# Analysis and genotoxicity of the isoflavones genistein, daidzein and equol, and risk assessment for consumption in human diet

**PhD thesis to be submitted to the**

Faculty of Chemistry,

University of Dortmund

by

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Dortmund 2005

This thesis has been performed with support from the Deutsche Forschungsgemeinschaft by the International Graduate College “Molecular Mechanisms in Food Toxicology” (Düsseldorf-Dortmund-Maastricht-Utrecht-Wageningen). Supervisors within the International Graduate College were Prof. Dr. Dr. H. M. Bolt, Dortmund (experimental part) and Prof. Dr. J. C. S. Kleinjans, Maastricht (discussion of risk assessment). The teaching programme, as part of the International Graduate College, was coordinated by Prof. Dr. R. Kahl, Heinrich-Heine University, Düsseldorf.

## Acknowledgements

I thank the Deutsche Forschungsgemeinschaft for the financial support of my thesis work, by a stipend, via the International Graduate College “Molecular Mechanisms in Food Toxicology” (GRK 738), located at the Heinrich-Heine University of Düsseldorf.

Special and sincere thanks to Prof. Dr. Dr. H.M. Bolt for giving me the possibility of carrying out this project at IfADo (Institut für Arbeitsphysiologie, University of Dortmund).

I am also very grateful for the highly valuable discussions with Prof. Dr. G.H. Degen and for her helpful comments and suggestions for this thesis work.

I would like to acknowledge Prof. Dr. R. Kahl for the dedicated hours and valuable motivation during the Graduate College Lectures at the Heinrich-Heine University of Düsseldorf.

I am very thankful to Dr. P. Diel, Institut für Kreislaufforschung und Sportmedizin der Deutschen Sporthochschule, Köln, who provided me with the rodent plasma samples for isoflavone analysis.

I thank all my colleagues from IfADo for the wonderful time I spent with them and for the friendly support and help. Especially, I gratefully thank Dr. D. Bonacker, from whom I learnt the Micronucleus assay and Stefan Moors who helped me with an introduction into the analytical techniques. I would like to thank all my colleagues in Düsseldorf, with whom I shared many lectures and graduate courses in Germany and in The Netherlands.

Last but not least, I would like to thank my parents and my sister for the unconditioned support during the time I stayed in Germany, and to my beloved husband for his constant understanding and encouragement.

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## Abbreviations

| | |
|---|---|
| A. dest. | aqua destillata (distilled water) |
| AcOH | acetic acid |
| ATP | adenosine triphosphate |
| ATPase | adenosine triphosphatase |
| AUC | area under the curve (dose parameter in toxicokinetics) |
| BCA | bicinchoninic acid |
| BSA | bovine serum albumin |
| b.w. | body weight |
| C max | maximal plasma concentration (toxicokinetics) |
| Cl | clearance (toxicokinetics) |
| Cl/F | systemic clearance normalized to the bioavailable |
| $cm^2$ | square centimeter |
| Conc. | concentration |
| CREST | **C**alcinosis, **R**aynaud phenomenon, **E**sophageal dismotility, **S**clerodactyly and **T**elangiectasia (disease leading to centromere antibodies) |
| CV% | coefficient of variation, % |
| D oral | oral administered dose (toxicokinetics) |
| D i.v. | intravenous administered dose (toxicokinetics) |
| DAPI | 4`6`diamidino-2-phenylindole |
| DMEM | Dulbecco`s modified Eagle`s medium (for cell culture) |
| DMSO | dimethylsulphoxide |
| DNA | deoxyribonucleic acid |
| EDTA | ethylenediaminetetraacetic acid |
| ER | estrogen receptor |
| F | bioavailability |
| FCS | fetal calf serum (for cell culture) |
| FITC | fluorescein-5-isothiocyanate |
| GC-MS | gas chromatography-mass spectrometry |
| h | hour |
| HPLC | high performance liquid chromatography |
| $IC_{50}$ | inhibition concentration of 50 % |

| | |
|---|---|
| Ig | immunoglobulin |
| i.v. | intravenous |
| MeOH | methanol |
| MMS | methylmethanesulfonate |
| MN | micronucleus |
| NR | Neutral Red |
| OVX | ovariectomized |
| PBS | phosphate- buffered saline |
| PI | propidium iodide |
| p.o. | per os |
| rpm | revolutions per minute |
| SD | standard deviation |
| t ½ | half-life |
| t max | mean time to attain peak plasma concentration |
| UV | ultraviolet light |
| v/v | volume/volume |
| V79 cells | Chinese hamster cell line (for cell culture) |
| VCR | vincristine |
| Vd | volume of distribution (toxicokinetics) |
| Vd/F | average volume of distribution normalized to apparent bioaiavailability |
| $x_i$ | each independent measurement |
| $\bar{x}$ | mean value |

# 1. PREFACE

The issue of environmental chemicals with hormonal activity, being addressed as "endocrine disruptors", has been debated since the beginning of the nineties (Bolt and Degen 1996/97). Initially, this debate had been focused on polychlorinated organic compounds, plasticizers and other man-made chemicals. Later, the impact of naturally occurring and hormonally active (estrogenic) compounds in human food has been discussed. The impact of man-made environmental chemicals with hormonal/endocrine effects has been compared with that of natural food ingredients, in order to set priorities for governmental regulations (Bolt et al. 2001). This has initiated new interest in the biological activities of estrogenic, hormonally active compounds found in plants, most of which have an isoflavone structure in common. The phytoestrogens daidzein and genistein are two such naturally occurring isoflavones, which are found in edible plants, especially in soybeans (structural formulae: see Fig. 2). A significant part of human exposure to natural phytoestrogens is, therefore, dependent on the type of diet.

In whole animal models and in experimental systems in vitro, phytoestrogens appear capable of acting both as partial agonists and as antagonists, the primary effects of which are mediated via interaction with the estrogen receptor. Phytoestrogen-rich diets have the potential to exert adverse as well as beneficial effects in humans (Cassidy, 1996). This is related to the hormonal activity, which is therefore relevant for the assessment of associated risks to humans.

Apart from the intrinsic hormonal activities of these compounds, the question of genotoxicity and the associated carcinogenicity has been raised from a toxicological point of view (Kulling and Metzler, 1997). This thesis project, elaborated as part of the International Graduate College "Molecular Mechanisms in Food Toxicology", intended to confirm or reject the claim of Kulling and Metzler (1997) of a relevant genotoxicity of genistein and daidzein and to accomplish a genotoxicity risk assessment for soy isoflavones in human diets.

The work was therefore based on input from the fields of analytical chemistry, toxicology and risk analysis.

# 2. INTRODUCTION

## 2.1. Phytoestrogens: definition

Phytoestrogens are defined as estrogenic compounds found in plants. They can be divided into three main classes: isoflavones, lignans and coumestans. They have common chemical characteristics as phenolic substances, with some structural similarities to the natural estrogen estradiol (Figs. 1 and 2). The presence of a phenolic ring allows binding to the estrogen receptor (ER), which is followed by induction of specific estrogen-responsive gene products that finally lead to biological effects such as stimulation of cell growth (Kurzer and Xu, 1997).

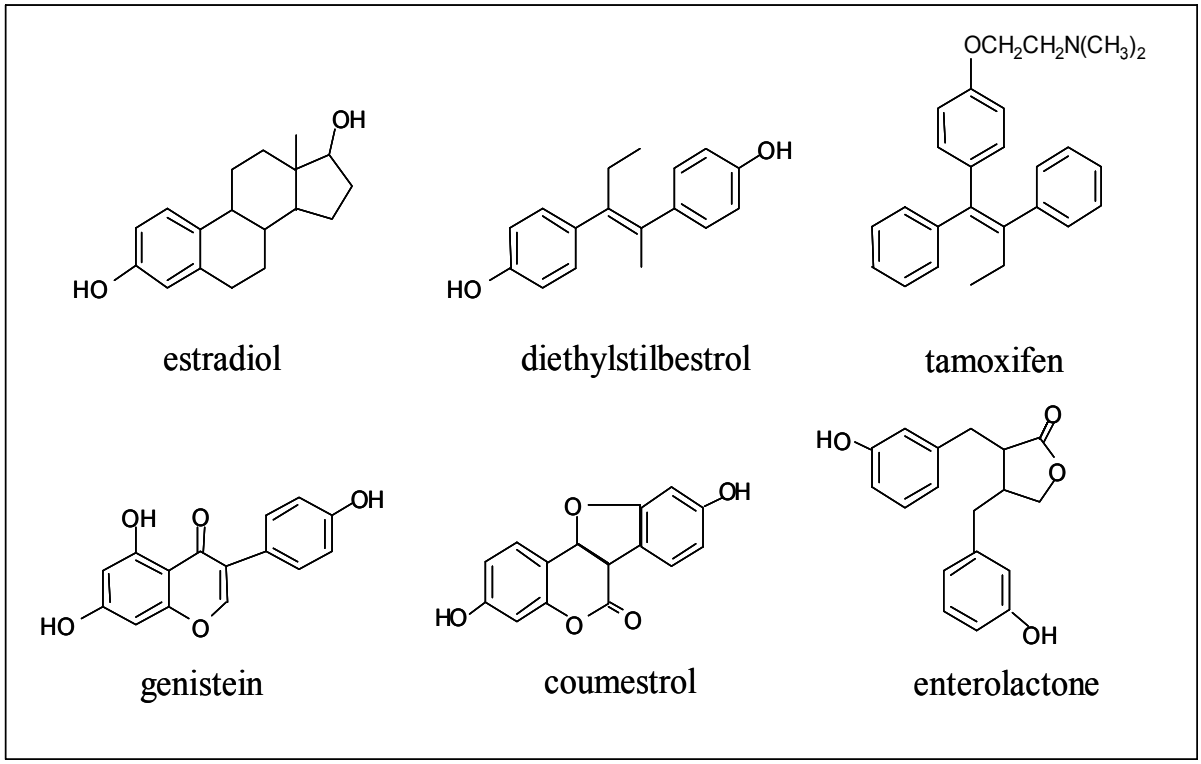



Fig. 1. Examples of chemical structures of some estrogens/antiestrogens. Genistein (isoflavone), coumestrol (coumestan) and enterolactone (lignan) in comparison with estradiol (natural estrogen), diethylstilbestrol (synthetic estrogen), and tamoxifen (synthetic antiestrogen).

## 2.2. Phytoestrogens: dietary sources

The phytoestrogens genistein (5,7,4´trihydroxyisoflavone) and daidzein (7,4´dihydroxyisoflavone), as well as their methylated derivatives (biochanin A and formononetin) (Fig. 2) are naturally occurring isoflavones present in a number of plants, especially in soybeans and in soy-derived products. Genistein, daidzein and their ß-glucoside conjugates (Fig. 2) are found at high concentrations (up to 3mg/g) in soy beans (Coward et al., 1993, see Table 1 and 2). Chick peas and other legumes, as well as clover and bluegrass, have also been identified as relevant isoflavone sources. Food processing is known to influence the conjugation state (conjugated vs. aglycones) of isoflavones in soy foods. Non-fermented soy foods (e.g. tofu) contain higher levels of glucosides, while fermented soy foods (e.g. tempeh) contain higher levels of aglycones, as a result of enzymatic hydrolysis during fermentation (Wang and Murphy, 1994).

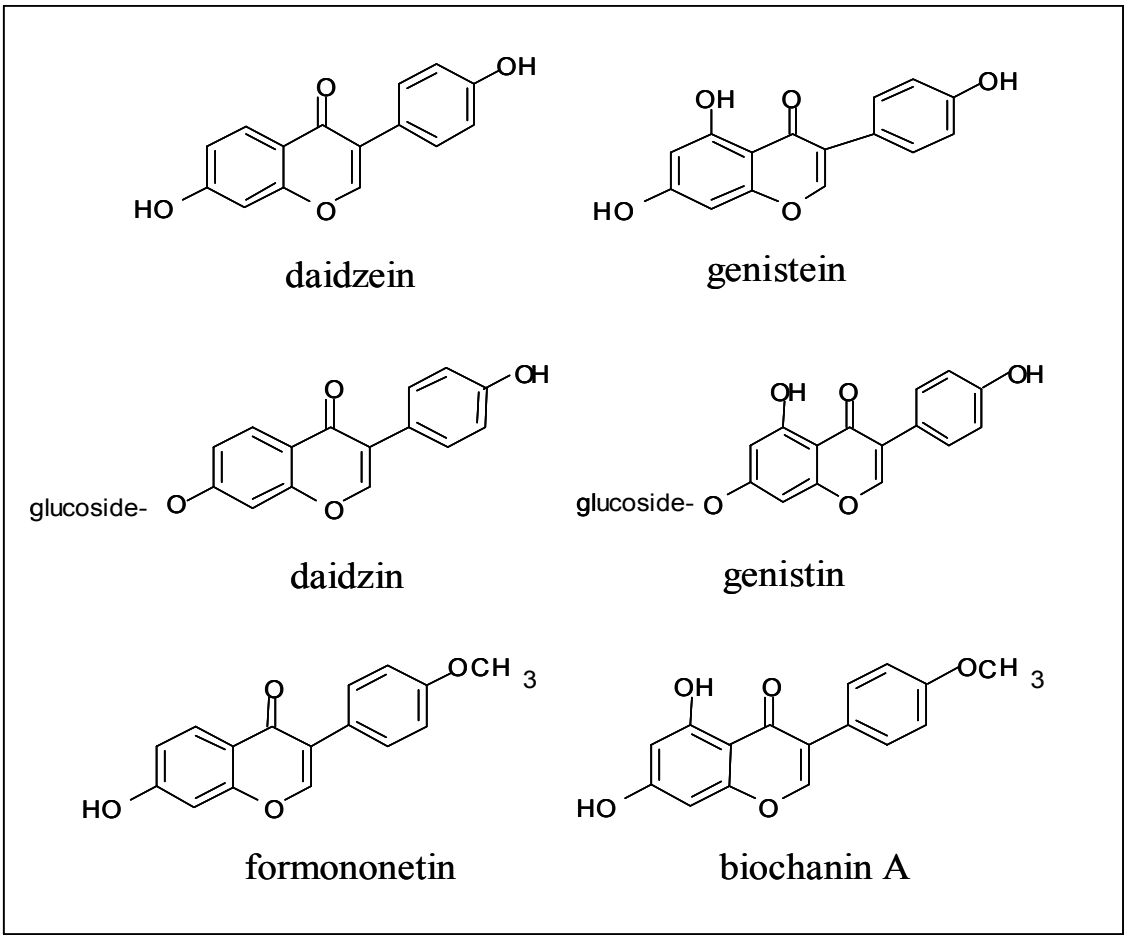



Fig. 2. Chemical structures of isoflavones. Genistein differs from daidzein by an additional hydroxyl group at position 5. Also ß-glucoside conjugates (daidzin and genistin) and methylated forms of isoflavones (formononetin and biochanin A) occur in plants, which are demethylated in the human and animal organism.

| | Conjugated | | Aglycones | | | | Aglycones % | |
|---|---|---|---|---|---|---|---|---|
| Food | genistin | daidzin | genistein | daidzein | Total | D/G ratio | genistein | daidzein |
| soy milk | 1.68 | 1.34 | 0.09 | 0.14 | 3.25 | 0.83 | 5 | 10 |
| tofu* | 1.21 | 0.59 | 1.51 | 0.01 | 2.03 | 0.49 | 11 | 12 |
| tofu** | 2.08 | 1.51 | 0.11 | 0.11 | 3.83 | 0.74 | 5 | 7 |
| soy flour | 0.74 | 0.58 | 0.01 | nd | 1.34 | 0.77 | 2 | 0 |
| soy powder | 1.15 | 0.58 | 0.01 | nd | 1.75 | 0.5 | 1 | 0 |
| soy nuts | 1.39 | 0.85 | 0.06 | 0.05 | 2.36 | 0.62 | 5 | 6 |

Table 1. Isoflavone concentrations (expressed as mg/g dry weight; mean values of triplicate analysis) in Asian primary soy materials. * Tree of Life Tofu; **Mori-Nu Tofu; D/G means daidzein+daidzin/genistein+genistin; nd, not detected. Data from Coward et al., 1993.

| | Conjugated | | Aglycones | | | | Aglycones % | |
|---|---|---|---|---|---|---|---|---|
| Soy product | genistin | daidzin | genistein | daidzein | Total | D/G ratio | genistein | daidzein |
| tempeh | 0.29 | 0.1 | 0.43 | 0.29 | 1.13 | 0.55 | 59 | 74 |
| miso | 0.06 | 0.05 | 0.74 | 0.51 | 1.38 | 0.7 | 92 | 91 |
| rice miso | 0.35 | nd | 0.24 | 0.12 | 0.72 | 0.21 | 41 | 100 |
| barley miso | 0.26 | 0.23 | 0.39 | 0.31 | 1.19 | 0.83 | 61 | 57 |
| soybean paste | 0.16 | 0.09 | 0.51 | 0.4 | 1.17 | 0.73 | 76 | 82 |
| soybean paste/rice | 0.11 | 0.14 | 0.17 | 0.17 | 0.58 | 1.08 | 62 | 55 |
| soybean paste/wheat | 0.22 | 0.19 | 0.25 | 0.21 | 0.87 | 0.85 | 53 | 53 |

Table 2. Isoflavone concentrations (expressed as mg/g dry weight; mean values of triplicate analysis) in Asian soy products. D/G means daidzein+daidzin/genistein+genistin; nd, not detected. Data from Coward et al., 1993.

Apart from the differences in isoflavone contents of soy beans and derived products, the consumption of these compounds varies with the type of human diet: many Asian populations (with low rates of breast and prostate cancer) consume 30-50 times more isoflavones (almost entirely derived from soy) than American or European populations (Bolt and Degen, 2000, Setchell, 1998). In contrast to Western diets, traditional Oriental diets consist of much higher proportions of food derived from vegetation, especially of soy products.

## 2.3. Metabolism and disposition of isoflavones

### *2.3.1. Absorption and reductive metabolism of isoflavones*

The chemical form in which isoflavones occur is an important issue because it may influence the biological activity, the bioavailability, and therefore the physiologic effects of these dietary constituents.

Metabolic pathways of daidzein and genistein in humans were originally proposed by Setchell and Adlercreutz (1988), based on the metabolites found in human urine (Fig.3). As already mentioned, isoflavones occur in soy products mostly in the glycoside forms (daidzin and genistin). After ingestion, soybean isoflavones are hydrolysed by intestinal glycosidases, which release the aglycones, daidzein and genistein. These may be absorbed or further metabolised to specific metabolites (Fig. 3).

The extent of absoption may be dependent on the chemical form of the isoflavones, in that the unconjugated isoflavones, because of their liphophilic properties, may be more readily absorbed and, therefore, be more bioavailable than the highly-polar conjugated species (Cassidy, 1996). There is evidence that hydrolysis of the isoflavones glycosides occurs before their absorption from the gut since the glycosides have not been detected in plasma (Andlauer et al., 2000; Setchell et al., 2002). The site of hydrolysis has not been fully elucidated, but it is known that glycosidase enzymes are produced in the small intestine and by the gut microflora (Day et al., 1998; Rowland et al., 2003). It seems likely that mammalian beta –glycosidase activity, associated with the small intestinal mucosa, is involved in glycoside hydrolysis prior to absorption. Beta-glycosidase activity shows developmental expression early in life. This facilitates the absorption of isoflavones contained in soy infant formulae, resulting in very high plasma concentrations in infants fed with “soy milk” products (Setchell et al., 1997).

After hydrolysis of the sugar moiety in the gastrointestinal tract, isoflavones can be absorbed, or undergo additional biotransformation by the gut microflora to reductive metabolites (Fig. 3): genistein is metabolically transformed to dihydrogenistein (4′,5,7 trihydroxy-isoflavanone) and 6′hydroxy- O-desmethylangolesin (6′-hydroxy-O-DMA) (2′,4′,6′,4′′tetrahydroxy-α-methyldeoxybenzoin, Fig. 3, right panel), and finally degraded to 4-hydroxyphenyl-2-propionic acid (HPPA) and 1,3,5-trihydroxybenzene (THB) (not shown, Joannou et al., 1995; Coldham et al., 2002).

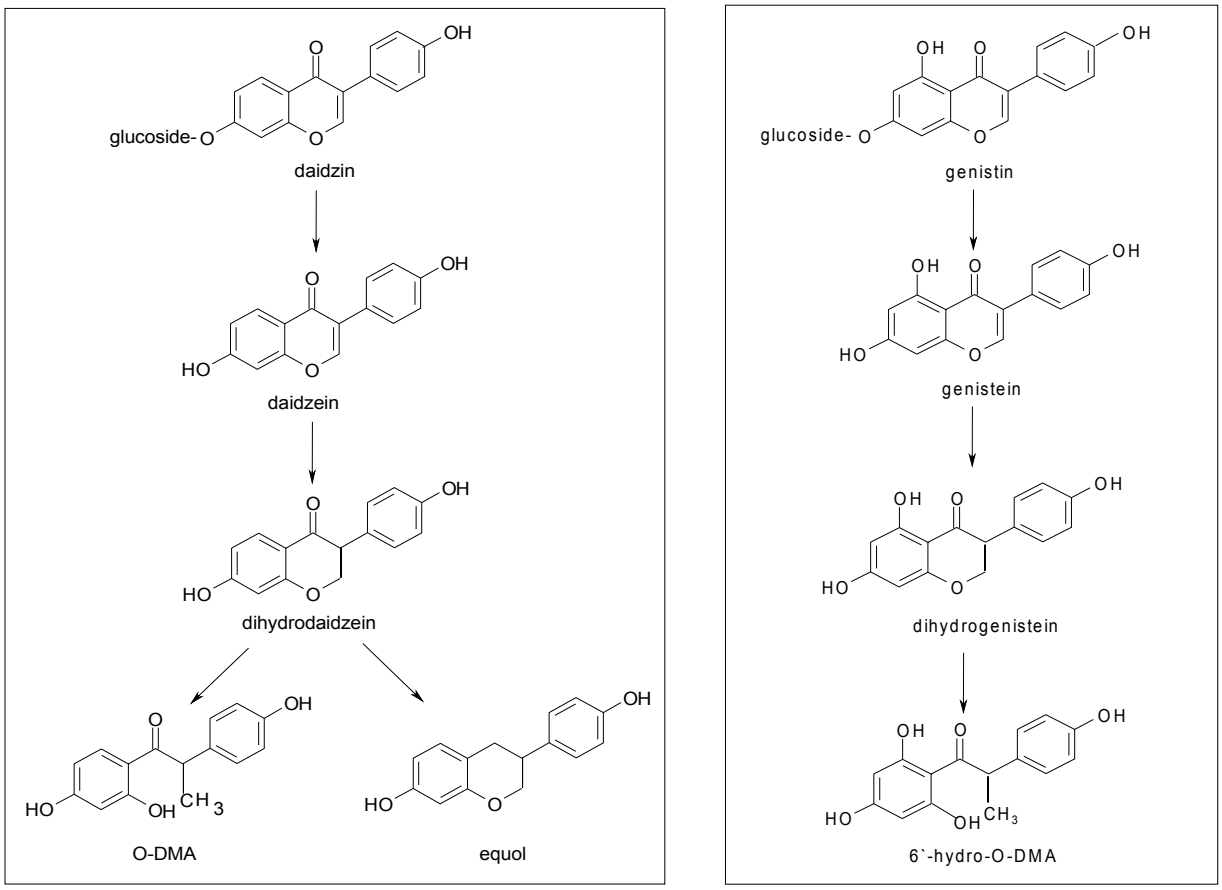



Fig. 3. Metabolic pathways for daidzein and genistein. The glycosidic forms present in plants are cleaved in the gastrointestinal tract and metabolised by gut bacteria and mammalian enzymes.

Daidzein is reduced to dihydrodaidzein (4′,7 dihydroxy-isoflavanone), which is further metabolised to O-desmethylangolesin (O-DMA) (4′,6′,4′′trihydroxy-α-methyldeoxybenzoin) and/or the isoflavan equol (Fig. 3, left panel). The ring structures and numbering of atoms within these structures are shown in Fig. 4.

The extent of this metabolism appears to be highly variable among individuals and is influenced by other components of the diets (Setchell et al., 1984). At high levels of carbohydrate intake, causing increased intestinal fermentation, a more extensive biotransformation of phytoestrogens is seen, with an increase of the formation of equol.

