the HPLC chromatograms of the resulting incubations (Fig. 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 24 h. 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 (Singh et al., 2010) compared to human (about 1–2%) (Gu et al., 2006; Hosoda et al., 2010). Only one publication (Setchell et al. (2011)) 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. (2010) and Shimoi (2004) 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 postmenopausal 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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Conflict of Interest

The authors have declared no conflict of interest.

Transparency Document

The Transparency document associated with this article can be found in the online version.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.tiv.2015.01.013.

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