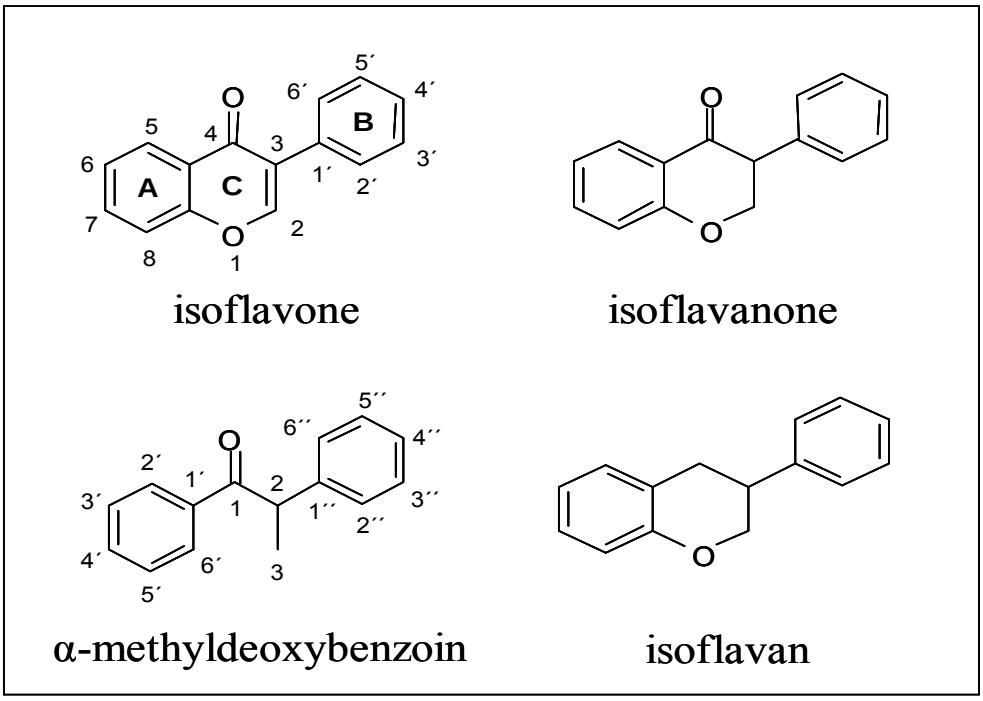



Fig. 4. Ring structures of isoflavones and related compounds.

Human urinary excretion of isoflavone metabolites is variable. For instance, only about 30-40% of subjects excrete significant quantities of equol after isoflavone consumption (Kelly et al., 1995; Lampe et al., 1998; Rowland et al., 2000). This ability to convert daidzein into equol has led to the term "converters" to describe persons who have the necessary (bacterial intestinal) enzymes to effect this biotransformation. The gut microflora plays a decisive role in determining the levels of circulating isoflavones and their metabolites (Xu et al., 1995). Antibiotic treatment of human subjects decreases the excretion of metabolites of isoflavones, again showing the importance of the bacterial flora (Adlercreutz et al., 1986). Equol, first described by Setchell et al. (1984), may be a biologically important isoflavone metabolite since it is of higher estrogenic activity than the parent compound (Sathyamoorthy and Wang, 1997; Willard and Frawley, 1998), and it is found to be the predominant phytoestrogen in breast tissue (Maubach et al. 2003).

*2.3.2. Conjugation and enterohepatic circulation of isoflavones*

As explained above, after ingestion, the isoflavone glycosides are hydrolysed in the gut under the action of beta-glycosidases to release unconjugated daidzein and genistein.
Absorbed isoflavones are transported to the liver via the hepatic portal vein (Fig. 5), where they are rapidly conjugated with glucuronic acid, or to a much lesser extent with sulphuric acid, in the liver by phase-II enzymes (Setchell et al., 1981, 1984 and reviewed by Setchell and Cassidy, 1999; Fig. 5). Thus, in both blood and urine, isoflavones are primarily found as conjugates. In plasma, the free and sulphate fractions are interconvertible and biologically active, while the glucuronide fraction is considered biologically inactive (Adlercreutz et al. 1993a).
Like endogenous estrogens, these conjugates are excreted through both urine and bile. After excretion into bile, conjugated isoflavones undergo "enterohepatic circulation" (Fig. 5) and can then be deconjugated by gut bacteria. Deconjugation may promote reabsorption, further metabolism, and once again degradation in the intestine (Setchell and Adlercreutz, 1988; Xu et al., 1995).
It has also been observed that portal venous blood of rats contained almost exclusively glucuronide conjugates (Axelson and Setchell, 1981), indicating that conjugation may also take place in enterocytes, prior to release into the portal blood and transport to the liver.

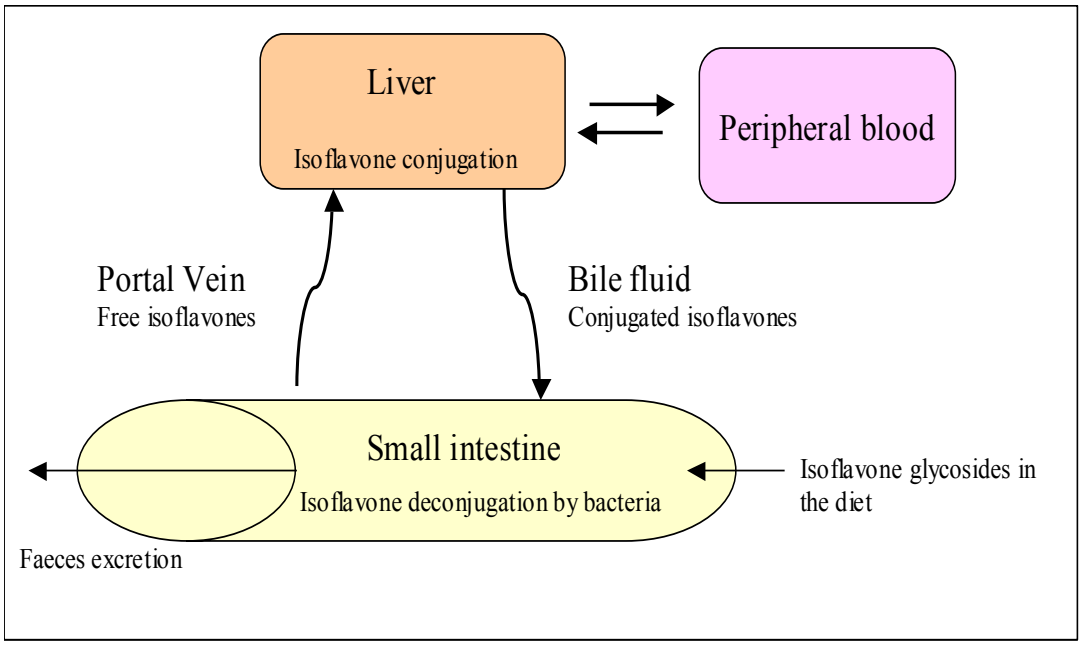



Fig. 5 . Schematic representation of the enterohepatic circulation of isoflavones.

*2.3.3. Oxidative metabolism of isoflavones*

Isoflavones, in addition to reductive and conjugation ways of metabolism, are prone to oxidative (hepatic) biotransformation (Kulling et al., 2000; Kulling et al., 2001). Daidzein and genistein are metabolised by cytochrome P-450 enzymes to hydroxylated metabolites, with additional hydroxy group(s) vicinal to the original hydroxy group.
After incubating daidzein with hepatic rat microsomes, major products identified were 3´-OH-daidzein, 6-OH-daidzein, 8-OH-daidzein, 5,6-diOH-daidzein, 3´,6-diOH-daidzein, 3´,8 diOH-daidzein and 6,8-diOH-daidzein. Incubation of genistein with rat microsomes gave rise to the monohydroxylated metabolites 6-OH-genistein, 3´-OH-genistein and 8-OH-genistein, and to the dihydroxylated metabolites 3´,6-diOH-genistein and 3´,8-diOH-genistein.
The oxidative metabolism of the soy isoflavones was also studied in human volunteers (Kulling et al., 2001). After intake of soy products, several hydroxylated metabolites of daidzein and genistein were identified in urinary extracts after conjugate hydrolysis: 3´-OH-daidzein, 6-OH-daidzein, 8-OH-daidzein, 3´-OH-genistein and 8-OH-genistein were observed in HPLC profiles, and 6-OH-genistein, 3´,6-diOH-daidzein, 3´,8 diOH-daidzein, 3´,6-diOH-genistein and 3´,8-diOH-genistein were additionally identified by GC-MS.
Also, some metabolites of daidzein of an isoflavanone, α-methyldeoxybenzoin, or an isoflavan structure, with additional hydroxyl groups in the phenolic rings, were identified from fecal fermentation extracts of the corresponding isoflavone or from human urine collected after soy supplementation (Heinonen et al., 2003; Heinonen et al., 2004). The analogous compounds derived from genistein could not be determined. Genistein, which has a hydroxyl substituent at the 5-position, appears more susceptible to C-ring cleavage by intestinal microflora (Xu et al., 1995), being converted to 4-hydroxyphenyl-2-propionic acid (HPPA) and to 1,3,5-trihydroxybenzene (THB) (Coldham et al., 2002).
Thus, it appears likely that in human subjects the isoflavone aglycones are in fact first converted to reduced metabolites by gut microflora prior to the oxidative metabolism in the liver, as suggested Kulling et al. (2002). However, also a small fraction of oxidized isoflavone metabolites may enter the enterohepatic circulation, may be excreted in bile, and may be reduced by the gut microflora (Heinonen et al., 2004).
Formononetin and biochanin A represent the 4´-methyl ethers of daidzein and genistein, respectively (Fig. 2). For these phenolic methyl ethers, the oxidative demethylation at C-4´ is

an additional metabolic pathway, and the oxidative demethylation of formononetin and biochanin A to daidzein and genistein appears to be favoured over a ring hydroxylation of formononetin and biochanin A. This was confirmed after the intake of dietary supplements containing formononetin and biochanin A, which resulted in high plasma concentrations of daidzein and genistein (but not of ring-hydroxylated metabolites of formononetin and biochanin A, Setchell et al., 2001).

## 2.4. Toxicokinetics of isoflavones

### *2.4.1. Toxicokinetics of isoflavones in rodents*

The toxicokinetic behaviour of isoflavones in the organism of rodents has been characterized by Janning et al. (2000) and by Supko and Malspeis (1995). The former reported on the toxicokinetics of daidzein in female DA/Han rats, and the latter studied the plasma kinetics of genistein in male Harlan CD2F1 mice.

The time-course of daidzein plasma levels after a single intravenous dose, can be fitted by a three-exponential decline function (Derendorf and Garett, 1987): immediately after i.v. injection of 10 mg/kg b.w. daidzein in rats, daidzein plasma concentrations were in the order of 30 µg/ml, which decreased rapidly within the first hour. After 6 h, the daidzein concentrations were below 50 ng/ml. The elimination half-life ($t_{1/2}$) of the ultimate γ-period, as derived from plasma concentration curves, was approximately 4.2 h. When the same dose was administered orally (by gavage), the compound was detected in blood after 10 min, indicating a rapid absorption of daidzein (or at least of a relevant part of the dose). The major feature of the plasma level-time curve has been the occurrence of fluctuations, along with marked individual deviations. This behaviour is very characteristic of extensive enterohepatic circulation (Fig. 5; Löffler and Bolt, 1980; Back et al., 1980).

Compared to the lower dose of 10 mg/kg b.w., the plasma levels of daidzein after a higher dose of 100 mg/kg b.w. remain within a similar order of magnitude, pointing to a lower bioavailability at higher doses. Based on the calculated AUC (area under the curve) and the respective doses, the oral bioavailability of daidzein was calculated to be 9.7% and 2.2%, for the doses of 10 and 100 mg/kg b.w., respectively. Due to the enterohepatic recirculation, the toxicokinetics are complex.

The oral bioavailability (F) of a compound (drug or, in this case, daidzein) is calculated from the dose and area under the plasma concentration vs. time curve (AUC) values, upon comparative intravenous and oral administration of the same dose, according to:

$$F = \frac{AUC_{oral} \times Dose_{iv}}{Dose_{oral} \times AUC_{iv}}$$

Along with plasma levels of free daidzein, also the daidzein conjugates decline rapidly after i.v. administration of daidzein. Conjugates (glucuronides and sulfates) are also the main circulating metabolites when daidzein is given orally (e.g., by gavage). Daidzein is efficiently excreted in the bile as glucuronide conjugate.

There seems to be an accumulation of daidzein in certain tissues, since concentrations of total daidzein (free plus hydrolysed conjugates) in liver and kidney tissues were three- to five fold higher than in plasma.

The administration of genistein to mice by i.v. injection at doses of 9, 26 and 54 mg/kg b.w. has led to initial plasma levels in the range of 15 to 60 µg/ml (Supko and Malspeis, 1995). After declining more than 250-fold over a period of 50 min, the plasma levels of genistein again gradually increased until a plateau near 90 min; the appearance of this secondary peak was due to enterohepatic cycling. The apparent terminal half-life was 4.7 h, and the mean apparent total plasma clearance of genistein was about 66.5 ml/min/kg b.w., a value similar to that of the hepatic blood flow. The systemic bioavailability of orally administered genistein (180 mg/kg b.w.), which resulted in a genistein plasma concentration of 1.1 µg/ml, was only 12%.

Another study (Degen et al., 2002b) reported a rapid transplacental transfer of daidzein in pregnant DA/Han rats to the fetus. After 10 min of a single i.v. administration of 10 mg/kg b.w., the placenta contained one-tenth the hepatic daidzein concentration (31 µg/g in maternal liver). This shows a rapid transfer from mother to fetus.

Overall, the toxicokinetics of both isoflavones, daidzein and genistein, present clear similarities. Such data are relevant to extrapolate biological effects and toxicities from experimental rodents to the human situation and are therefore indispensible for a toxicological risk assessment.

*2.4.2. Toxicokinetics of isoflavones in humans*

The pharmacokinetic behaviour of naturally occurring isoflavones has been determined in human volunteers after oral intake of daidzein, genistein and their beta-glycosides as a single bolus dose (Setchell et al., 2001). Several parameters were determined to characterize the toxicokinetics of the isoflavones in humans, including area-under-the-curve (AUC), half-life ($t_{1/2}$), peak plasma isoflavone levels ($C_{max}$), time required to achieve the peak levels ($t_{max}$), systemic clearance normalized to the bioavailable fraction (Cl/F) and volume of distribution, normalized to the bioavailable fraction (Vd/F). AUC, $t_{max}$, $t_{1/2}$ of elimination and Vd/F characterize the systemic exposure, rate of absorption, rate of elimination and the extent of isoflavone distribution in the body, respectively.
The results from this toxicokinetic study using pure isoflavones in premenopausal women confirm that humans absorb isoflavones rapidly and efficiently. The fates of daidzein, genistein and their respective beta-glycosides are similar. Table 3 summarizes the relevant toxicokinetic parameters (Setchell et al., 2001):

| | Daidzein | Genistein | Daidzin | Genistin |
|---|---|---|---|---|
| Dose | 50 mg | 50 mg | 50 mg | 50 mg |
| $t_{max}$ | 6.6 h | 5.2 h | 9 h | 9.3 h |
| $C_{max}$ | 194 ng/ml | 341 ng/ml | 394 ng/ml | 341 ng/ml |
| $t_{1/2}$ | 9.3 h | 6.8 h | 4.6 h | 7 h |
| AUC | 2.9 µg/(ml x h) | 4.5 µg/(ml x h) | 4.5 µg/(ml x h) | 4.9 µg/(ml x h) |
| Cl/F | 17.5 L/h | 18.3 L/h | 11.6 L/h | 10.8 L/h |
| Vd/F | 236 L | 161 L | 77 L | 112 L |

Table 3. Toxicokinetic parameters reported after the oral ingestion of 50 mg of isoflavones, as a single-bolus dose, in healthy premenopausal women (Setchell et al., 2001).

The shapes of the plasma appearance and disappearance curve showed an early peak before $C_{max}$ ; this is typical of substances that undergo enterohepatic circulation. When premenopausal women receive a single bolus dose of 50 mg of glycosides daidzin and genistin, the curves displayed characteristics similar to those of the corresponding aglycones. However, the time to attain maximum plasma daidzein and genistein concentrations was

longer at 9 and 9.3 h, respectively, after the glycosides were ingested. This indicates that the rate-limiting step for absorption is the initial hydrolysis of the sugar moiety.
The systemic bioavailability of genistein is much greater than that for daidzein as indicated by the high AUCs values. Moreover, the greater bioavailability of the isoflavones determined from the AUC when the glycosides are ingested, is explained by the glycoside moiety acting as a protective group to prevent biodegradation of the isoflavone structure.
The average volume of distribution normalized to apparent bioaiavailability fraction (Vd/F) is large for both daidzein and genistein, indicating extensive tissue distribution. Daidzein presents a much large Vd/F than genistein, and this explains why genistein levels in plasma always exceed daidzein concentrations when equal amounts of the two isoflavones are given (Setchell et al., 2001).
Compared to the levels of conjugates, only a relatively small proportion of the aglycone isoflavones appears in plasma, even when high doses of isoflavones were ingested.

## 2.5. Main biological effects

### *2.5.1. Hormonal effects*

Phytoestrogens have been shown to bind the estrogen receptor (ER), although this binding is only weak in comparison to that of the endogenous hormone, estradiol. As an example, genistein competes with [$^3$H]-estradiol for binding to the ER (Wang et al. 1996), and the concentration required to produce a 50% inhibition of estradiol binding ($IC_{50}$) was $5x10^{-6}$ M. Daidzein, equol and O-DMA (see Fig. 3) also bind to the ER: equol ($IC_{50}=1.0x10^{-6}$) has the highest affinity for the ERα , two-fold higher than daidzein ($IC_{50}=2.0x10^{-6}$) and six-fold higher than O-DMA ($IC_{50}=6.0x10^{-6}$) (Schmitt et al. 2001).
Phytoestrogens exert estrogenic effects both in cell culture and in vivo. For instance, phytoestrogens stimulate the growth of estrogen-dependent MCF-7cells and elicit an uterotrophic response in rodents (Diel et al. 2000). Interestingly, genistein and daidzein showed a biphasic behaviour on cellular proliferation. Concentrations between 0.01-10 μM stimulated the growth of MCF-7 cells (Wang et al. 1996, Hsu et al. 1999). However, at concentrations >10 μM, these phytoestrogens inhibited cell proliferation. Genistein has been shown to stimulate expression of estrogen-responsive pS2 mRNA at concentrations as low as 0.01 μM. Since the range for stimulation of cell proliferation was the same that stimulates

expression of pS2, it indicates that stimulation of growth was mediated through the ER. Genistein did not stimulate growth in ER-negative MDA-MB-231cells; in these ER-negative cells the inhibitory effects of genistein (>10μM) on proliferation remained. These data show that the inhibitory effect on the growth of MCF-7 cells observed with high concentrations of genistein is independent of binding to the ER.
Phytoestrogens may also act as anti-estrogens. This may occur through various mechanisms: at concentrations 100-1000 times those of endogenous estradiol (probable levels in human plasma after high phytoestrogen consumption), phytoestrogens may be able to compete effectively with endogenous estrogens, bind to the ER, and prevent estrogen-stimulated growth in mammals (Adlercreutz et al. 1995).

### *2.5.2. Non-hormonal effects*

Isoflavones may exert some effects through an antioxidant mechanism. Genistein has been shown to decrease the hydrogen peroxide production in tumour cells ($IC_{50}$ = 25 μM). Daidzein displayed weaker effects ($IC_{50}$ = 150 μM). Furthermore, genistein is a potent inhibitor of superoxide anion generation by xanthine oxidase ($IC_{50}$ = 1-2.5 μM ), but daidzein has a moderate inhibitory effect ($IC_{50}$ = 5 μM).
Genistein has also been shown to increase activities of antioxidant enzymes. Feeding 250 ppm (in the diet) of genistein to 6- to 7-week old female CD-1 mice for 30 days enhanced the activities of catalase, superoxide dismutase, glutathione peroxidase, and glutathione reductase by 10-30% in skin and small intestine (Wei et al. 1995).

### *2.5.3. Genotoxicity*

The question of genotoxicity of estrogenic compounds of plant origin has been raised for lignans, coumestrol, and for the isoflavones, genistein and daidzein (Kulling and Metzler 1997, Kulling et al. 1999, Tsutsui et al. 2003). A lack of knowledge in this field is apparent. The discussion is focused on chromosomal genotoxicity, and preliminary data by Kulling and Metzler (1997) have pointed to genistein, the hydroxy derivative of daidzein, being more genotoxic than the parent compound daidzein. In this context, several potential mechanisms were discussed, such as interference with cytoskeletal macromolecules, with topoisomerase

II, and effects on intracellular generation of reactive oxygen species (Kulling and Metzler 1997). Also, possible mechanisms of mutagenicity by direct interaction of the compounds with DNA have been mentioned (Tsutsui et al. 2003).
As the genotoxicity of genistein and daidzein and associated mechanisms of action are of key relevance for the regulatory handling of these compounds as components of human food, it was of importance to establish dose-effect-relationships, related to genotoxicity endpoints, in a suitable test system. As the preliminary data of Kulling and Metzler (1997) have demonstrated that the chromosomal endpoint of induction of micronuclei in the V79 cell line could be relevant for the consideration of genotoxicity of genistein and daidzein, the second part of the thesis presented here establishes the dose-genotoxicity response relations in this experimental test system; these data also incorporated genistein, daidzein and its metabolite equol.

### 2.6. Aim of the present study

The aim of the present thesis work was to characterize the genotoxicity risk of the isoflavones daidzein, genistein and the daidzein metabolite, equol. There are two aspects of risk assessment: the profile of biological effects of the agent (hazard) and the quantity exposure to that compound of humans.
Thus, an **assessment of the human exposure** was performed, based on literature data and on an own additional investigation of the toxicokinetics. For the latter, rodents were used as an in vivo model.
On the other hand, a **hazard identification** was done: a qualitative/quantitative description of the possible effects of the substances. Again, the information on the known hormonal effects of the compounds was taken from the literature, whereas experimental data on the dose-response of genotoxicity and a characterization of the mode of action were generated as part of this thesis.
Finally, both aspects, possible human exposure and compound-related effects were put into a perspective to arrive at a characterization of the possible risk of endocrine activities and of genotoxicity for humans, due to phytoestrogen contents in the human diet.

# 3. EXPERIMENTAL PART

## 3.1. Materials and Methods

### *3.1.1. Chemicals*

| | |
|---|---|
| Acetic acid | Merck (Darmstadt, Germany) |
| Acetone | Merck (Darmstadt, Germany) |
| Acetonitrile | Merck (Darmstadt, Germany) |
| Acridine orange | Sigma-Aldrich (Taufkirchen, Germany) |
| Agarose (normal melting) | Gibco (Eggenstein, Germany) |
| Agarose (low melting) | Gibco (Eggenstein, Germany) |
| Ammonium acetate | Fluka (Sima-Aldrich, Taufkirchen, Germany) |
| Ascorbic acid | Merck (Darmstadt, Germany) |
| BCA-reagent A “Uptima” | Interchim (Mannheim, Germany) |
| BCA-reagent B “Uptima” | Interchim (Mannheim, Germany) |
| BCA-standard 2 mg/ml | Interchim (Mannheim, Germany) |
| Biochanin A | Sigma-Aldrich (Taufkirchen, Germany) |
| Daidzein | Biomol Feinchemikalian GmbH |
| DAPI | Sigma-Aldrich (Taufkirchen, Germany) |
| Dichloromethane | Merck (Darmstadt, Germany) |
| Dimethylsulphoxide | Merck (Darmstadt, Germany) |
| Dulbecco´s modified Eagle´s medium | Gibco (Eggenstein, Germany) |
| $Na_2$-EDTA | Sigma Aldrich (Taufkirchen, Germany) |
| Equol | Sigma Aldrich (Taufkirchen, Germany) |
| Ethanol | Merck (Darmstadt, Germany) |
| Ethidium bromide | Sigma Aldrich (Taufkirchen, Germany) |
| Fetal calf serum (FCS) | Biochrom (Darmstadt, Germany) |
| Formononetin | Fluka (Sima-Aldrich, Taufkirchen, Germany) |
| β-Glucuronidase enzyme | Roche Diagnostics GmbH (Mannheim, Germany) |
| Genistein | Sigma Aldrich (Taufkirchen, Germany) |
| HCl | Merck (Darmstadt, Germany) |

| | |
|---|---|
| $H_2SO_4$ | Merck (Darmstadt, Germany) |
| $KH_2PO_4$ | Merck (Darmstadt, Germany) |
| $K_2Cr_2O_7$ | Merck (Darmstadt, Germany) |
| N-Laurylsarcosinate (sodium salt) | Sigma Aldrich (Taufkirchen, Germany) |
| Methanol (Gradient grade) | Merck (Darmstadt, Germany) |
| Methyl methane sulfonate | Merck (Darmstadt, Germany) |
| Neutral red | Sigma-Aldrich (Taufkirchen, Germany) |
| NaCl | Merck (Darmstadt, Germany) |
| $Na_2HPO_4$ | Merck (Darmstadt, Germany) |
| NaOH | Merck (Darmstadt, Germany) |
| $NaOAc.3H_2O$ | Sigma-Aldrich (Taufkirchen, Germany) |
| Phosphate buffered saline (PBS), 10X | Gibco (Eggenstein, Germany) |
| Pig plasma | Fiebig-Nährstofftechnik (Idstein, Germany) |
| Potassium chloride | Fluka (Sima-Aldrich, Taufkirchen, Germany) |
| Propidium iodide | Sigma-Aldrich (Taufkirchen, Germany) |
| Triethylamine buffer substance | Merck (Darmstadt, Germany) |
| Tris (hydroxylmethyl)aminomethane | Merck (Darmstadt, Germany) |
| Triton X | Fluka (Sima-Aldrich, Taufkirchen, Germany) |
| Trypanbue, steril | Serva (Heidelberg, Germany) |
| Trypsin-EDTA | Gibco (Eggenstein, Germany) |
| Tween20 | Merck (Darmstadt, Germany) |
| Vincristine | Sigma-Aldrich (Taufkirchen, Germany) |

The primary antibody for the CREST analysis (CREST Serum) was obtained from DPC Biermann GmbH, Bad Nauheim, Germany.

The second FITC conjugated anti-human IgG F $(ab)_2$ was from Sigma-Aldrich (Taufkirchen, Germany).

### *3.1.2. Instruments*

| | |
|---|---|
| Analysis balance AE 240 | Mettler (Bergisch Gladbach, Germany) |
| Autoclave "Varioklav Type 500" | H+P Labortechnik (Oberschleißheim, Germany) |
| Cell culture flask, 25, 75, 185 cm² | Greiner GmbH (Frickenhausen, Germany) |
| Centrifuge tubes, steril (12 ml, 50 ml) | Greiner GmbH (Frickenhausen, Germany) |
| $CO_2$-Incubator Type BB16 Function Line | Heraeus (Hanau, Germany) |
| Coverslips | Menzel-Gläser (Braunschweig, Germany) |
| Digital Camera DSC-S85 | Sony (Köln, Germany) |
| Disposable syringes (10ml/25ml) | Millipore (Eschborn, Germany) |
| Disposable steril 0.22 µm filters Unit Millex-GP | Millipore (Eschborn, Germany) |
| Electrophoresis unit | Bio-Rad Laboratories GmbH (München, Germany) |
| Filters: I3, blue/N2.1, green/A, UV | Leitz (Wetzlar, Germany) |
| Fluorescent microscope DM LB | Leitz (Wetzlar, Germany) |
| Fluorescent microscope DM RB | Leitz (Wetzlar, Germany) |
| Freezer, -20°C, "Öko Plus" | Siemens (München, Germany) |
| Freezer, -70°C, "Ult Freezer 994" | Heraeus (Hanau, Germany) |
| Heating Surface | Karl Hecht Assistant KG (Sondheim/Röhn, Germany) |
| Laminar Flow, Air ELB 2448 | Heraeus (Hanau, Germany) |
| Light microscope 403028 | Zeiss (Jena, Germany) |
| Magnet bubbler | Heidolph (Kelheim, Germany) |
| Megafuge 1,0 R | Heraeus (Hanau, Germany) |
| Microtiter Plates, 96 well | Costar (VWR International, Bruchsal, Germany) |
| Neubauer Chamber | Karl Hecht Assistant KG (Sondheim/Röhn, Germany) |
| Microscope slides: Chamber Slide System, 16 well | Merck (Darmstadt, Germany) |
| Microscope slides (76 x 26 mm) | Menzel-Gläser (Braunschweig, Germany) |
| pH-Meter MR 2000 | Heidolph (Kelheim, Germany) |
| pH-Meter MP 225 | Metter Toledo (Schwerzenbach, Switzerland) |
| Plate photometer340 ATC | STL Labinstruments (Craiilsheim, Germany) |
| Pipettes and tips (2µl, 10µl, 20µl, 100µl, 200µl, 1000µl) | Eppendorf (Hamburg, Germany) |
| Precision balance PB 602 | Mettler (Bergisch Gladbach, Germany) |
| Quadriperm trays | Viva Science (Hannover, Germany) |
| Shaker "Orbital Shaker S03" | Stuart Scientific, Dunn Labortechnik (Asbach, Germany) |
| Shaker MTS2 | IKA (Staufen, Germany) |
| Software "Tecan" | Tecan Austria GmbH (Grödig, Salzburg, Austria) |
| Sonication bath "Sonorex RK 102H" | Beyer-Enders (Düsseldorf, Germany) |

| | |
|---|---|
| Thermomixer "Comfort" | Eppendorf (Hamburg, Germany) |
| Transfer pipettes, steril | Sarstedt (Nümbrecht, Germany) |
| Tubes (2.0 ml; 1.5 ml) | Eppendorf (Hamburg, Germany) |
| Tubes LiChrolut® RP18 | Merck (Darmstadt, Germany) |
| Vortex "Reamix 2789" | Hartenstein (Würzburg, Germany) |

### *3.1.3. Test solutions and buffers*

The test solutions of the three isoflavones were prepared in dimethylsulphoxide (DMSO) and stored at –20°C in the dark.

#### *3.1.3.1. Analytics of isoflavones*

*Hydrolysis buffer*

Stock solution (10X): 13.6 g $NaOAc.3H_2O$, 1 g ascorbic acid and 0.1 g $Na_2EDTA$ are dissolved in 20 ml Aq. dest. The pH is adjusted with acetic acid to pH = 5, and the volume is filled up until 100 ml.
Applied solution: dilution 1:10 of the stock solution.

*Triethylammonium sulfate buffer (TEAS) 3 M, pH 7*

30.4 g Triethylamine is dissolved in 30 ml A.dest. (under safety cabinet), 5 ml $H_2SO_4$ are added very slowly and gently and constantly swirled for 1 h. When the organic phase becomes thinner (~ 5 mm) the pH can be adjusted with $H_2SO_4$. Afterwards, the volume is completed to 100 ml.

*Ammonium acetate buffer 1 M, pH 7*

7.708 g ammonium acetate are dissolved in 100 ml A.dest.

*Ammonium acetate buffer 10 m M, pH 5*

Stock solution: 7.708 g ammonium acetate are dissolved in ~ 70 ml A.dest. The pH can be adjusted with acetic acid until pH = 5. The volume is completed to 100 ml.
Applied solution: dilution 1:100 of the stock solution.

*Compounds used as internal standards*

Biochanin A, stock solution: 5 mg biochanin A is dissolved in 10 ml ethanol (0.5 mg/ml)

Formononetin, stock solution: 2.5 mg formononetin is dissolved in 50 ml methanol (50 µg/ml)

*3.1.3.2. Cell culture*

*Trypan Blue solution*

Trypan Blue 1:10 in PBS

*Acridine Orange solution*

Stock solution: 1mg/ml A. dest.

Applied solution:

- 7 ml 0.3 M $Na_2HPO_4$ * 2 $H_2O$
- 7 ml 0.3 M $KH_2PO_4$
- 5 ml stock solution
- 80 ml A. dest

*Methanol-acetic acid fixation solution*

Methanol-acetic acid (3:1) is prepared 1 day before use and is stored at –20°C.

*KCl*

A 0.4% KCl in A. dest. solution is prepared a day before use, sterilized and stored at 4°C.

*Micronucleus positive control substances*

MMS stock solution (10mg/ml): 0.1 g MMS is dissolved in 1 ml medium and then is diluted 1:10 in medium.

VCR stock solution (1 µM): 0.9 mg VCR is dissolved in 1 ml medium and then is diluted 1: $10^3$ in medium.

*Neutral red solution*

Stock solution: 40 mg Neutral Red in 10 ml A. dest. is shaken for 1 h at 37°C and then stored at 4°C.

Applied solution (NR medium): 100 ml medium + 1250 µl stock solution is prepared and stored for 18 h at 37°C in an incubator. Before usage, the solution is filtered using a 0.22 µm filter Unit Millex-GP.

*Neutral red fixation solution*

50 % Ethanol, 1 % acetic acid

*BCA reagent*

50 parts BCA reagent A "Uptima" and 1 part BCA reagent B "Uptima"

*Soerensen buffer (pH 6.8)*

5 ml 0.3 M $Na_2HPO_4$ * 2 $H_2O$ + 5 ml 0.3 M $KH_2PO_4$. The volume is completed until 100 ml with A. dest.

*CREST dye solution*

- 1 µg/ml DAPI
- 0.1 mg/ml PI
- DAPI/PI (3:1)

*Lysing solution*

Stock solution: 131.5 g NaCl, 33.5 g $Na_2EDTA$, 1.08 g Tris, and 7.2-10.8 g NaOH are dissolved in 900 ml A.dest. and the pH is adjusted in 10.

1 g Na-laurylsarcosinate is dissolved in 100ml A.dest. and is added to the previous solution.

This stock solution can be stored at 4°C for a month.

Applied solution: 178 ml stock solution is warmed up to room temperatute and is mixed with 20 ml DMSO and 2 ml Triton X.

*Electrophoresis buffer*

150 ml of a 10 N NaOH solution (400 g/L) and 25 ml of a 200 mM $Na_2EDTA$ solution (14.9 g/200 ml) are diluted in 5 L A. dest.

This stock solution can be stored at 4°C for a month.

*Neutralization buffer*

48.4 g/L Tris is dissolved in A. dest. (pH = 7.5).

*Ethidium bromide solution*

Stock solution (10X): 10 mg ethidium bromide in 50 ml A. dest. (200 µg/ml). It is stored in the dark at room temperature.

Applied solution: 1:10 dilution (20 µg/ml).

*Agarose preparation*

0.5 % low melting temperature (LMP) agarose is prepared dissolving 100 mg in 20 ml PBS. It is boiled twice and stored in sterile Eppendorf tubes. For the COMET assay the agarose is warmed up to 95°C and kept at 37°C.

### *3.1.4. Determination of genistein and daidzein concentrations in rat chow and rat plasma by HPLC*

#### *3.1.4.1. Chromatographic principles*

##### *3.1.4.1.1. Principles and characteristic values of chromatographic separations*

The principle of chromatography is based on the passage of the constituents to be separated between two immiscible phases. For this, the sample is dissolved in the mobile phase and moves across a stationary phase.

In the case of column chromatography, all the constituents travel the same route through the separation bed, and due to the specific interactions with the stationary phase they appear at the end of the column at different times, where they are detected.

The sample is dissolved in the mobile phase and is introduced at the head of the column. Then, using the mobile phase, elution is undertaken until the substances are separated and detected at the end of the column. Once the sample has been injected, the constituents distribute between the mobile and stationary phases. If the mobile phase is then continuosly supplied, the substances distribute along the column between the new mobile and the stationary phase. Compounds retained more strongly on the stationary phase take longer to be separated than substances, which are less strongly bound.

The *migration rate* by which the analyte molecules travel through the column can be defined as: $v = L/t_R$ (1)

where L is the length of the column and $t_R$ is the *total retention time* of the analyte. This value can be obtained directly from the chromatogram (Fig. 6), and it corresponds to the time required for the molecules of the analyte to pass through the column. The hold-up time ($t_M$) is the retention time of a compound that is not retained at all (mobile phase).

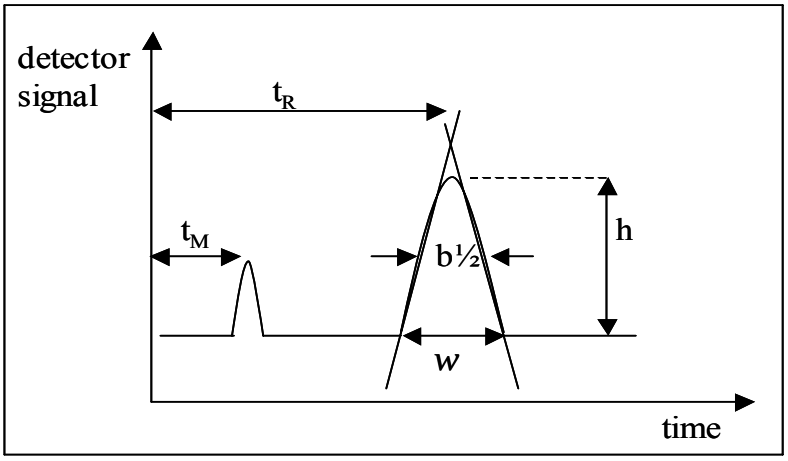



Fig. 6. Tipical chromatogram.

The *capacity factor* k´ is defined as:

$k' = (t_R - t_M)/ t_M$ (2)

where $t_R - t_M$ is the adjusted retention time of the analyte ($t'_R$).

The capacity factor can be established directly from the chromatogram based on the total retention times of the constituents of interest and the hold-up time (Fig.6). Capacity factors should be conveniently within the range 1 to 5. If the k´ is lower than 1, then the compounds are being eluted too quickly, since their retention time hardly differs from the retention time of the mobile phase. If the capacity factor is much higher than 5, then in practice there are very long retention times.

The *selectivity factor* ($\alpha$) is a measure of the separation of two substances. Using the capacity factors for calculating the selectivity factor of two substances, A and B, one obtains:

$\alpha = k'_B/k'_A$ (3)

Determination of $\alpha$ from an experimental chromatogram is feasible by substitution by (2) in equation (3) giving the ratio of adjusted retention times:

$\alpha = (t'_R)_B / (t'_R)_A$ (4)

The width of a peak (peak broadening) is in direct relation to the separation efficiency or column efficiency. In order to explain this, the concept of *plate height* (H) (i.e., the height equivalent to a theoretical plate) and the *number of theoretical plates* (N) are introduced. On every theoretical plate, equilibration for the substance takes place between the stationary and the mobile phases. Thus, N results in:

$N = L/H$ (5)

H can also be understood as the relation between the variance of the peak $\sigma^2_L$ and the migration distance (L):

$H = \sigma^2_L/L$ or $H = \sigma^2_t L / t^2_R$ (6)

The smaller the plate height, the higher the column efficiency and thus the resolution obtained.

To determine N from the chromatogram, the base-line width, *w*, can be used. The *w* can be determined from the intersection of the tangents of the inflection points with the base line (Fig.6). The base width can be written as:

$w = 4\,\sigma_t$ (7)

Thus, $H = w^2\, L/16\, t^2_R$ (8)

and $N = 16\,(t^2_R/w)^2$ (9)

Since equilibrium is hardly ever attained due to the movement of the mobile phase, the peak broadening can be derived from a kinetic theory, which is caused by the finite rate at which mass-transfer processes occur during migration of the analyte in the column. The extent of this effect depends on the length of possible passages between the mobile and the stationary phase and is thus directly in proportion to the flow rate of the mobile rate (μ).

The *van Deemter equation* establishes a dependence of H with μ:

$H = A + B/\mu + C_S\,\mu + C_M\,\mu$ (10)

In Table 4 the individual terms of the van Deemter equation are explained in more detail: the plate height is described by the constants A as a measurement for the Eddy diffusion, which describes the distance a flowing stream that moves before its velocity is changed by the packing; the B term describes the influence of the longitudinal diffusion, which is created by the diffusion of the particles away from the peak centre towards or against the direction of the flow of the mobile phase. The mass transfer term in the mobile phase, $C_M\,\mu$, represents the convective component of flow dispersion. This mass transfer term is inversely proportional to

the diffusion coefficient in the mobile phase and is directly dependent on the particle diameter of the packing material, as well as on the column diameter. The $C_S\,\mu$ term represents the mass transfer in the stationary phase. The effect increases with the increasing thickness of the liquid film and with decreasing diffusion coefficient in the stationary phase.

| Influence | Term in *van Deemter* equation |
|---|---|
| Longitudinal diffusion | $B/\mu = 2\,k_D D_M/\mu$ |
| Mass transfer in liquid stationary phase | $C_S\mu = \frac{q\,k'd_f^2\,\mu}{(1+k')^2\,D_S}$ |
| Mass transfer in mobile phase | $C_M\,\mu = \frac{f(d_p^2,\,d_c^2)\,\mu}{D_M}$ |

Table 4. Kinetic influences on peak broadening: μ, linear velocity of mobile phase; $D_M$, diffusion coefficient in mobile phase; $D_S$, diffusion coefficient in stationary phase; $d_p$, diameter of the packing material; $d_f$, thickness of the liquid coating on the stationary phase; $d_c$, column diameter; $k_D$ and q, constants; f ( ) characterizes a functional dependence.

In principle, for efficient separations a low plate height (minimum H) should be reached and this can be obtained by: (a) low film thickness of liquid coating of stationary phases, (b) homogeneous packing of the stationary phase using closely destributed packing materials, (c) small column diameter, (d) large diffusion coefficients in the stationary phase and small diffusion coefficients in the mobile phase.

*3.1.4.1.1.1. Basis of liquid chromatography*

The basis of liquid chromatography is a liquid mobile phase. In classic liquid chromatography, glass columns were employed, which had inner diameter of 1-5 cm, a length of 50-500 cm and the particle diameter of 150-200 mm.
In *high-performance liquid chromatography* (HPLC), particles with diameters of up to 3 or 10 μm are used; this results in shorter separations, without losing resolution (H increases with μ, but decreases with smaller particle size of the support material through the $C_M$).

Separations using a liquid mobile can be based on four different principles: ***adsorption***, in the form of liquid-solid chromatography; ***ion exchange***, to be understood as the transfer of classic ion exchange in the batch process to column chromatography; the ***exclusion*** principle is based on the molecular sieve effect. The principle of ***partition*** is currently one of the most frequently used analysis methods and is the methodology used in this work, and thus will be explained in more detail: liquid immobilized on a carrier, which can be silica gel or alumina, can act as stationary phase. The liquid is adhered to the carrier by physical adsorption. Chromatography in which a polar stationary phase and a less polar or non-polar mobile phase is used, is termed *normal-phase chromatography*. In contrast, when the stationary phase is relatively non-polar and a polar mobile phase is used, is termed *reversed-phase chromatography*.

*Reversed phases*

Silica gel is used as a uniform, porous, mechanically sturdy material in particles sizes of 3, 5 or 10 µm. The surface of fully hydrolysed silica gel consists of silanol-(hydroxyl-) groups, which can react with siloxanes (Si-O-Si groups). The alkyl group most frequently used is $C_{18}$ (n-octadecyl); these long chains are aligned in parallel and perpendicular to the particle surface. They form a brush-like surface, and the longer the alkyl chain is, the longer the retention times are.

### *3.1.4.1.1.2. Instrumentation in liquid chromatography*

The components of a HPLC unit, which are shown in Fig. 7, are:

- Reservoir for the solvents in the mobile phase
- Pump system
- Syringe or an injection system to feed the sample
- Separation column, preceded by a precolumn
- Detector

The *solvents* used as mobile phase are stored in a resevoir in glass bottles. Dissolved gases, which can lead to the formation of bubbles and might interference in the detector, are

removed by sucking through a degasser, installed before the pumps. Also, a millipore filter is used to remove suspended matter in the inlet pipe.

A distinction is made between isocratic and gradient elution. In the former method, one works with a single solvent of constant composition; in gradient elution, better separation is achieved, because the composition of the eluents is constantly altered according to a particular programme.

The *pumping system* for HPLC generates high pressures, this is obtained using sapphire valves; the system must have only slight residual pulsation, chemical resistence and good control of the flow. Reciprocating pumps are preponderant today and were employed for this study. They operate as double piston pumps, which work with a phase shift of 180° to suppress pulsation.

Gradients can be produced on the low or high pressure side. If the two solvents are mixed on the suction side of the pump, one refers to a low pressure gradient, the overall flow rate is controlled by a single pump. To produce a high pressure gradient two pumps are required and the solvent constituents are mixed on the pressure side of the pumps; in this work this form was employed.

The *sample injection* system must allow volumes of 50-500 µl to be introduced and pressure must be kept within the system. A sample loop is suitable for sample introduction. Using a microlitre syringe, the sample solution is fed in through a needle inlet into the loop of a six-way valve.

A standard separation column initially used was 250 mm long, with an inner diameter of 4.6 mm, and was filled with 5 or 10 µm particles. A number of theoretical plates of approximately 50,000 per metre can be obtained. The columns are integrated into the system in the form of cartridges. The connections to the stainless steel tubes are fittings (conical metal sealing rings).

In order to reduce the volume of solvents, a smaller column with inner diameter of 1mm and 150 mm long was employed in this work. A number of theoretical plates up to 100,000 per metre can be attained using 3 µm particles (microbore HPLC).

Short precolumns are employed to protect the separation column (4.5 mm inner diameter x 30 mm length).

For *detection,* a UV detector is most frequently used and was also employed for the detection of isoflavones in this study at 260 nm, the absorption maximum of the analytes according to Franke et al. (1995). The single wavelength UV detector employed here uses a deuterium

lamp as its light source (polychromatic broad spectrum source), a grating or prism as dispersive unit, and a sample cell made of quartz.

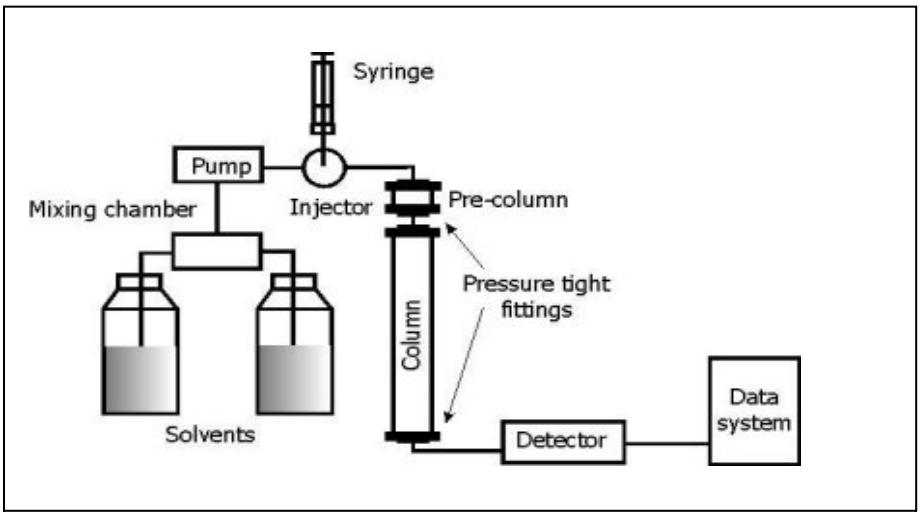



Fig. 7. Structure of a HPLC unit.

*3.1.4.2. Experimental procedures*

*3.1.4.2.1. Study design on exposure evaluation*

An in vivo study was performed in rats to measure the blood levels of isoflavones, in relation of the dose that was given orally. This relation is crucial for the calculation of effective blood concentrations in relation to food intake.

The animal experimentations were performed in cooperation with the Institut für Kreislaufforschung und Sportmedizin, Deutsche Sporthochschule, Köln. Animals were used for a series of experiments studing physiological effects of isoflavones under different conditions of exercise. For the present purpose, the blood levels resulting from different dose regimens of isoflavones were of interest.

The animals were seventeen juvenile female Wistar rats (101-125 g). These were obtained from Janvier (Janvier, Le Genest-St-Isle, France) and were maintained under controlled conditions of temperature (20°C ± 1, relative humidity 50-80 %) and illumination (12 h light, 12 h dark). All the rats had free access to a standard rodent chow (Ssniff R/M H), which

contained an expected total isoflavone concentration of ~ 400 µg/g (according to Degen et al., 2002a) and to drinking water.

Animals were ovariectomized to remove the source of endogenous estrogens, and after 14 days, when the endogenous hormonal level had declined, the diet was changed. At this time point, the animals were divided: 6 rats were part of the first group, so-called ILD group. These rats were fed with an isoflavone-low diet (Ssniff GmbH, Soest, Germany), during the whole further period (12 weeks). The other eleven rats were fed with an isoflavone-rich diet (Harlan Winkelmann, Borchen, Germany) and were part of the so-called IRD group. Five of these animals were subjected to a running activity treatment ("Phyto runners"), whilst the other six rats were considered a control ("Phyto control"). Blood samples were collected after 0, 6 and 12 weeks of changing diet, frozen and analysed as described Janning et al. (2000). The different rodent diets used, were analysed using the method described by Degen et al., 2002a. Figure 8 shows a schematic description of the overall study design of the experiment conducted at the Deutsche Sporthochschule, Köln.

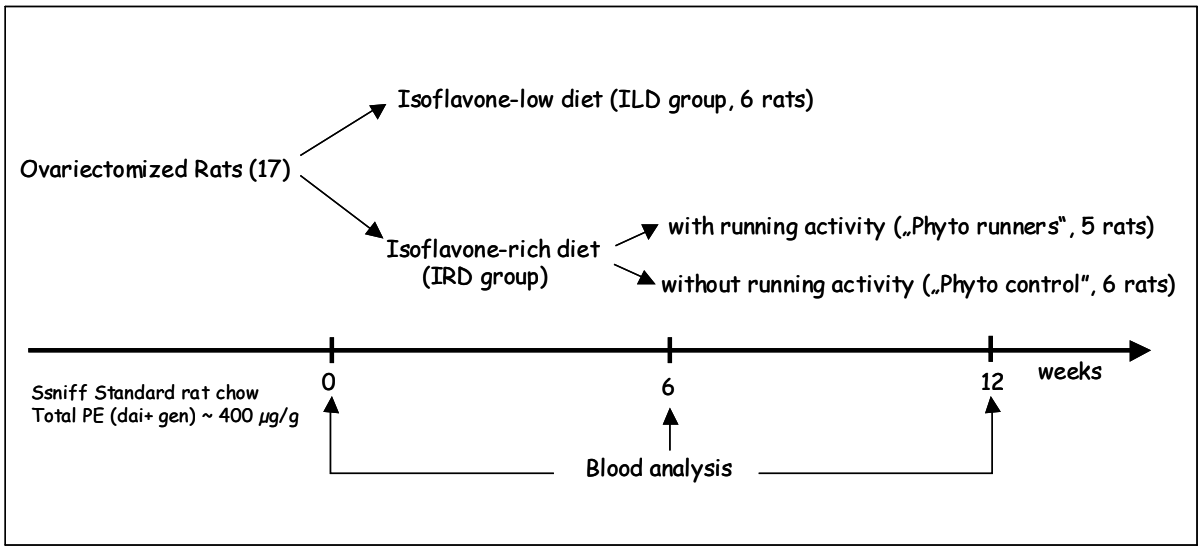



Fig. 8. Schematic description of the animal study design (Deutsche Sporthochschule, Köln).

### *3.1.4.2.2. Analysis of isoflavones in rodent diet*

The rat chows (isoflavone-low and isoflavone-rich diet), which were used in the animal experiment were analyzed for the daidzein and genistein contents by microbore HPLC, after acid hydrolysis of samples which had been ground to a homogenous powder.

The basic procedure followed the method of Franke et al. (1994, 1995). Briefly, 1 g of the powdered dry material was dispersed in a mixture of 10 ml of 10 M HCl and 40 ml of 96% ethanol. Biochanin A was used as internal standard, 50 µl (stock solution of 0.5 mg/ml in ethanol). The mixture was sonicated for 10 minutes, followed by refluxing at 80° C for 3 h and cooling to room temperature. About 4 ml of this mixture was centrifuged at 4300 rpm for at least 10 minutes. Aliquots (100 µl) of the supernatant were diluted with 900 µl of water containing 10% methanol in 1% acetic acid, and 10 µl of this dilution were injected into the microbore HPLC system (described below).
Separation and quantification of the isoflavones followed procedures of Degen (1990) and Franke et al. (1998), with modifications for the microbore-HPLC system: a time gradient was used mixing eluent B (10:5:1 v/v/v of methanol/acetonitril/dichloromethane) and eluent A (90:10 v/v of 10% aqueous acetic acid /eluent B): 0-5 minutes 15% B in A; 5-16 min 15-30% B in A; 16-35 min 30-100 % B in A for separation of the analytes, and a re-equilibration phase at 35-36 min 100-15% B in A; 36-45 min 15% B in A; the gradient was delivered at a flow rate of 40 µl/min (the eluent flow from the pumps of 260 µl/min was reduced by means of a splitter). The effluent was monitored continuously at 260 nm (UV detection). The limit of detection was 10 µg isoflavone/g chow (the same as described by Degen et al., 2002a).

*3.1.4.2.3. Analysis of isoflavones in rat plasma*

The HPLC analysis was based on the method of Franke et al. (1998). Plasma samples from rats were thawed and subjected to conjugate hydrolysis according to Gamache and Acwoth (1998): aliquots of 100 µl plasma were mixed with 100 µl of "hydrolysis buffer" (0.1 M sodium acetate pH 5 with 0.1% (w/v) ascorbic acid and 0.01% (w/v) EDTA), 10 µl of internal standard (formononetin; stock solution of 50 µg/ml in methanol), 8 µl glucuronidase solution and 4 µl sulfatase solution and were allowed to hydrolyse (glucuronide and sulfate metabolites) while shaking (Thermomixer) at 37 °C overnight. Then, 119.7 µl water, 75 µl ammonium acetate buffer (1 M, pH 7), and 83.3 µl triethylammonium sulfate buffer (3 M, pH 7) were added, and the samples were heated at 60 °C (Thermomixer) for 10 minutes to facilitate dissociation of isoflavones from plasma proteins. The deproteinized samples were afterwards extracted by solid-phase at room temperature using LiChrolut® RP18 tubes, which were first washed with 7.5 ml methanol for 30 min, 5 ml water and 5 ml ammonium acetate

buffer (10mM, pH 5). After the plasma samples had passed over the tubes, these were rinsed with 5 ml ammonium acetate buffer (10mM, pH 5) and 5 ml water. The absorbed isoflavones were eluted with 1.5 ml methanol. The eluates were evaporated to dryness under a gentle stream of nitrogen at 45 °C, dissolved in 100 µl of (40:60 v/v) methanol/aqueous acetic acid (1%), and stored at –20 °C until HPLC analysis. Separation and quantitation was carried out on a microbore HPLC system. Samples were chromatographed with a gradient mixed from eluent B (10:5:1 v/v/v of methanol/acetonitril/dichloromethane) and eluent A (90:10 v/v of 10% aqueous acetic acid /eluent B): 0-5 minutes 25% B in A; 5-25 min 25-50% B in A; 25-40 min 50-100 % B in A and 40-41 min 100-25% B in A; 41-50 min 25% B in A; the gradient was delivered at a flow rate of 260 µl/min (from the pumps), and after split at 40 µl/min (to the column). The effluent was monitored continuously at 260 nm (UV detection). The limit of detection was 20 ng isoflavone/ml plasma (Janning et al., 2000).

#### *3.1.4.2.4. Instrumentation*

For the analysis of daidzein and genistein in rat chow and in rat plasma, microbore HPLC analysis was used on a gradient system (pumps: LC10 AD, Shimadzu), equipped with a SCL-10 A VP system controller (Shimadzu, Diusburg, Germany), a LCP flowsplitter (LC Packings, Amsterdam, the Netherlands), and a SPD-10 AV VP spectrophotometrical UV detector (Shimadzu, Duisburg, Germany) with a micro flow cell (LC-Packings, Amsterdam, the Netherlands). Data processing was carried out using Class VP 4.2 chromatography data system (Shimadzu, Duisburg, Germany). Samples were chromatographed on a reverse-phase analytical column (150 x 1 mm, with Luna 3 µm C18(2)), and a precolumn (30 x 1 mm, same reverse-phase material) both from Phenomex (Aschaffenburg, Germany).

#### *3.1.4.2.5. Peak identification*

HPLC peaks were identified by comparison of the retention times of standard mixtures with those of samples. Some samples were spiked with standards solutions of isoflavones to see whether the retention times change by matrix influences. Both matrixes (plasma and rodent

chow) were analyzed to confirm that there were no interfering substances at the retention times of the compounds of interest under the conditions described.

*3.1.4.2.6. Calibration curves*

For calibrating the system for rat chow analysis, the *method of standard additions* was used, where the analyte itself is employed for internal calibration. The sample (after the acid hydrolysis described in section 3.1.4.2.2.) was split into subsamples before the analysis. One subsample (S0) was diluted with 900 µl of water containing 10% methanol in 1% acetic acid, and 10 µl of this dilution were injected into the microbore HPLC system, while the other subsamples were treated with increasing amounts of stock solutions of daidzein or genistein (c = 5 µg/ml MeOH). Then the analytical HPLC determination was carried out on all subsamples (S0-S7), and the resulting data were plotted as peak areas vs. added concentration. The measurement on the unkown sample was evaluated by:
**c = b/a**; where **b** is the independently determined blank signal that applies for the particular measurement (axis interception) and **a** is the slope of the curve.
Table 5 shows an example of a protocol of a calibration curve performed for dietary rat chow:

| Subsample | Hydrolysed sample | Daidzein (C=5µg/ml) | Genistein (C=5µg/ml) | Solvent (10%MeOH, 1%AcH) | Final added conc. |
|---|---|---|---|---|---|
| S0 | 100 µl | 0 µl | 0 µl | 900 µl | 0 ng/ml |
| S1 | 100 µl | 10 µl | 10 µl | 880 µl | 50 ng/ml |
| S2 | 100 µl | 20 µl | 20 µl | 860 µl | 100 ng/ml |
| S3 | 100 µl | 30 µl | 30 µl | 840 µl | 150 ng/ml |
| S4 | 100 µl | 40 µl | 40 µl | 820 µl | 200 ng/ml |
| S5 | 100 µl | 50 µl | 50 µl | 800 µl | 250 ng/ml |
| S6 | 100 µl | 60 µl | 60 µl | 780 µl | 300 ng/ml |
| S7 | 100 µl | 70 µl | 70 µl | 760 µl | 350 ng/ml |

Table 5. Design of a calibration curve for rat food samples.

In the case of the plasma samples, an *external calibration* was done, that means that the samples containing the unkown amounts and the standards containing the known amounts were treated separately as described above for plasma samples (section 3.1.4.2.3.). Table 6 shows an example of a protocol of a calibration curve performed with pig plasma as matrix:

| Final conc. | Plasma | Daizein | Genistein | Formononetin C = 50 µg/ml |
|---|---|---|---|---|
| 0 ng/ml | 100 µl | 0 µl | 0 µl | 10 µl |
| 25 ng/ml | 100 µl | 5 µl of C=0.5 µg/ml | 5 µl of C=0.5 µg/ml | 10 µl |
| 50 ng/ml | 100 µl | 10 µl of C=0.5 µg/ml | 10 µl of C=0.5 µg/ml | 10 µl |
| 75 ng/ml | 100 µl | 15 µl of C=0.5 µg/ml | 15 µl of C=0.5 µg/ml | 10 µl |
| 100 ng/ml | 100 µl | 20 µl of C=0.5 µg/ml | 20 µl of C=0.5 µg/ml | 10 µl |
| 500 ng/ml | 100 µl | 10 µl of C=5 µg/ml | 10 µl of C=5 µg/ml | 10 µl |
| 1000 ng/ml | 100 µl | 20 µl of C=5 µg/ml | 20 µl of C=5 µg/ml | 10 µl |
| 1500 ng/ml | 100 µl | 30 µl of C=5 µg/ml | 30 µl of C=5 µg/ml | 10 µl |

Table 6. Design of a calibration curve for plasma samples.

### *3.1.4.2.7. Reproducibility of isoflavone measurements by HPLC*

*Accuracy* and *precision* are the most important criteria for defining the quality of an analytical method: accuracy is the agreement between the average of the measured values and the accepted reference value. The (arithmetic) mean value is considered the best estimate of the true (unknown) value for the measured quantity and is calculated by averaging the experimental data:

$$\bar{x} = 1/n \, \Sigma \, x_i$$

where n is the number of replicate measurements and $X_i$ is each independent measurement. Precision is the scatter of the measured values around the average value and can be estimated by using the sample standard deviation:

$$SD = \{ \frac{\Sigma(x_i - \bar{x})^2}{(n-1)} \}^{1/2}$$

To assess the reproducibility of the HPLC method employed, several samples prepared with known amounts of daidzein and genistein were independently processed and analysed as a group on three separate occasions. Both mean values and variances were calculated and were expressed as coefficients of variation (%):

$$CV\ \% = (SD/\bar{x}) \times 100$$

*3.1.4.2.8. Analytical recovery of isoflavones*

For recovery experiments using HPLC method, known amounts of the isoflavones were spiked into 100 µl aliquots of pig plasma and were treated as described for plasma samples before. The same was done with 100 µl aliquots of hydrolysed rodent diet to study the recovery rates of the method in this matrix.

*3.1.5. Cell culture procedures*

*3.1.5.1. Cell line*

The studies on the cytotoxicity and genotoxicity of genistein, daidzein and the daidzein metabolite equol were carried out in the estrogen receptor-negative male Chinese hamster lung fibroblast cell line (V79) (Fig. 9). These cells grow with a doubling time of 12 h or less, have a stable diploid karyotype and possess 22 chromosomes (Schempp and Vogel, 1979; Speit et al., 1994). Moreover, V79 cells are very robust, easy to passage, and do not have special medium requirements.

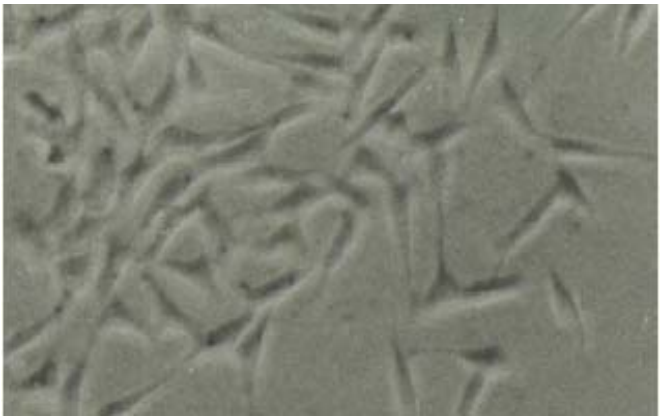


Fig. 9. V79 Cells attached to solid surface.

The V79 cell line was chosen to avoid stimulatory effects of the test compounds on cell proliferation: MCF-7 (ER-positive) cells, but not MDA (ER-negative) cells, treated with estradiol showed an increase in the micronuclei frequency which correlated with the estradiol-

induced cell proliferation (Fischer et al., 2001). Genistein and daidzein are known to stimulate the growth of ER-positive cells in concentration ranges between 0.01 and 10 µM and inhibit growth at higher (>10 µM) concentrations (see section 2.5.1). Thus, with the reported (biphasic) effects on cell growth, an assessment on the genotoxicity of phytoestrogens may be complex.

*3.1.5.1.1. Handling of V79 cells*

Continous cultures are comprised of a single cell type that can be serially propagated in culture for a limited number of cell divisions (approximately 30).
The cultivation of V79 cells was carried out in Dulbecco´s modified Eagle´s medium (DMEM) supplemented with 10% fetal calf serum (FCS). This cell culture medium contains 3.7 g/l $NaHCO_3$, 1 g/l D-glucose as well as L-glutamine. This composition allows an optimal cell proliferation. The fetal calf serum contributes with amino acids, inorganic salts, albumin, and growth factors. The cells were cultured in special plastic flasks in a chamber (incubator) that provides a humidified atmosphere with 5% $CO_2$ in air at 37°C. The flasks allow a continued air supply to the cells.

*3.1.5.1.2. Passaging of V79 cells*

Most cells will only survive and grow in culture when attached to a suitable solid surface. Once the cells have filled the available space, the rate of cell division decreases (confluent culture). Under conditions which are not optimal, the cells tend to peel off the surface. Thus, it is essential to transfer the cells to fresh bottles regularly, to prevent them becoming overcrowded. The first step in sub-culturing is to detach the cells from the surface of the culture vessel. This is done by exposing the cells to trypsin (proteolytic enzyme), this works best if $Ca^{+2}$ and $Mg^{+2}$ ions are removed by chelating with EDTA. Hence, the medium is poured off, the V79 cells are then rinsed with 5 ml of trypsin-EDTA solution (0.25% trypsin, 1mM EDTA), the flask bottom must be completely covered to rinse out the medium, otherwise the trypsinisation is not optimal. Afterwards, the cells are treated again with 5 ml trypsin for 3-5 min until the cells are detached (this is examined using a phase contrast

microscope). The reaction is stopped with medium supplemented with 20% FCS, and the cells are transferred to centrifugation tubes and centrifuged at 1000 rpm at room temperature for 10 min. The pellet is resuspended in 5 ml medium.

The second step in sub-culturing V79 cells is counting of viable cells: 100 µl of cell suspension is mixed with 900 µl Trypan Bue dye solution. This azo dye acts as an indicator for cell viability, and colours the dead cells that can be distinguished from the vital, non-coloured cells. The mixture of Trypan Blue and cells is placed in a Neubauer Chamber (Fig. 10) with a defined volume (0.1 µl), and the viable cell number is calculated with the help of a light microscope.

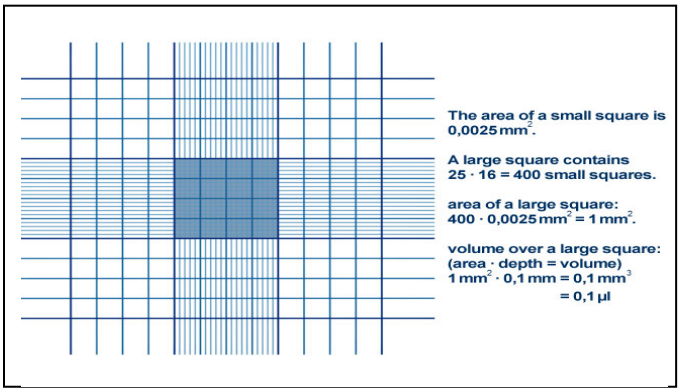



Fig.10. Counting chamber. The depth of the counting chamber is 0.1 mm, and the area of the big squares is 1 mm². There are 9 sets of big squares; each one has 16 small squares.

The cells from the 4 large squares in the edges are counted, but cell clots as well as dead cells are not taken into account. The mean value is calculated from the cell number of the 4 squares and the viable cell number is calculated according:

$$\text{Cell number/ml} = \text{mean value} \times \text{dilution factor} \times 10^4$$

The last step is to re-plate the cells at an appropriate concentration in new culture flasks: $3 \times 10^5$ cells from the resuspended pellet are placed in a 75 cm² flask and 25 ml DMEM is added. The flask are mixed by gentle swirling and incubated at 37°C in 5% $CO_2$ air.

*3.1.5.1.3. Cryopreservation and storage of V79 cells*

The aim of cryopreservation is to enable stocks of cells to be stored to prevent the need to have the cells in culture at all times. Thus, V79 cells are frozen in sterile Eppendorf 1 ml tubes with 10 % DMSO for 2 h at –20°C and then at –70°C.

*3.1.5.2. Cytotoxicity assays*

*3.1.5.2.1. Principles of cytotoxicity assays*

Two methods for determining the cytotoxicity of daidzein, equol and genistein on cell cultures were used. These methods are the Neutral Red Uptake assay, based on the method of Borenfreund and Borrero (1984) and Riddell et al. (1986) and the determination of total cellular protein (BCA).

The Neutral Red Uptake assay can be used to measure the growth of a population of cultured mammalian V79 cells. Viable cells take up the Neutral Red dye and transport it to a specific cellular compartment, the lysosome. The uptake, transport, and storage of Neutral Red dye occurs via active biological processes that require energy, as well as intact cellular and lysosomal structures. Damage to any of the systems involved or a reduction in cell number due to cell death would result in a decreased uptake of the Neutral Red in treated cells (compared to untreated controls).

The BCA assay is a colorimetric assay that involves the reduction of $Cu^{2+}$ to $Cu^{+}$ by proteins in an alkaline medium. The bicinchoninic acid chelates $Cu^{+}$ ions with very high specificity, to forms a water-soluble purple coloured complex. The macromolecular structure of proteins, the number of peptide bonds and the presence of four amino acids (cysteine, cystine, tryptophan and tyrosine) are responsible for the color formation in protein samples when assayed with BCA. This reaction is measured by the high optical absorbance of the final $Cu^{+}$ complex at 570 nm. Absorbance is directly proportional to the protein concentration, with a broad linear range between 5-20 μg/ml to 1-2 mg/ml. The protein concentration can be calculated with a reference curve obtained from a standard protein.

*3.1.5.2.2. Experimental procedures*

In order to evaluate the cytotoxicity of the isoflavones, V79 cells were cultured in DMEM medium supplemented with 10% FCS in a humidified atmosphere with 5% $CO_2$ in air at 37°C for 48 h. Cells were removed from the 75 cm² flasks with 0.25% trypsin, 1mM $Na_2$-EDTA and harvested by centrifugation at 1000g for 10 min; the number of viable cells/ml was determined by Trypan Blue dye exclusion. A total of $5x10^4$ cells/ml were plated in 96 well tissue-culture plates (Fig. 11), and after 24 h medium was changed and the substances were added as DMSO solutions. The concentrations used were 0, 25, 50, 75, 100, 125, 150 µM daidzein; 0, 5, 10, 25, 50, 75, 100 µM genistein; and 0, 5, 10, 18, 25, 50, 100 µM equol. The final concentration of DMSO in the culture medium was kept below 0.2 % (v/v). The cells were treated for 18 h (1.5 cell cycle) at 37° C in 5% $CO_2$ air. These experiments were carried out in a ninety-six well plate (Fig. 11), using eight wells (one row) for each concentration, medium and DMSO control and growth control (row 12; only cells that receive no treatment at all and whose absorbance were used to evaluate the cell growth), and were repeated in several independent assays.

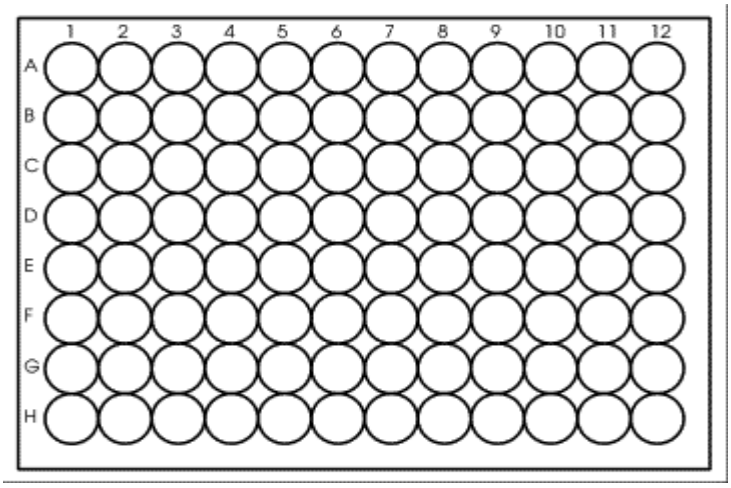
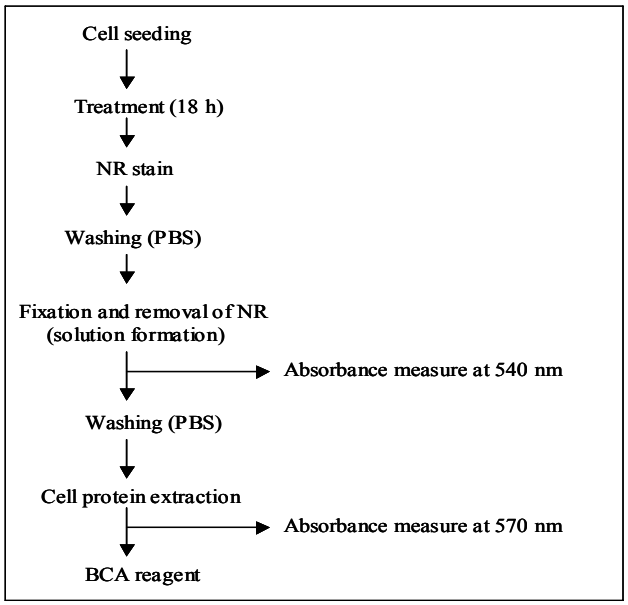



Fig. 11. Ninety-six well plate. The cells in each row (8 wells) were treated with the same substance concentration. Row 3 (A3-H3): medium; row 6 (A6-H6): solvent control; rows 4, 5, 7, 8, 9, 10, 11: different concentrations of daidzein, equol or genistein; row 1, 2: different concentrations of bovine albumin for the standard curve for the BCA assay (see below) and row 12 was used to seed cells that receive no treatment (growth control).

For the Neutral Red Uptake Assay a 0.4 % aqueous stock solution of Neutral Red (NR) was prepared and added to DMEM medium to obtain a final concentration of 0.05 mg/ml (designated NR medium). This solution was pre-incubated overnight at 37°C and then filtrated using a 0.22 µm filter Unit Millex-GP to remove precipitated crystals. The NR medium was added to the treated cells. After 3 h, neutral red solution was discarded, the cells were rinsed five times with warm (37° C) phosphate-buffered saline (PBS; pH = 7.4) to remove the excess non-incorporated dye and 200 µl of destain solution (50% ethanol, 1% acetic acid) were added to each well to fix the cells and remove the NR into solution. The plates were shaken for 20 min, and the absorbance of the solution in each well was measured in a 96-well plate spectrophotometer at 540 nm, against wells with untreated cells (Tecan software).
The BCA assay was performed on the same cells that were rinsed five times with warm (37°C) PBS, and a cell protein "extraction" was carried out with a solution of 0.1% Triton. The plates were kept at -70° C for 30 min. Then the plates were thawed, and the lysis of the cells was checked with the help of a light microscope.

The BCA reagent was prepared with cupric sulfate and bicinchoninic acid (1:50) (BCA: protein quantitation kit Uptima), and 200 µl per well was added to the cells. They were incubated for 30 min at 37° C. The absorbance was measured at 570 nm.
For the standard curve, different known concentrations of bovine serum albumin were used: 2.0, 1.0, 0.8, 0.6, 0.4 and 0.2 mg/ml; each concentration was added in two wells in rows 1 and 2 (where no cells were seeded). These standard solutions were also mixed with the BCA reagent. The Tecan software compares the absorbances of the albumin with the cell protein solutions.
The following is a schematic description of the procedures conducted for both cytotoxicity assays:




### *3.1.5.3. Micronucleus assay*

#### *3.1.5.3.1. Principles of micronucleus assay*

The term "micronucleus" describes the appearance of nuclear substructures of varying size and number which can occur in cells following DNA damage or mitotic spindle damage. Micronucleus formation can occur during S phase (*interphase*) of the cell cycle, for example as a route for the expulsion of chromosome breaks or during *mitosis* when whole chromosomes are unable to travel to the spindle poles (spindle damage). Possible mechanisms of spindle-related micronucleus formation include the reformation of the nuclear envelope around chromosomes which have failed to attach to the spindle (Sorger et al., 1997); attachment failure leads to non-disjunction (two sister chromatids migrating to a single pole) or lagging (chromatids do not migrate towards either pole). Similarly, defects in duplication of the centrosomes at S phase (giving rise to more than two centrosomes) can also give rise to segregation problems, for example the formation of tripartite spindle, which in turn may give rise to micronuclei (Meek, 2000). On the other hand, micronuclei may result from lesions/adducts at the level of DNA or chromosomes, giving rise to acentric fragments, which are excluded from the two daughter nuclei at the late stages of mitosis.
Formation of micronuclei originating from chromosome fragments or chromosome loss events requires a mitotic division.

In this thesis, the induction of micronuclei was used to study the genotoxicity properties of isoflavones in comparison with two known MN inducers. Vincristine (VCR) and methylmethanesulfonate (MMS) were used as positive controls in the MN assay and in CREST analysis.

VCR (Fig. 12) is a dimeric alkaloid isolated from the periwinkle plant, Catharanthus roseus, which is used for treatment of several forms of malignancy (Ferguson and Pearson, 1996). Many studies have shown that VCR induces a wide spectrum of divitional aberrations which result in mitotic arrest, polyploidy and aneuploidy (Miller and Adler, 1989). VCR binds to tubulin, inhibiting the tubulin polimerization and assempbly of mitotic spindle microtubules. In addition this drug inhibits cell proliferation, producing an accumulation of cells in the mitotic phase.

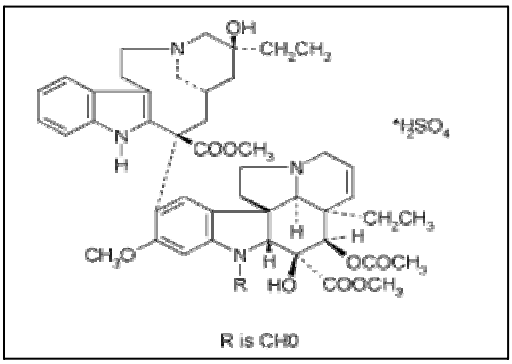



Fig. 12. Chemical structure of VCR.

MMS (Fig. 13) is an alkylating agent, which induces an increase in the frequency of micronuclei in lung fibroblast in vivo (Tao et al., 1993). MMS produces DNA damage (clastogen effect) in concentrations below cytotoxic concentrations in V79 and in blood cells (Kreja and Seidel, 2002).

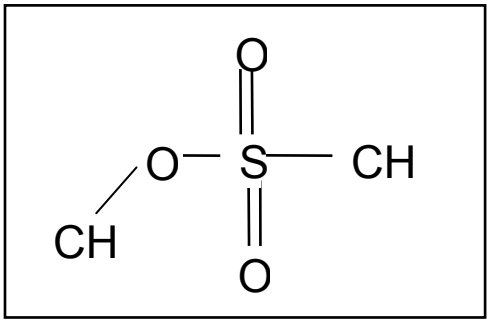



Fig. 13. Chemical structure of MMS.

*3.1.5.3.2. Experimental procedures*

V79 cells were cultured in DMEM medium supplemented with 10% FCS in a humidified atmosphere with 5% $CO_2$ in air at 37° C for 48 h. Cells were removed from the 75 cm² flasks with 0.25% trypsin, 1mM $Na_2$-EDTA and harvested by centrifugation at 1000 g for 10 min; the number of viable cells/ml was determined by Trypan Blue dye exclusion. A number of $3x10^5$ cells were seeded in 25 cm² flasks in 5 ml medium and grown for two days. Then the medium was changed and the isoflavones were added as DMSO solutions. The concentrations used were 0, 25, 50, 75, 100 µM daidzein; 0, 5, 10, 18, 25, 50, 75 µM genistein and 0, 5, 10, 18, 25, 50 µM equol. The final concentration of DMSO in the culture medium was kept below 0.1% (v/v). Two positive controls were carried out: MMS, 50 µg/ml and VCR, 10 nM. A solvent (DMSO), and a medium control with 10% FCS were performed as well. The cells were treated during 18 h (1.5 cell cycles) at 37° C in 5% $CO_2$ air. Following disaggregation with trypsine/EDTA and resuspension in complete medium, cells were subjected to hypotonic conditions with 0.4% KCl and fixed with methanol-acetic acid (3:1). Cells were mounted onto slides, air-dried and observed under a light microscope to confirm the integrity of the cytoplasm. Afterwards cells were stained with a solution of 50 µg/ml of acridine orange and immediately observed by fluorescence microscopy using blue excitation (see Table 7). The slides were analyzed within 2 weeks, and 1000 cells/slide were scored. This assay was carried out twice, and 3 slides per concentration in each assay were analysed. The criteria for the scoring of micronuclei were: (1) only cells with intact cytoplasm and main nucleus were counted, (2) diameter less than 1/3 the main nucleus, (3) colour the same as or lighter than the main nucleus, and (4) location within 3 or 4 nuclear diameters of the nucleus not touching the nucleus and no bridges in between.

*3.1.5.4. Kinesin-driven microtubule gliding assay*

Kinesin belongs to the group of motorproteins known to convert chemical energy (preferentially from ATP) into mechanical energy (Kuznetsov and Gelfand, 1986). A characteristic feature of kinesin proteins is its capacity for moving along microtubules of several micrometers length without dissociating (Block et al. 1990). With the „kinesin gliding assay“, it is possible to mimic intracellular movement and transport processes in vitro by the

gliding of taxol-stabilised microtubules across a kinesin-coated glass surface (Vale et al., 1985; von Massow et al., 1989; Schnapp et al.,1990, see Fig. 14). Thus, with this motility assay, the effects of phytoestrogens on the cytoeskeleton were studied. The microtubule gliding assay was essentially carried out according to Böhm et al. (2000): 3 µl kinesin (270 µg/ml) were mixed with 13.4 µl dithiothreitol (IEM) buffer, 2 µl 1M sodium chloride (in IEM buffer), 0.2 µl 2 mM taxol (in DMSO), and 1 µl 10 mM magnesium ATP (in IEM buffer) and incubated for 5 min at room temperature. After adding 0.4 µl taxol-stabilized microtubules, formed from 2 mg/ml phosphocellulose-purified tubulin in IEM buffer, 10 µl of this mixture (assay mixture) were transferred onto glass slides, pretreated with bovine serum albumin (BSA; 5 mg/ml), and covered by a coverslip of 18 mm x 18 mm. The gliding assay was performed for daidzein and genistein, that were added as DMSO solutions. The concentrations used were 100 and 500 µM for both phytoestrogens and two solvent controls were carried out as well (1 and 5% DMSO). The gliding activity was monitored at 22-25°C by video-enhanced differential interference contrast microscopy, using an Axiophot microscope (Zeiss) equipped with the image processing system Argus 50 (Hamamatsu). Gliding velocities were determined from video records by measuring the distance the microtubules migrated within a defined time and the mean value was calculated as the motility-characterizing parameter.

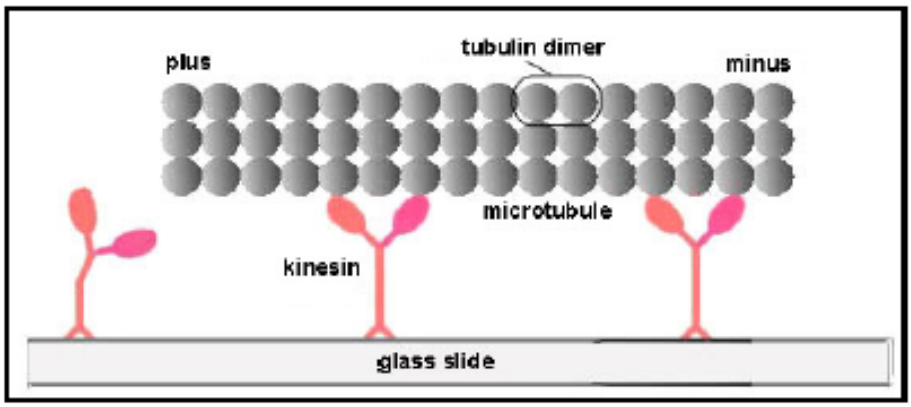



Fig. 14. Gliding assay: taxol-stabilised microtubules across a kinesin-coated glass surface (www.imb-jena.de).

*3.1.5.5. CREST analysis*

Micronuclei (MN) are formed when an entire chromosome or a chromosome fragment fails to migrate in one of the two daughter nuclei during mitosis. Conventional microscopic analysis, however, does not discriminate MN with respect to their "contents". It has been shown that the presence or absence of kinetochore proteins in a MN can be used to identify MN deriving from chromosome loss (kinetochore-positive), produced by aneugenic substances or deriving from chromosome breakage (kinetochore-negative), produced by clastogenic substances. A large number of studies have demonstrated that the MN assay can distinguish clastogens from aneugenic agents using immunofluorescent antikinetochore (CREST) staining of MN following treatment with agents with different mechanisms of action. The anti-kinetochore antibody is obtained from CREST patients` serum (CREST syndrome: *c*alcinosis, *R*aynaud phenomenon, *e*sophageal dismotility, *s*clerodactyly and *t*elangiectasia). The commercial CREST serum contains antibodies to a specific protein of the kinetochore region of chromosomes (Fig. 15). These antibodies bind not only to kinetochores of human chromosomes but also to those of mammalian cells in general (Tan et al. 1980) and they can be identified by binding a secondary anti-human antibody which has been labelled with fluorescein-5-isothiocyanate (FITC). Thus, analysis of MN which contain whole chromosomes shows the interphase nuclei containing many discrete bright-yellow speckles that were distinct and distributed throughout the nucleus.

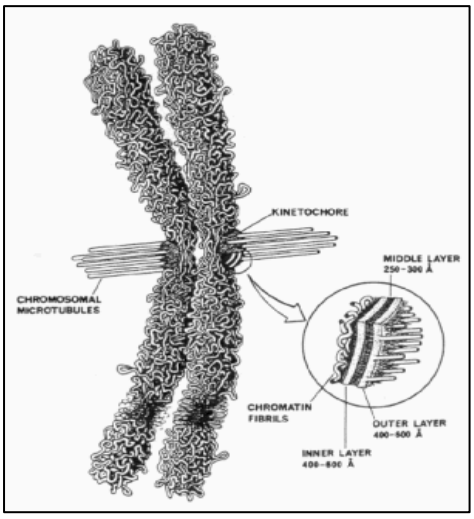



Fig. 15. Kinetochore proteins in the chromosome.

The CREST analysis was performed as the "standard" MN assay, except that $1x10^4$ V79 cells were seeded onto slides placed in "Quadriperm trays" in 5 ml 10% FCS DMEM medium that was changed the following day. Twenty-four hours later the test substances as DMSO solutions were added. The concentrations were 20 µM for genistein, 100 µM for daidzein and 25 and 50 µM for equol. After 18-hour-treatment, cells were washed with warm sterile PBS (1X) and were then subjected to hypotonic conditions with 0.075 M KCl for 15 min. Fixation with cold (-20°C) methanol were carried out for 30 min and with acetone for 10 min. Afterwards cells were washed during 5 min with PBS-0.1 % Tween 20 (pH 7.4). Two drops (~50 µl) of CREST serum as the first antibody (diluted 1:50 with PBS-0.2 % Tween 20) were added to each slide, and the cells were incubated at 37° C. After 24 h cells were washed twice with PBS-0.1 % Tween 20 and covered with 80 µl of secondary FITC-labelled goat anti-human IgG for 1 h. Then the slides were rinsed twice with PBS-0.1 % Tween 20 and rehydrated with Soerensen buffer (pH 6.8). Counterstaining was performed with a solution containing 0.1 mg/ml propidium iodide (PI):1 µg/ml 4´6´diamidino-2-phenylindole (DAPI); (3:1). The induction of MN was evaluated by scoring a total of 1000 cells /slide with well preserved cytoplasm at 400 X magnification. The analysis of CREST staining was restricted to areas where nuclei showed an adequate speckled pattern of bright, fluorescent, yellow-green spots. MN were located by DAPI fluorescence (UV excitation), checked with a PI filter if necessary, and finally classified using the FITC filter (blue excitation, Table 7). MN were classified as CREST *positive*, when bright spots were clearly observed; *negative*, when no spots were observed or *unclear*, when either opaque spots or a bright background were observed.

*3.1.5.6. COMET assay*

The COMET assay is a technique for studying DNA damage (strand breaks) and repair. In this assay, a small number of cells suspended in a thin agarose gel on a microscope slide is lysed, electrophoresed, and stained with a fluorescent DNA binding dye. In the "neutral version" DNA damage in cells due to double-strand breaks becomes apparent as increased migration of chromosomal DNA from the nucleus toward the anode, which resembles the shape of a comet. In the "alkaline version", also DNA single-strand breaks and alkali-labile

sites become apparent, and the amount of DNA migration indicates the amount of DNA damage in the cell.

The COMET assay in the alkaline version was used, based on the method of Singh et al. (1988): $1x10^4$ V79 cells were seeded in 1 ml of DMEM medium supplemented with 10% FCS and kept in a humidified atmosphere with 5% $CO_2$ in air at 37° C. After 24 h the medium was changed, and the test substances were added as DMSO solutions. Two independent experiments were carried out. The concentrations of isoflavones used for the first study were: 0, 25, 50, 75, 100 µM daizein; 0, 5, 10, 18, 25, 50 µM genistein and 0, 5, 10, 18, 25, 50 µM equol. The final concentration of DMSO in the culture medium was kept below 0.1% (v/v). The cells were treated for 3, 12 and 18 h at 37° C in 5% $CO_2$ air with daidzein and genistein and for 3 h with equol. For the second experiment, the cells were treated for 3 hours, and the concentrations used were: 50, 100, 150 and 200 µM for the three compounds. In this case the final concentration of DMSO was 0.2% (v/v). A positive control ($K_2Cr_2O_7$) at a final concentration of 50 or 100 µM was carried out, and medium and solvent controls were performed as well. After the treatment period, the cells were removed with 200 µl of 0.25% trypsin, 1mM $Na_2$-EDTA and harvested by centrifugation at 1000 g for 3 min. The pellet was resuspended in 100 µl PBS, and $4x10^4$ cells were mixed with 500 µl of 0.5 % „low melting-temperature agarose“ at 37° C. Then, approximately $2x10^4$ cells were placed on a precleaned microscope slide which was already covered with a thin layer of 1.5 % normal melting agarose to promote a firm attachment of a second layer. The cell suspension was immediately covered with a coverglass, and the slides were placed horizontally in a steel-made, cold tray for 5 min to allow solidification of the agarose. Then, the slides were immersed overnight in a „lysing solution“ at 4°C (1% sodium laurylsarcosinate, 2.23 M NaCl, 90 mM $Na_2$-EDTA, 8.8 mM Tris, 0.25 M NaOH to reach pH 10, and 1% Triton X-100, 10% DMSO, added freshly) in order to lyse the cells, to remove cellular proteins and to permit DNA unfolding. The slides were then removed from the lysing solution and placed on a horizontal electrophoresis unit. The unit was filled with fresh electrophoresis buffer (1 mM $Na_2$-EDTA and 300 nM NaOH). The slides were set into this high-pH buffer for 30 min to allow unwinding of DNA before electrophoresis. Electrophoresis was conducted for the next 30 min at 25 V and 300 mA. After electrophoresis, the slides were washed gently to remove alkali and detergents, which would interfere with ethidium bromide staining with 0.4 M Tris, pH 7.5 during 15 min. Then the slides were dehydrated in 50%, 75%, 100% ethanol (5 min in each solution). Afterwards,

the slides were stained by placing 50 µl of a 20 µg/ml ethidium bromide solution on each slide. For each substance concentration a total of 100 cells from two slides were analysed under the green light of a fluorescence microscope (Table 7) and using image analysis software (Comet Assay II, Perspective Instruments Suffolk, England).
The tail length was the final parameter used. Figure 16 shows a schematic description of the different steps in the COMET assay.

| Dye | Absorption spectra (nm) | Light | Leica Filter |
|---|---|---|---|
| Acridine Orange | 450-490 | blue | I3 |
| PI | 515-560 | green | N2.1 |
| DAPI | 340-380 | UV | A4 |
| FITC | 450-490 | blue | I3 |
| Ethidium Bromide | 515-560 | green | N2.1 |

Table 7. Dyes and filters used for MN assay, CREST analysis and COMET assay.

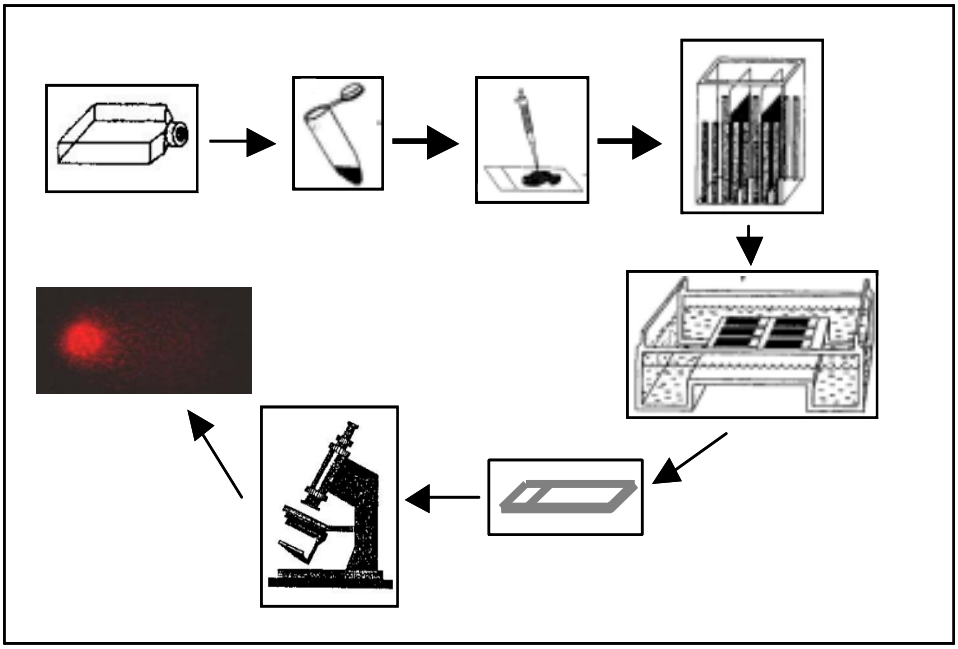


Fig. 16. Schematic description of the COMET assay.

In collaboration with the laboratory of Prof. Dr. Regine Kahl, Heinrich-Heine University, Düsseldorf, two other experiments were carried out. The induction of DNA damage by much higher concentrations of genistein was detected by the COMET assay in V79 cells. The experimental procedures were the same as those described above, except for the lysis solution, that was slighly different (2.5 M NaCl, 10mM Tris, 100mM $Na_2$-EDTA, 1 % Triton X-100, pH 10). V79 cells were incubated with different concentrations of genistein (100, 250 and 500 µM) for 3 h and the induced DNA damage was evaluated in 50 cells as the average image length.

In order to study an antioxidative capacity of genistein, the COMET assay was applied with hydrogen peroxide. This causes oxidative damage to DNA, and pre-treatment of V79 cells with antioxidative compounds was expected to decrease hydrogen peroxide-induced DNA-damage. Cells ($3x10^6$) were seeded in a six-well plate; after 24 h, cells were pre-incubated for 1 h with 50 µM genistein and then treated with 500 µM $H_2O_2$ for 2 h. After treatment, formation of DNA strand breaks was assessed with the COMET assay as described above.

## 3.2. Results

### *3.2.1. Evaluation of dietary isoflavone exposure*

#### *3.2.1.1. Parameters of the HPLC analysis*

Figures 17 and 18 show typical chromatograms to quantify daidzein and genistein in rat diet or rat plasma. The isoflavones in these matrixes were identified by comparison of the retention times with respective standards. The retention times in analysis of rodent diet samples were 20.5 min for daidzein and 26.4 min for genistein, while in analysis of plasma these were 22.3 min for daidzein and 29.4 min for genistein.

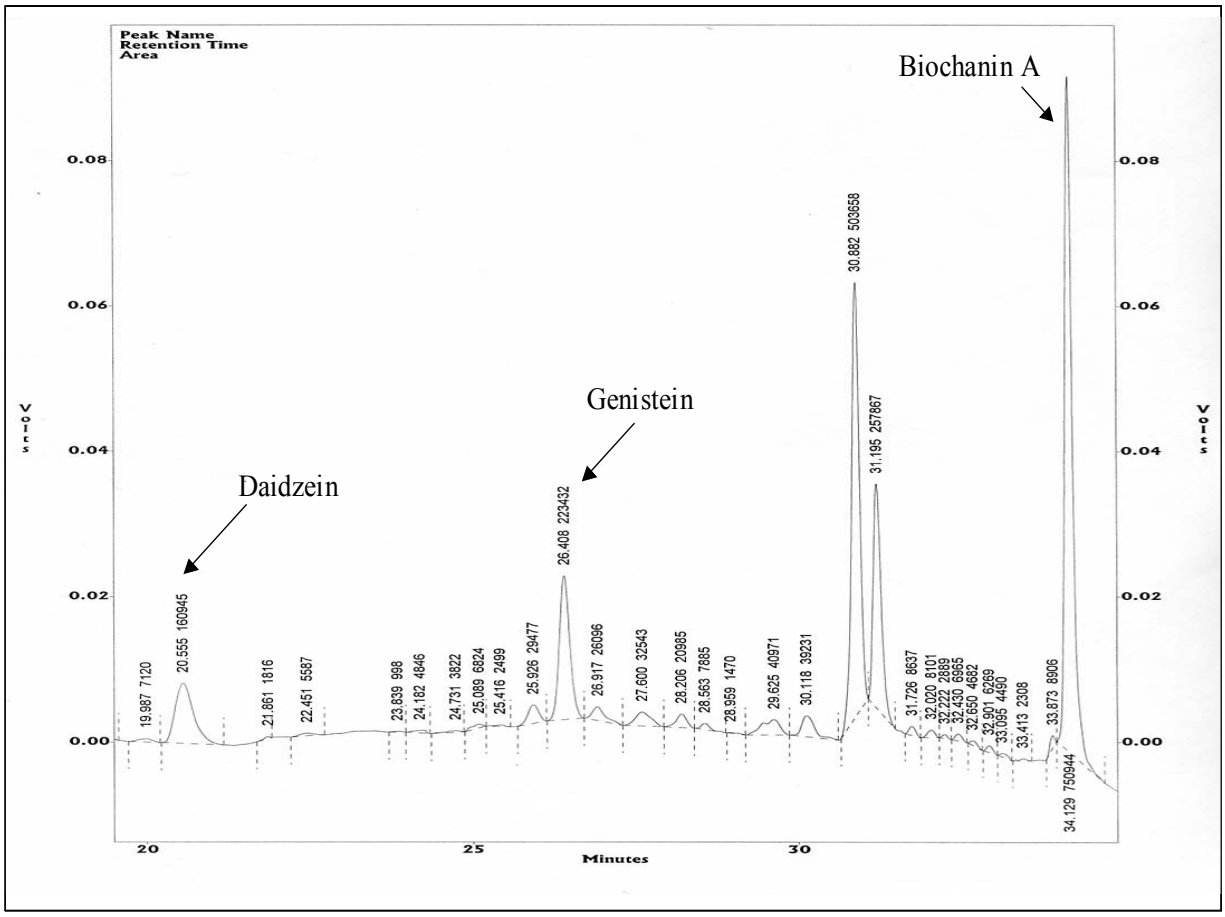



Fig. 17. Example of a chromatogram of a determination of daidzein and genistein in rodent diet (HPLC).

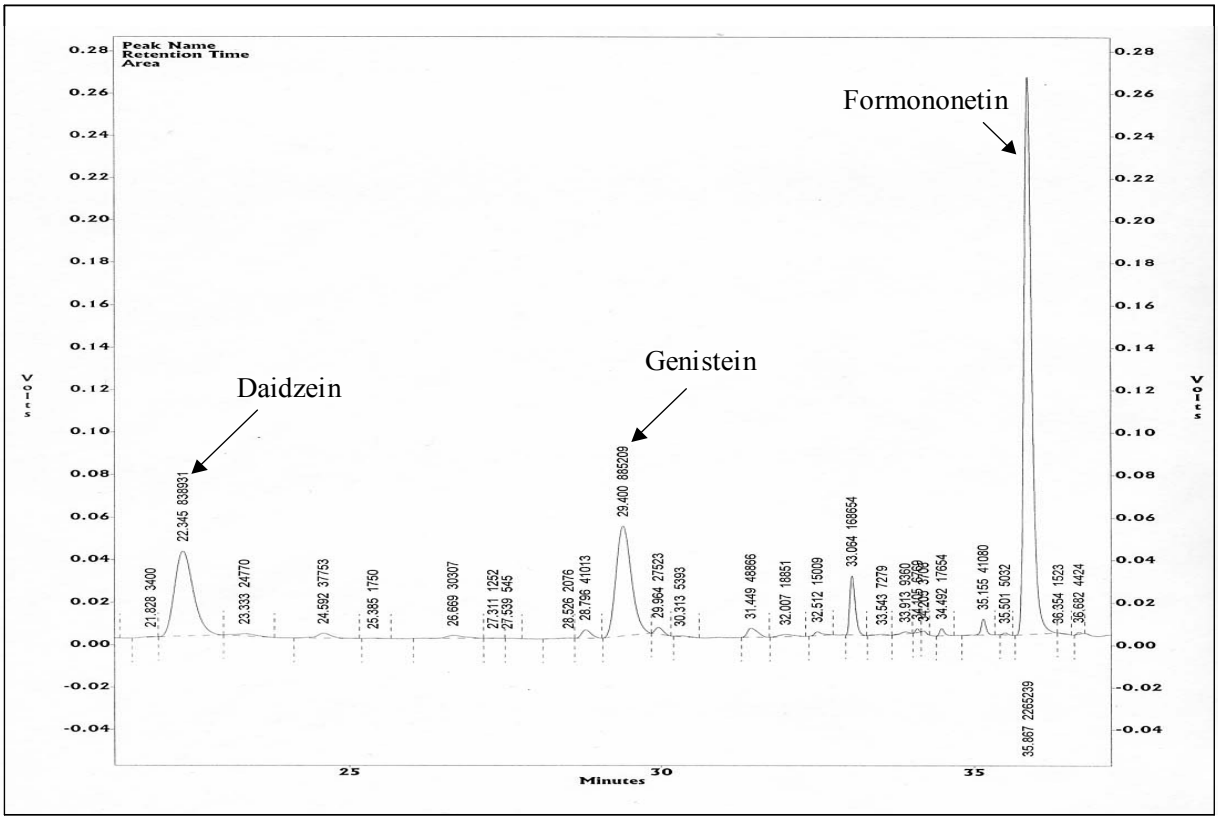



Fig. 18. Example of a chromatogram of a determination of daidzein and genistein in plasma (HPLC).

The reproducibility of the overall analytical procedure (including sample processing, extraction and chromatography) was evaluated. Three samples with known amounts of isoflavones were independently processed and analysed. Both mean values and variances were calculated and were expressed as variation coefficients (%). Tables 8 and 9 show the data for daidzein and genistein in rodent diet (three different concentrations of 50 ng/ml, 100 ng/ml and 250 ng/ml). Tables 10 and 11 show the reproducibility data for daidzein and genistein in plasma (at concentrations of 50 ng/ml, 100 ng/ml and 500 ng/ml).

| Daidzein conc. [ng/ml] | Determined daidzein conc. [ng/ml] | Mean value [ng/ml] | Standard deviation [ng/ml] | CV % |
|---|---|---|---|---|
| 50 | 41.7<br>36.5<br>28.6 | 35.6 | 5.4 | **15.2** |
| 100 | 84.3<br>80.5<br>81.1 | 81.9 | 1.7 | **2.1** |
| 250 | 239.6<br>274.9<br>213.5 | 242.7 | 25.1 | **10.3** |

Table 8. Reproducibility of the microbore HPLC method for determination of daidzein in rodent diet.

| Genistein conc. [ng/ml] | Determined genistein conc. [ng/ml] | Mean value [ng/ml] | Standard deviation [ng/ml] | CV % |
|---|---|---|---|---|
| 50 | 20.4<br>24.6<br>24.9 | 23.3 | 2.1 | **8.7** |
| 100 | 65.4<br>95.3<br>60.9 | 73.9 | 15.2 | **20.6** |
| 250 | 238.9<br>215.1<br>238.7 | 230.9 | 11.2 | **4.8** |

Table 9. Reproducibility of the microbore HPLC method for determination of genistein in rodent diet.

| Daidzein conc. [ng/ml] | Determined daidzein conc. [ng/ml] | Mean value [ng/ml] | Standard deviation [ng/ml] | CV % |
|---|---|---|---|---|
| 50 | 39.9<br>49.4<br>39.1 | 42.8 | 4.6 | **10.8** |
| 100 | 111.4<br>98.3<br>94.9 | 101.5 | 7.1 | **6.9** |
| 500 | 478.9<br>498.2<br>471.9 | 483.1 | 11.1 | **2.3** |

Table 10. Reproducibility of the microbore HPLC method for determination of daidzein in plasma.

| Genistein conc. [ng/ml] | Determined genistein conc. [ng/ml] | Mean value [ng/ml] | Standard deviation [ng/ml] | CV % |
|---|---|---|---|---|
| 50 | 32.4<br>49.7<br>56.2 | 46.1 | 10.1 | **21.8** |
| 100 | 94.6<br>105.9<br>98.9 | 99.8 | 4.7 | **4.7** |
| 500 | 499.3<br>458.1<br>496.5 | 484.6 | 18.8 | **3.9** |

Table 11. Reproducibility of the microbore HPLC method for determination of genistein in plasma.

For the analysis of recovery rates, three determinations were done for each concentration of isoflavones. The recoveries of genistein and daidzein from plasma were calculated at 50, 100 and 500 ng/ml for each compound after spiking known amounts of the isoflavones in 100 µl aliquots of plasma. The standard method chosen to analyse isoflavones in plasma started with diluting the plasma in 150 mM ammonium acetate buffer, pH 7, containing 500 mM TEAS buffer. This markedly increased the recovery of genistein (Coward et al., 1996). Known standard solutions of genistein and daidzein were added to plasma resulting in concentrations varying from 50- 500 ng/ml, and extraction using solid phase cartridges was performed. The mean recovery rates ranged from 69.2 - 94.1% for daidzein and 78.3 - 96.8% for genistein (Tables 12 and 13).

| Daidzein conc. [ng/ml] | Determined daidzein conc. [ng/ml] | Recovery rates [%] | Mean recovery rates [%] |
|---|---|---|---|
| 50 | 42.9<br>33.7<br>26.9 | 85.9<br>67.5<br>54.1 | 69.2 |
| 100 | 79.6<br>65.3<br>68.1 | 79.6<br>65.3<br>68.1 | 71.1 |
| 500 | 503.9<br>483.1<br>423.9 | 100.7<br>96.6<br>84.8 | 94.1 |

Table 12. Recovery rates for the determination of daidzein in rodent plasma.

| Genistein conc. [ng/ml] | Determined genistein conc. [ng/ml] | Recovery rates [%] | Mean recovery rates [%] |
|---|---|---|---|
| 50 | 39.8<br>57.3<br>46.4 | 79.6<br>114.5<br>92.8 | 95.4 |
| 100 | 84.8<br>113.4<br>92.2 | 84.8<br>113.4<br>92.2 | 96.8 |
| 500 | 413.3<br>381.5<br>379.3 | 82.7<br>76.3<br>75.9 | 78.3 |

Table 13. Recovery rates for the determination of genistein in rodent plasma.

Recoveries for genistein and daidzein determined in rodent diet were calculated at concentrations of 50, 100 and 250 ng/ml. Known amounts of each isoflavone were added to 100 µl aliquots of hydrolysed isoflavone-free diet. The recoveries varied from 84.5- 93.7% for daidzein and from 81.6- 93.7% for genistein (Tables 14 and 15).

| Daidzein conc. [ng/ml] | Determined daidzein conc. [ng/ml] | Recovery rates [%] | Mean recovery rates [%] |
|---|---|---|---|
| 50 | 44.8<br>54.2<br>39.6 | 89.6<br>108.4<br>79.2 | 92.4 |
| 100 | 83.6<br>86.9<br>83.1 | 83.6<br>86.9<br>83.1 | 84.5 |
| 250 | 214.5<br>240.3<br>247.9 | 85.8<br>96.1<br>99.2 | 93.7 |

Table 14. Recovery rates for the determination of daidzein in rodent diet.

| Genistein conc. [ng/ml] | Determined genistein conc. [ng/ml] | Recovery rates [%] | Mean recovery rates [%] |
|---|---|---|---|
| 50 | 42.2<br>37.6<br>42.5 | 84.5<br>75.2<br>85.1 | 81.6 |
| 100 | 77.3<br>102.9<br>73.5 | 77.3<br>102.9<br>73.5 | 84.4 |
| 250 | 226.1<br>226.3<br>250.6 | 90.5<br>90.5<br>100.2 | 93.7 |

Table 15. Recovery rates for the determination of genistein in rodent diet.

In order to quantitate total daidzein and genistein in rodent chow samples, the calibration was performed using the method of standard addition for each unknown sample. Table 16, as well as Fig. 19 and 20 show an example of a calibration curve for the analysis of these isoflavones in rodent diet. The calibration curves were linear in the concentration range chosen (50-350 ng/ml) with regression equations of $y = 675.9\ x + 16461$ ($r^2 = 0.9794$) for genistein and $y = 582.9\ x + 4979.6$ ($r^2 = 0.9687$) for daidzein. When the peak areas were corrected to the internal standard, a linear regression equation of $y = 0.0008\ x + 0.053$ ($r^2 = 0.9863$) was obtained for genistein and $y = 0.0007\ x + 0.03$ ($r^2 = 0.9146$) for daidzein.

| [isoflavone] ng/ml | Area biochanin A | Area daidzein | Area genistein | Area ratio daidzein/biochanin A | Area ratio genistein/biochanin A |
|---|---|---|---|---|---|
| 0 | 530201 | 17689 | 28354 | 0.033 | 0.053 |
| 50 | 615532 | 34800 | 50457 | 0.056 | 0.082 |
| 100 | 638294 | 51811 | 80764 | 0.082 | 0.126 |
| 150 | 609804 | 86352 | 113346 | 0.142 | 0.186 |
| 200 | 538418 | 125301 | 126062 | 0.233 | 0.234 |
| 250 | 735311 | 148937 | 196808 | 0.203 | 0.267 |
| 300 | 750944 | 160945 | 223432 | 0.215 | 0.297 |
| 350 | 800118 | 230191 | 258839 | 0.288 | 0.323 |

Table 16. Example of a calibration curve for determination of daidzein and genistein in rodent diet.

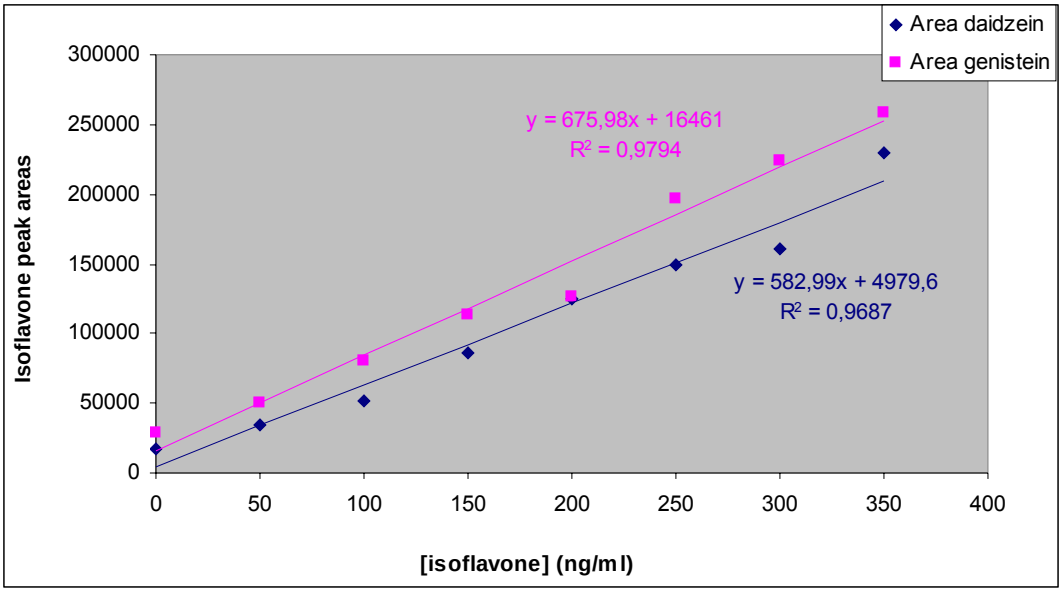



Fig. 19. Example of a calibration curve for determination of daidzein and genistein in rodent diet as a function of isoflavone peak areas vs. isoflavone concentrations (data from Table 16).

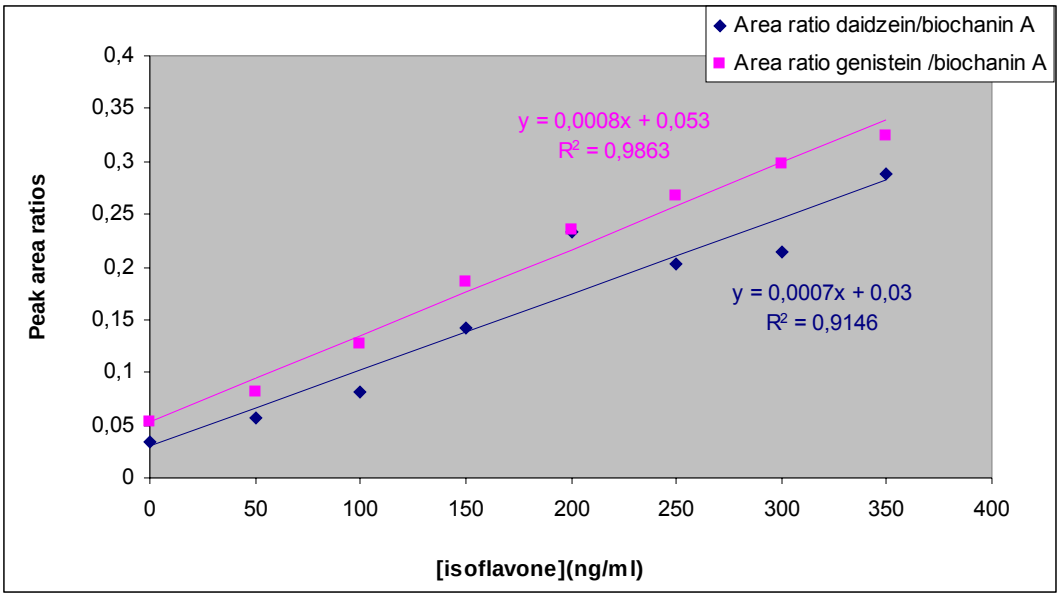



Fig. 20. Example of a calibration curve for determination of daidzein and genistein in rodent diet as a function of peak area ratios vs. isoflavone concentrations (data from Table 16).

Furthermore, calibration of the microbore HPLC to determine daidzein and genistein in plasma was carried out as an external calibration using pig plasma as matrix with known amounts of isoflavones. These solutions were treated in the same way as the unknown samples, as described in section 3.1.4.2.3. (Table 17, Fig. 21 and 22). This calibration also yielded linear regression equations: $y = 532.7\ x + 49761$ ($r^2 = 0.9967$) for genistein and $y = 437.5\ x + 37179$ ($r^2 = 0.999$) for daidzein. The correction of the peak areas gave equations of $y = 0.0002\ x + 0.0192$ ($r^2 = 0.9973$) for genistein and $y = 0.0002\ x + 0.0145$ ($r^2 = 0.9999$) for daidzein.

| [isoflavone] ng/ml | Area formononetin | Area daidzein | Area genistein | Area ratio daidzein/formononetin | Area ratio genistein/formononetin |
|---|---|---|---|---|---|
| 0 | 2295173 | 37591 | 11808 | 0.016 | 0.005 |
| 25 | 2505056 | 48844 | 66669 | 0.019 | 0.027 |
| 50 | 2643105 | 63944 | 81660 | 0.024 | 0.031 |
| 75 | 2604664 | 79628 | 101894 | 0.030 | 0.039 |
| 100 | 2543025 | 69018 | 119896 | 0.028 | 0.047 |
| 500 | 2447357 | 246451 | 313920 | 0.101 | 0.128 |
| 1000 | 2556920 | 481932 | 593012 | 0.188 | 0.232 |
| 1500 | 2480513 | 691857 | 840623 | 0.278 | 0.338 |

Table 17. Example of a calibration curve for determination of daidzein and genistein in rodent plasma.

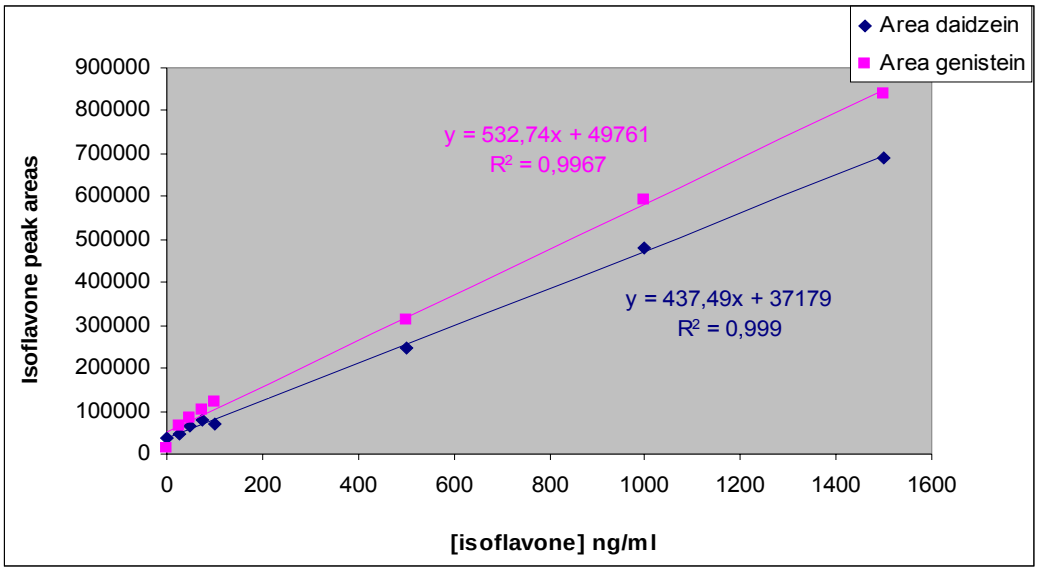



Fig. 21. Example of a calibration curve for determination of daidzein and genistein in rodent plasma as a function of isoflavone peak areas vs. isoflavone concentrations (data from Table 17).

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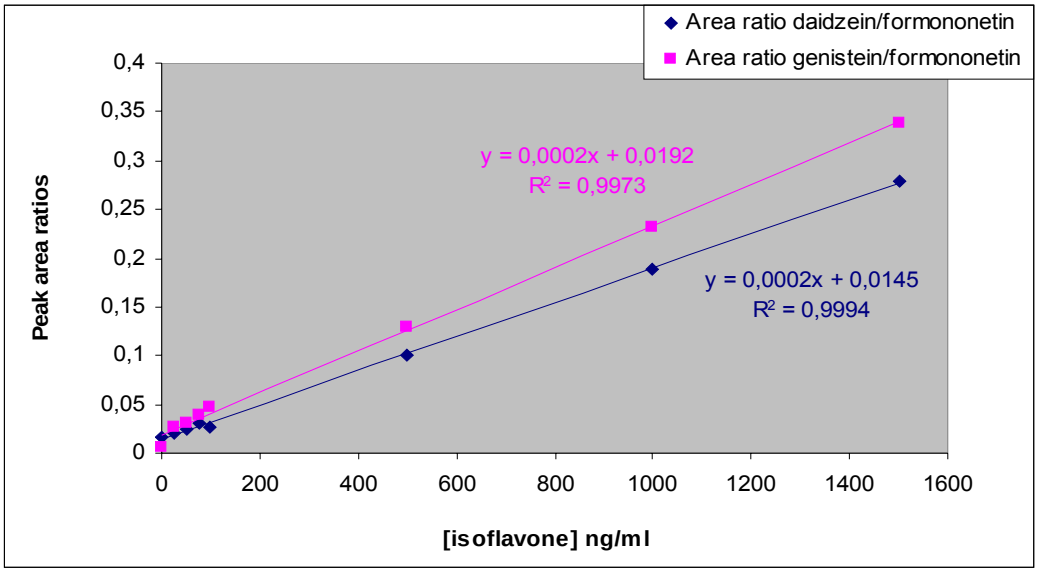


Fig. 22. Example of a calibration curve for determination of daidzein and genistein in rodent plasma as a function of peak area ratios vs. isoflavone concentrations (data from Table 17).

*3.2.1.2. Analysis of isoflavones in rodent diet by HPLC*

Two different rodent dietary samples were analysed for their total isoflavone contents by microbore HPLC. Total daidzein and genistein concentrations in the analyzed food samples are listed in Table 18.

| Rodent diet sample | Daidzein (µg/g) | Genistein (µg/g) | Ratio (gen/dai) |
|---|---|---|---|
| Isoflavone-low diet (ILD) | <10 | <10 | ---- |
| Isoflavone-rich diet (IRD) | 232 ± 10 | 240 ± 36 | 1.03 |

Table 18. Isoflavone analysis of rodent diets by HPLC, each value is representative of three independent experiments (mean value ± SD).

The total isoflavone (daidzein and genistein) concentrations in rodent diets, as used for this study, varied considerably between the two different suppliers. In the so-called "isoflavone-rich diet" (IRD), the mean concentration of total isoflavones was 472 µg/g chow and the ratio between genistein and daidzein contents was higher than 1. These data are in accordance with previous data which show higher genistein than daidzein levels (Degen et al., 2002a). The second rodent diet classified as "isoflavone-low diet" (ILD) presented non-detectable levels of daidzein and genistein (the limits of detection for both isoflanes were 10 µg/g chow).
The type of diet had no effect on the daily food consumption of the rats; this was approximately 20 g of feed per day. Animals on a isoflavone-rich diet (472 µg isoflavone/g chow) had a mean body weight of 350 g, and therefore an average oral intake of 26.8 mg isoflavones/kg b.w. (50:50; ratio of daidzein:genistein).

*3.2.1.3. Analysis of isoflavones in rodent plasma by HPLC*

As part of a larger project, aimed to study the effects of 17 ß-estradiol and isoflavones with respect to movement drive, body weight and bone mineral density, bioassays were conducted with ovariectomized Wistar rats at the Institut für Kreislaufforschung und Sportmedizin, Deutsche Sporthochschule, Köln. The experiments provided the opportunity to obtain blood samples for the analysis of the isoflavone levels produced by the dietary phytoestrogen

background. The results of the analysis for daidzein and genistein in these samples are shown in Table 19 (aglycone plus conjugates in ng/ml) and are also depicted graphically in Fig. 23 for daidzein and in Fig. 24 for genistein.

| | | | [genistein] ng/ml | | | [daidzein] ng/ml | | |
|---|---|---|---|---|---|---|---|---|
| | | | Beginning of study | After 6 weeks | After 12 weeks | Beginning of study | After 6 weeks | After 12 weeks |
| | Animal Nr | Body Weight | Ssniff Standard Diet | | | Ssniff Standard Diet | | |
| ILD group | 311 | 395.5 | 106.3 | < 20 | < 20 | 105.9 | 23.1 | < 20 |
| | 312 | 345.3 | 42.4 | < 20 | < 20 | 39.8 | 21.1 | < 20 |
| | 313 | 389.8 | 290.7 | < 20 | < 20 | 235.6 | 22.9 | nd |
| | 314 | 390.2 | 141.2 | < 20 | < 20 | 132.1 | 20.3 | < 20 |
| | 315 | 368.7 | 222.3 | < 20 | < 20 | 148.3 | 25.5 | < 20 |
| | 316 | 385.4 | 153.9 | < 20 | < 20 | 120.8 | 25.7 | < 20 |
| | | | | MV (n= 6) < 20 | MV (n= 6) < 20 | | MV (n= 6) = 23.1 SD = 2.2 | MV (n= 6) < 20 |
| IRD group | 322 | 436.4 | 133.4 | 59.8 | 152.1 | 193.3 | 87.4 | 126.2 |
| | 323 | 400.6 | 80.1 | 45.2 | 70.5 | 128.2 | 50.4 | 62.4 |
| | 324 | 387.0 | 170.3 | 42.3 | 34.8 | 170.4 | 34.1 | 43.7 |
| | 325 | 337.6 | 138.9 | 20.8 | 133.5 | 168.6 | 30.6 | 88.5 |
| | 326 | 406.7 | 94.1 | 65.3 | 137.4 | 116.3 | 140.4 | 121.1 |
| | 327 | 366,9 | 328.4 | 18.7 | 60.5 | 274.9 | 24.6 | 48.6 |
| | 328 | 354.2 | 292.8 | 94.9 | 27.8 | 213.5 | 107.4 | 22.5 |
| | 329 | 432.5 | 461.2 | 173.2 | 82.9 | 389.9 | 122.6 | 64.7 |
| | 330 | 359.5 | 290.9 | 88.7 | 48.8 | 245.5 | 93.9 | 25.5 |
| | 331 | 380.8 | 574.4 | 218.7 | 88.6 | 511.2 | 165.9 | 74.6 |
| | 332 | 383.2 | 242.7 | 275.9 | 25.5 | 233.6 | 171.9 | 23.5 |
| | | | MV (n=17) = 221.4 SD = 141.2 | MV (n = 11) = 100.3 SD = 85.2 | MV (n= 11) = 78.4 SD = 45.4 | MV (n=17) = 201.6 SD = 113.2 | MV (n = 11) = 93.6 SD = 53.6 | MV (n= 11) = 63.8 SD = 36.5 |

Table 19. Isoflavone daidzein and genistein analysis of rodent plasma by HPLC at different time points and expressed in ng/ml. The body weight was evaluated at the end of the study. MV, mean value; SD, standard deviation.

All the animals were kept on a Ssniff Standard diet containing ~ 400 µg total isoflavone/g chow (Degen et al., 2002a) and on tap water before the first blood extraction was done. The analysis of isoflavones in these blood plasma samples gave mean values of 221.4 and 201.6 ng/ml for genistein and daidzein, respectively.

After 6 weeks, the animals kept on an isoflavone-low diet (ILD) showed a decrease in isoflavone plasma levels, with a mean value of 23.1 ng/ml daidzein and non-detectable levels of genistein (< 20 ng/ml). After12 weeks, the ILD animals presented non-detectable levels for both isoflavones analysed. As expected, the ILD animals presented a decline in their isoflavone plasma levels, since the diet was changed from a Ssniff Standard diet (~ 400 µg total isoflavone/g feed) to an isoflavone-low diet (< 10 µg isoflavone/g feed, see Table 18).

The animals fed with an isoflavone-rich diet (IRD) showed a less pronounced decrease in isoflavone plasma concentrations: 100.3 ng/ml genistein and 93.6 ng/ml daidzein after 6 weeks and 78.4 ng/ml genistein and 63.8 ng/ml daidzein after 12 weeks of changing the diet, although no decline was expected at all, since these animals were kept on a Ssniff Standard diet (~ 400 µg total isoflavone/g) and then the diet was changed to the IRD (472 µg total isoflavone/g chow).

The data of plasma levels in all the groups of animals presented considerable variations among the animals of the same group, due to interindividual biological differencies (the coefficients of variation of the analytical method, at a concetration of 100 ng/ml, were only 6.9 % for daidzein and 4.7 % for genistein, see Tables 10 and 11).

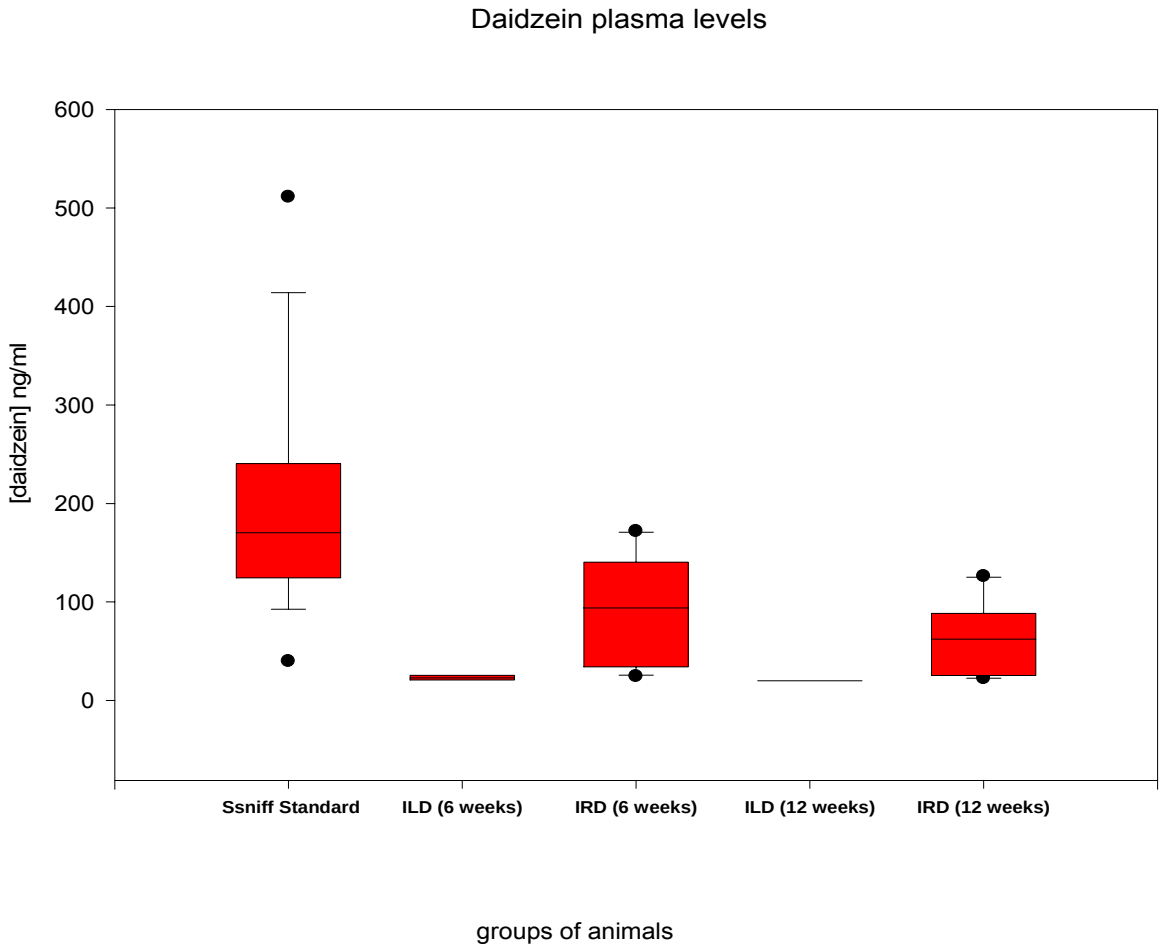


Fig. 23. Box Plot representation of the analysis of daidzein plasma concentrations in Wistar rats from a bioassay with oral administration of daidzein in the food. The median, the upper and lower quartiles as well as the outliers are represented. "Ssniff Standard" represents the daidzein plasma levels for all animals fed with standard rodent chow before changing diet. "ILD" represents the daidzein plasma levels for those animals on an isoflavone-low diet after 6 or 12 weeks. "IRD" represents the daidzein plasma levels for those animals on an isoflavone-rich diet after 6 or 12 weeks. This last group includes the "Phyto runners" and "Phyto control" groups. (Data from Table 19).

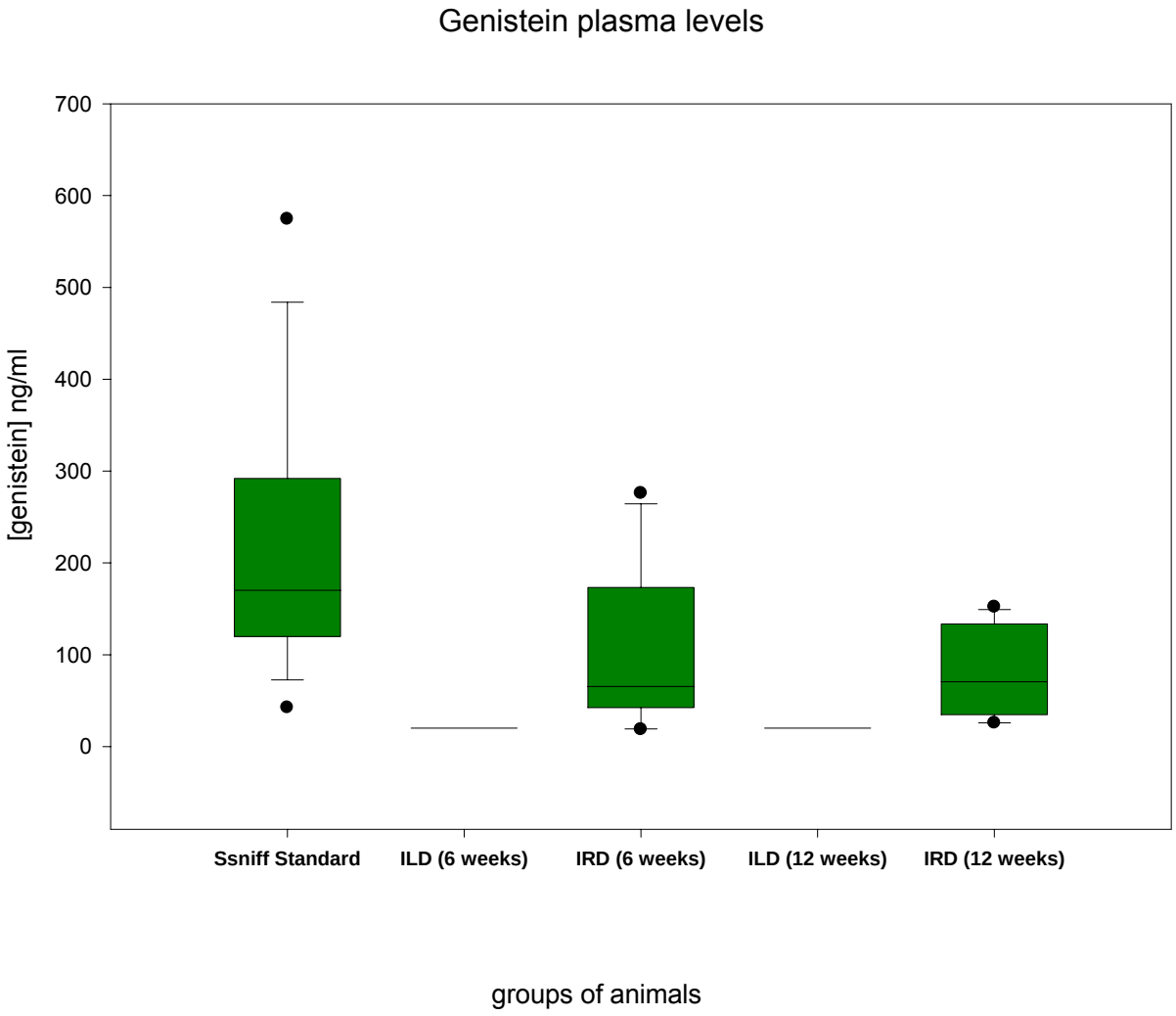


Fig. 24. Box Plot representation of the analysis of genistein plasma concentrations in Wistar rats from a bioassay with oral administration of daidzein in the food. The median, the upper and lower quartiles as well as the outliers are represented. “Ssniff Standard” represents the genistein plasma levels for all animals fed with standard rodent chow before changing diet. “ILD” represents the genistein plasma levels for those animals on an isoflavone-low diet after 6 or 12 weeks. “IRD” represents the genistein plasma levels for those animals on an isoflavone-rich diet after 6 or 12 weeks. This last group includes the “Phyto runners” and “Phyto control” groups. (Data from Table 19).

### *3.2.2. Dose-response assessment of the genotoxicity of isoflavones*

#### *3.2.2.1. Assessment of cytotoxicity*

Genotoxicity may result from specific toxic processes, but also from secondary events due to cytotoxicity. An assessment of cytotoxicity is needed for interpretation of genotoxicity results. Only if genotoxicity is found at concentrations that do not cause overt cytotoxicity, such genotoxicity response is considered positive and biologically significant.
The cytotoxicities of genistein, daidzein and equol were examined here using Neutral Red uptake and BCA assays, in order to assess the ranges of concentrations suitable for genotoxicity assays.

##### *3.2.2.1.1. Cytotoxicity of daidzein in V79 cells*

For the Neutral Red assay, concentrations of daidzein were chosen from 0 (solvent control) to 150 μM daidzein. Several sets of experiments were performed using a 96-well plate. A pre-condition was a sufficient cell growth during 24 h before the test substance was added as DMSO solution. The assessment was carried out considering the Neutral Red absorbance values of the growth control and the solvent control rows in the 96-well plate (see Fig. 11); the absorbance of the first measurement reflects the number of initial cells seeded, and the absorbance of the second one reveals the number of cells at the end of the experiment that did not receive an isoflavone treatment (See table 20 below). Figure 25 shows the data of one of these experiments (Assay No 3 in Table 20); each point represents the mean of n= 8 ± SD (8 wells for each concentration). Within the concentration range tested for daidzein (25-150 μM), a very slight reduction of Neutral Red uptake was observed compared to the control, resulting in an inhibition of 20 % at 100 μM ($IC_{20}$ = 100 μM).
The more sensitive BCA assay was applied using a concentration range up to 150 μM. In a typical experiment on one 96-well plate (Fig. 26 and Assay No 3 in Table 20) there was a reduction in the cell protein content of about 20 % at 95 μM daidzein ($IC_{20}$ = 95 μM), and a reduction of about 30 % at 150 μM daidzein.

This demostrates that daidzein does not cause an overt cytotoxicity in V79 cells at concentrations up to 100 µM. The positive and dose-dependent genotoxicity response of daidzein (see section 3.2.2.2.) can not be attributed to unspecific cytotoxicity.

| Assay No | Compound<br>Conc. Range (µM) | DMSO %v/v | Controls<br>mOD (NR) | Controls<br>prot mg/ml (BCA) | IC20 (NR) | IC20 (BCA) | Solvent effect |
|---|---|---|---|---|---|---|---|
| No 1 | daidzein<br>25-150 | 0.15 | M = 0.48<br>S = 0.42 | M = 1.28<br>S = 0.89<br>I = 0.52 | >150µM | ≈ 25µM | ▼ ▼ |
| No 1 | genistein<br>5-100 | 0.1 | M = 0.45<br>S = 0.39 | M = 0.69<br>S = 0.64<br>I = 0.52 } 25% | >100µM | >100µM | ▼ |
| No 2 | daidzein<br>25-150 | 0.15 | M = 1.16<br>S = 1.16 | M = 1.70<br>S = 1.39<br>I = 0.59 | >150µM | 100µM | ▼ |
| No 2 | genistein<br>5-100 | 0.1 | M = 1,11<br>S = 1,05 | M = 1.93<br>S = 1.83<br>I = 0.75 } 145% | >100µM | ≈ 75µM | ▼ |
| No 3 | daidzein<br>25-150 | 0.15 | M = 0.75<br>S = 0.68 | M = 1.85<br>S = 1.68<br>I = 0.65 | 100µM | ≈ 95µM | ▼ |
| No 3 | genistein<br>5-100 | 0.1 | M = 0.71<br>S = 0.73 | M = 1.61<br>S = 1.59<br>I = 0.37 } 330% | >100µM | ≈ 20µM | ▼ |
| No 4 | equol<br>5-100 | 0.1% (5-50µM)<br>0.2% (100µM) | M = 0.35<br>S (0.1%) = 0.33<br>S (0.2%) = 0.32 | M = 0.71<br>S (0.1%) = 0.87<br>S (0.2%) = 1.06<br>I = 0.30 | ≈ 100µM | ≈75µM (IC50) | S (0.1%) ▲<br>S (0.2%) ▲ ▲ |

Table 20. Overview of the cytotoxicity assays. M, medium control; S, solvent control; I, initial cells seeded; mOD, mili optical density

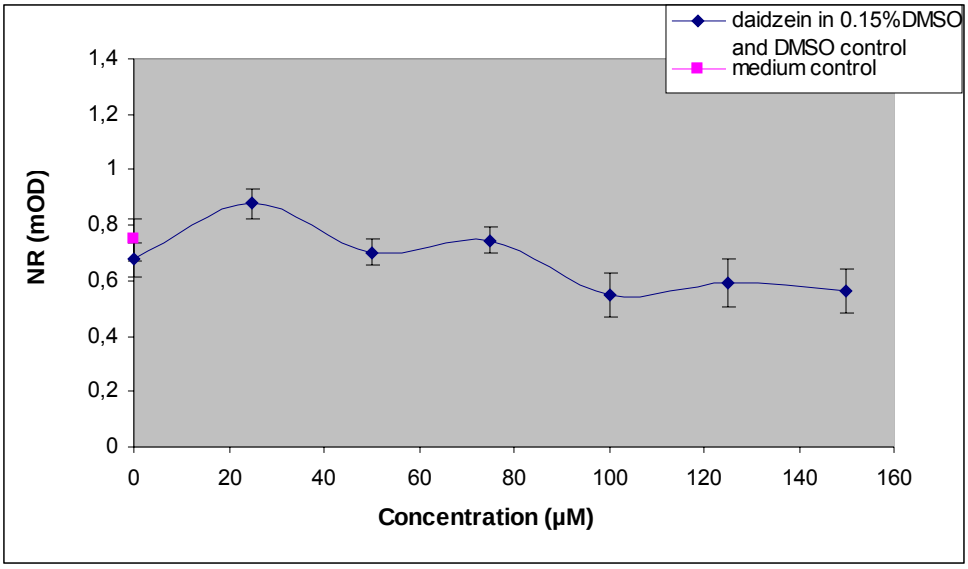


Fig. 25. Neutral Red assay for daidzein.

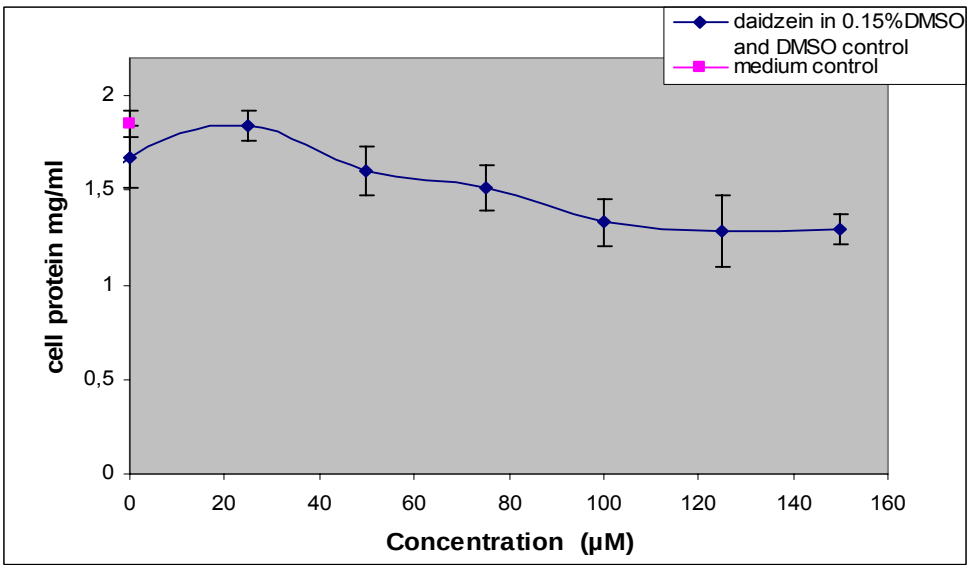


Fig. 26. BCA assay for daidzein.

### *3.2.2.1.2. Cytotoxicity of genistein in V79 cells*

Similar to the experiments on daidzein, several sets of experiments were performed for genistein using a 96-well plate, but only those were considered in which there was good cell growth during the previous 24 h before the substance was added (three set of experiments). Figure 27 shows one of the experiments (with n = 8 for each data point) for the Neutral Red

assay. In the case of genistein the highest concentration tested was 100 µM. There was no cytotoxicity, according to the Neutral Red assay, over the dose range studied ($IC_{20}$ > 100 µM). Hence, the more sensitive BCA assay was applied using a concentration range up to 100 µM genistein. In this case there were again 3 experiments where the cell growth was suitable for the cytotoxicity analysis. The respective data for the BCA assays are shown in Table 20. In the first experiment (Assay No1) there was only little cell growth (25 % comparing initial cells seeded and cells treated with medium), and there was no cytotoxicity in the dose range studied ($IC_{20}$ > 100 µM). In the second study (Assay No2), the cell growth was up to 145 % and showed an $IC_{20}$ ≈ 75 µM, and in the last example (Assay No 3) the cell growth was even higher (330%) and the cytotoxicity was more pronounced, resulting in an $IC_{20}$ of 20 µM. The data of this last experiment are depicted in Fig. 28, which also shows that the cell protein contents decrease further when the concentration of genistein increases ($IC_{50}$ ≈ 50 µM).

These results demonstrate that, using the BCA assay, there was some cytotoxicity caused by genistein in V79 cells at concentrations that did not cause reduction in the Neutral Red uptake. As the genotoxicity of genistein in the micronucleus assay starts already at a concentration of 5 µM (see section 3.2.2.2.), where no overt cytotoxicity can be found, this genotoxicity must be assessed as being clearly positive.

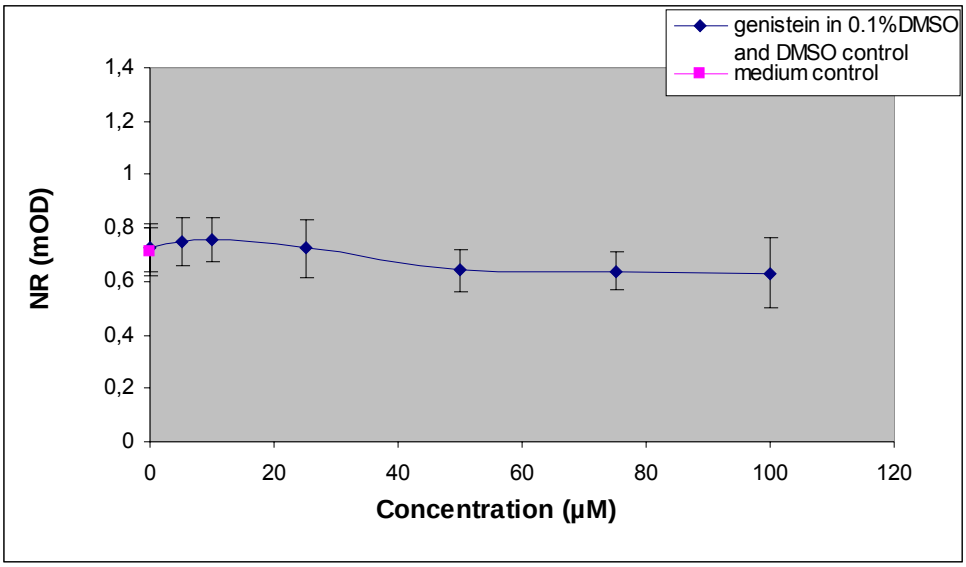


Fig. 27. Neutral Red assay for genistein.

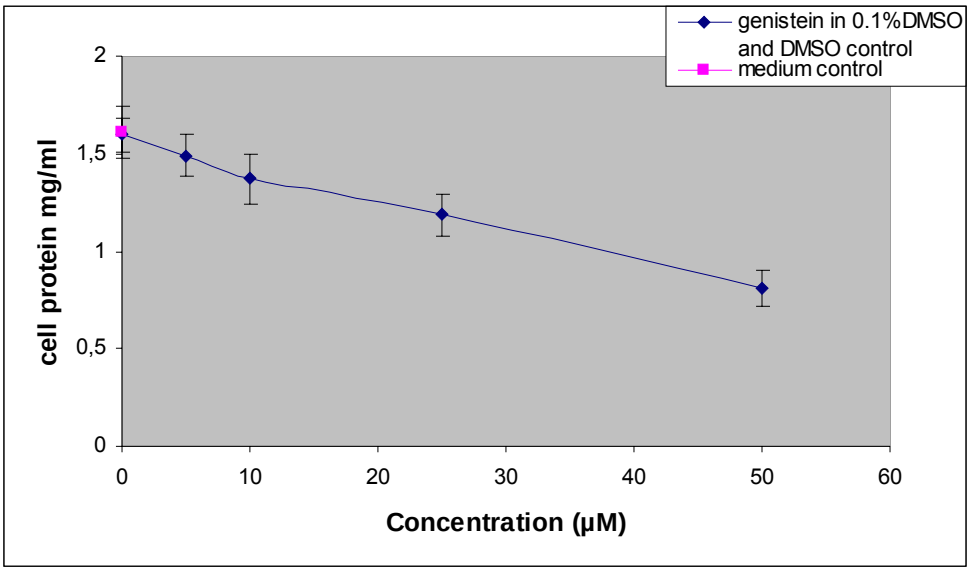


Fig. 28. BCA assay for genistein.

*3.2.2.1.3. Cytotoxicity of equol in V79 cells*

In the Neutral Red assay for equol, concentrations were chosen from 0 (solvent control) to 100 µM equol. Several sets of experiments were performed for this compound using a 96-well plate, and we took into account only those in which there was good cell growth during the previous 24 h before the substance was added. Figure 29 shows the data of such an experiment (with n = 8 ± SD for each data point). There was no overt cytotoxicity over the dose range studied, but a tendency of the Neutral Red uptake to decline when equol concentration increased ($IC_{20} \approx 100$ µM). It is necessary to underline that the treatment of V79 cells with 100 µM equol produced a final concentration of DMSO in the culture medium of 0.2 % (v/v), although this did not alter the Neutral Red uptake (see Table 20).
On the other hand, the BCA assay for equol shows some reduction in the cell protein amount of up to 50 % at 75 µM equol ($IC_{50} = 75$ µM) (depicted in Fig.30).
At concentrations of 20-25 µM equol, which appear slightly positive in the micronucleus test (see section 3.2.2.2.), no overt cytotoxicity is apparent.

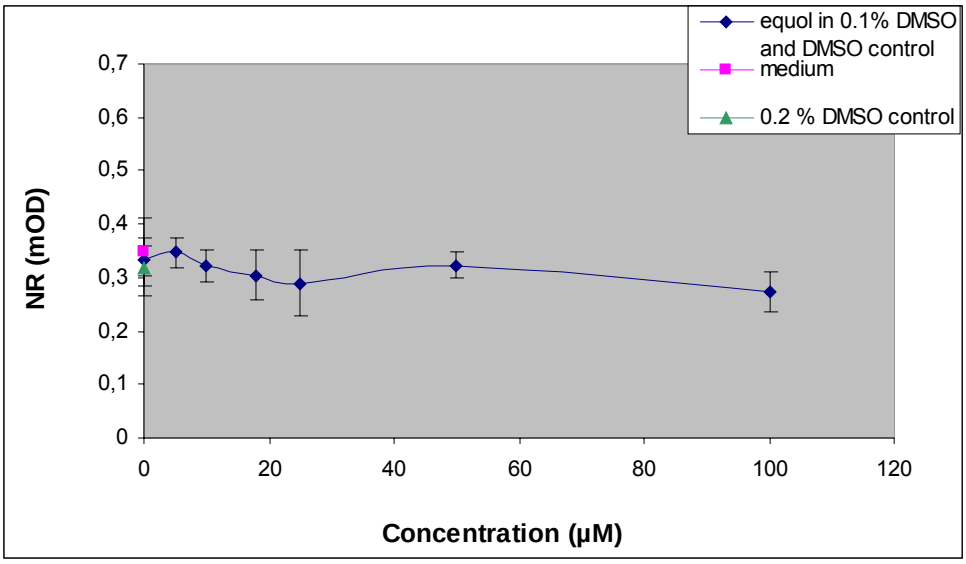


Fig. 29. Neutral Red assay for equol.

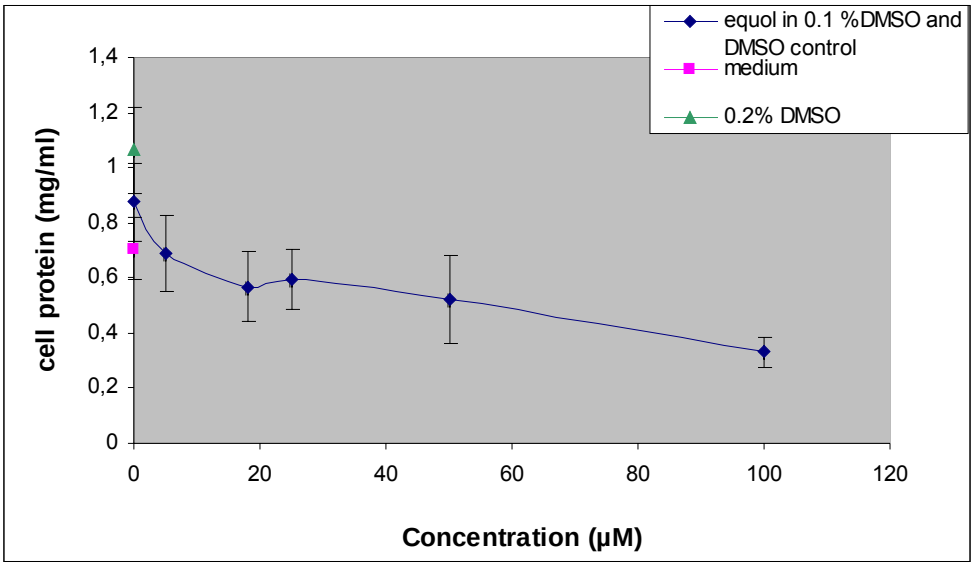


Fig. 30. BCA assay for equol.

*3.2.2.2. Induction of micronuclei*

Typical pictures of micronuclei in V79 cells are presented in Fig. 31: a micronucleus (MN) induced by MMS, a typical clastogen, is shown in the left panel, and one induced by VCR, an aneugen, is shown in the right panel. In general, clastogens, causing chromosome breaks, produce smaller micronuclei than aneugens that influence the distribution of whole chromosomes.

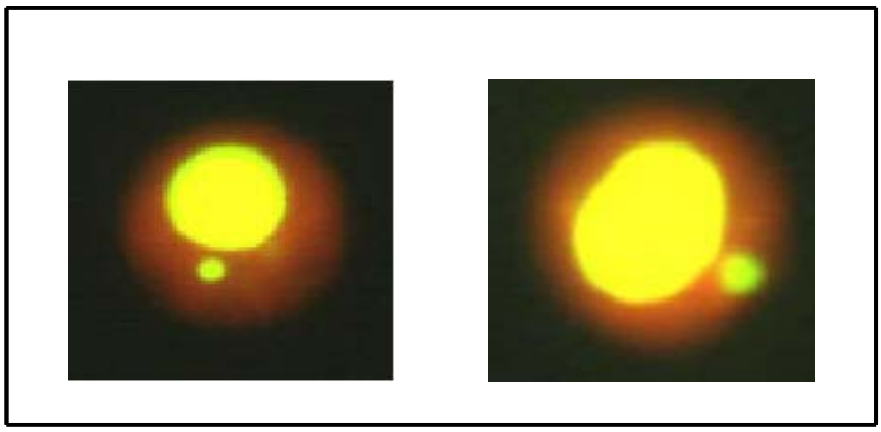

Fig. 31. Main nuclei and typical micronucleus, induced by MMS (left panel) at a 50 μg/ml and induced by VCR (right panel) at 10 nM.

The dose range chosen to assess the genotoxicity of daidzein with the Micronucleus assay in V79 cells (up to100 μM) was not associated with pronounced cytotoxicity (see *3.2.2.1.1.*). Figure 32 depicts a typical cell containing a micronucleus, induced by daidzein at 100 μM. Figure 33 shows the result for daidzein-treated cells; each data point is the mean of 6 individual evaluations. The medium and solvent (DMSO) controls are also depicted. There was a shallow increase of the number of the micronuclei with increasing concentrations of the test compound. At 25 μM, there was no significant increase in MN formation. Only at 75 μM, the MN frequency was doubled compared to the control and increased further at 100 μM. Thus, the genotoxicity of daidzein, in terms of the micronucleus assay results, can be assessed as weakly positive.

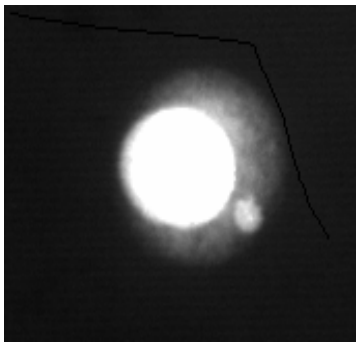

Fig. 32. Micronucleus induced by daidzein at 100µM.

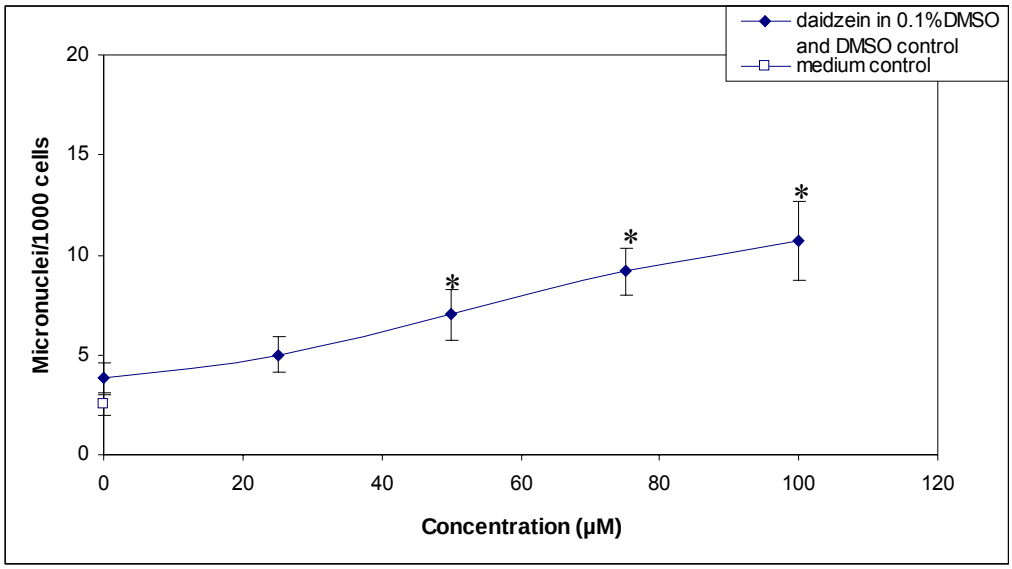


Fig. 33. Micronucleus induction by daidzein. V79 cells were treated for 18 h with different concentrations of daidzein and the MN frequency was determined as described in Section 3.1.5.3.2. MN rates significantly different ($P \leq 0.01$, using Student´s t-test) from the solvent control are marked with an asterisk.

Likewise, the genotoxicity of genistein was assessed, Fig. 34 depicts a typical cell containing a main nucleus and a micronucleus, induced by genistein at 50 µM. Figure 35 shows the dose-response curve. There is a steep increase in the number of micronuclei induced by genistein, up to a concentration of 25 µM, resulting in more than a triplication of the background (control) values. Beyond the concentration of 25 µM the number of micronuclei decrease. The reason for this "bell-shaped" dose-response curve for genistein must be related to the cytotoxicity of genistein: the decrease in cell viability/proliferation is related to the observed decrease in MN formation at higher genistein concentrations. This was demostrated by the reduction of cell protein by 50% at concentrations of 50 µM (Fig. 28).

Compared with daidzein, the genotoxicity response to genistein in the low dose range, up to 25 µM, was much more pronounced. In principle, this confirms the preliminary data on genistein genotoxicity by Kulling and Metzler (1997).

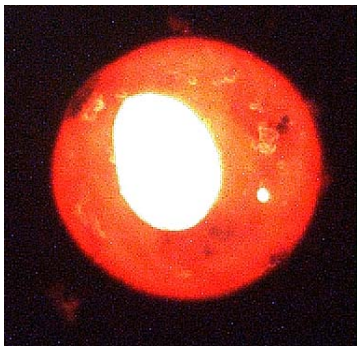

Fig. 34. Micronucleus induced by genistein at 50µM.

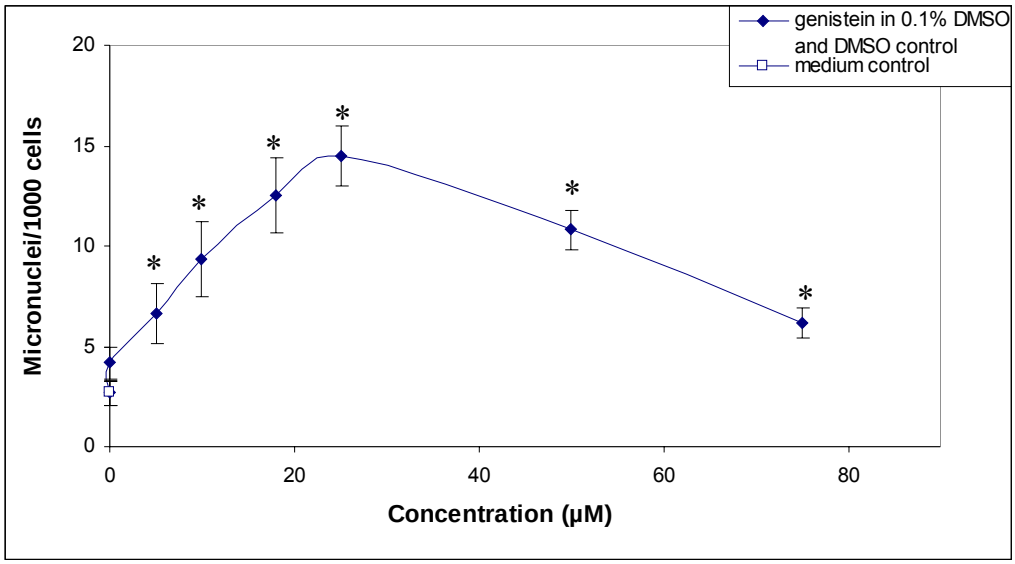


Fig. 35. Micronucleus induction by genistein. V79 cells were treated for 18 h with different concentrations of genistein and the MN frequency was determined as described in Section 3.1.5.3.2. MN rates significantly different ($P \leq 0.01$, using Student´s t-test) from the solvent control are marked with an asterisk.

As shown in Fig.36, treatment with the daidzein metabolite equol resulted in an increase by the factor of 2.5 of the frequency of MN at 25 µM with no further increase at 50 µM in this experiment.

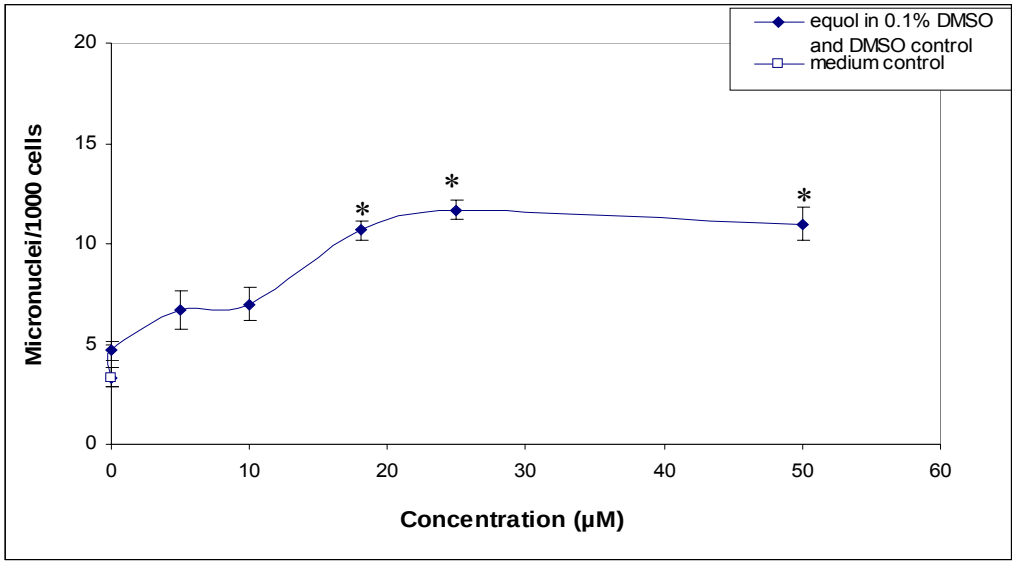


Fig. 36. Micronucleus induction by equol. V79 cells were treated for 18 h with different concentrations of equol and the MN frequency was determined as described in Section 3.1.5.3.2. MN rates significantly different ($P \leq 0.01$, using Student´s t-test) from the solvent control are marked with an asterisk.

Figure 37 shows a typical micronucleus induced by equol at 25 µM.

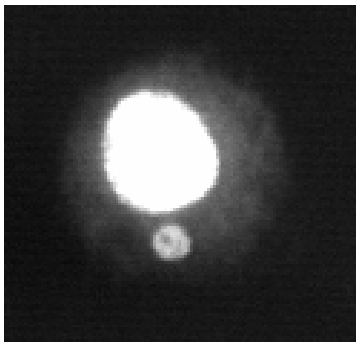

Fig. 37. Micronucleus induced by equol at 25 µM.

### *3.2.2.3. Assessment of microtubule gliding*

An important target for chemicals exhibiting a chromosomal genotoxicity is the microtubular network of cells. Interactions of chemicals with cytoskeletal proteins like tubulin may lead to chromosomal genotoxic damage. Micronuclei may be a consequence of disturbed microtubule assembly during mitosis (aneugenicity). The effects of daidzein and genistein on the

functional activity of the motor protein kinesin were examined in a cell-free assay by measurement of kinesin-driven motility: the gliding velocity of microtubules along immobilized kinesin molecules was not affected neither by daidzein nor genistein at 100 or 500 µM, compared to the respective controls (Fig. 38). This implies that these two compounds do not possess aneugenic properties related to disturbed motor protein functions.

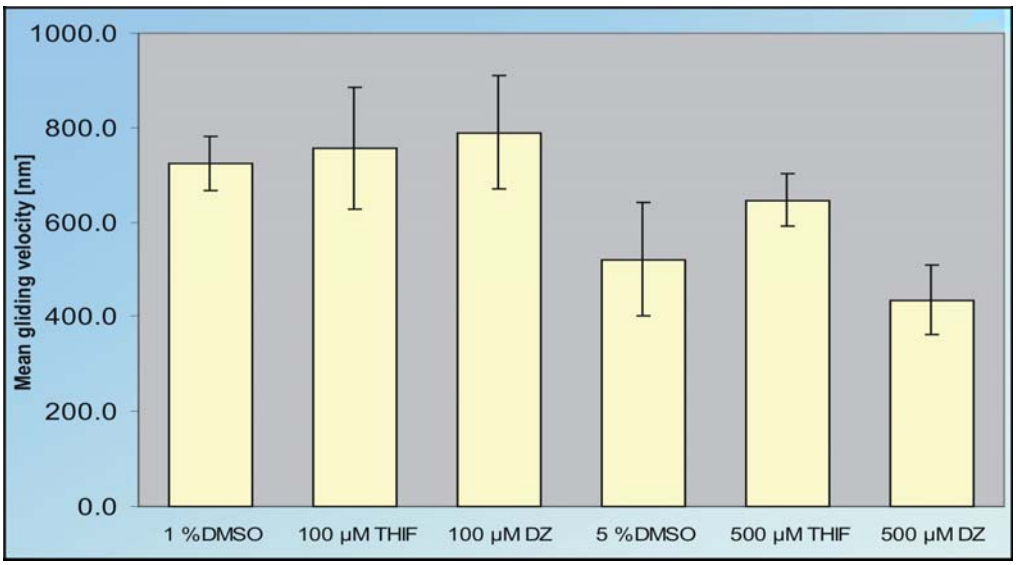


Fig. 38. Gliding assay results for daidzein (DZ) and for genistein (THF) at 100 and 500 µM. Solvent control was carried out as well at 1 and 5 % DMSO.

### *3.2.2.4. Characterization of micronuclei by CREST analysis*

The CREST analysis was performed to discriminate between clastogenic and aneugenic effects of the test compounds. This analysis was done for two positive controls as well: MMS (clastogen) at 50 µg/ml and VCR (aneugen) at 10 nM. Figure 39 shows the picture of the CREST-positive control VCR (an aneugenic compound), which gives rise to kinetochore-containing micronuclei.

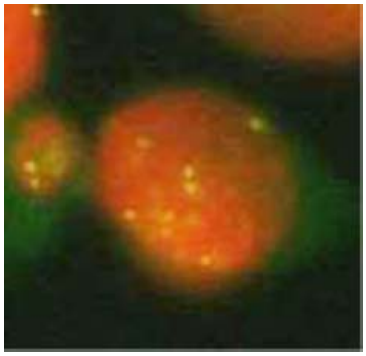

Fig. 39. Main nucleus and typical micronucleus (left), induced by VCR at 10 nM.

Figure 40 shows the quantitative results, each data point being the mean of 3 individual evaluations. Two positive controls (MMS, VCR), as well as medium and DMSO controls were also carried out. The concentrations used in this assay were 20 μM genistein, 100 μM daidzein, 25 and 50 μM equol. The concentrations for CREST analysis were chosen at the maximum of the dose-response curves in the standard micronucleus assay.

Genistein clearly induced mostly CREST-negative micronuclei, i.e. micronuclei containing only chromosomal fragments. This pattern of micronuclei induced by genistein resembles that seen with MMS, a known clastogen. This implies that genistein acts as a clastogenic compound.

Daidzein induced partly CREST (+) and CREST (-) micronuclei, and although daidzein slightly raised the frequency of micronuclei, it did not change the distribution of CREST (+) and CREST (-) micronuclei compared to the solvent control. These results are in agreement with the negative gliding assay outcome.

On the other hand, equol at 50 μM induced micronuclei with a pattern resembling that of VCR (a known aneugen). The equol effect was more pronounced at 50 μM in this experiment.

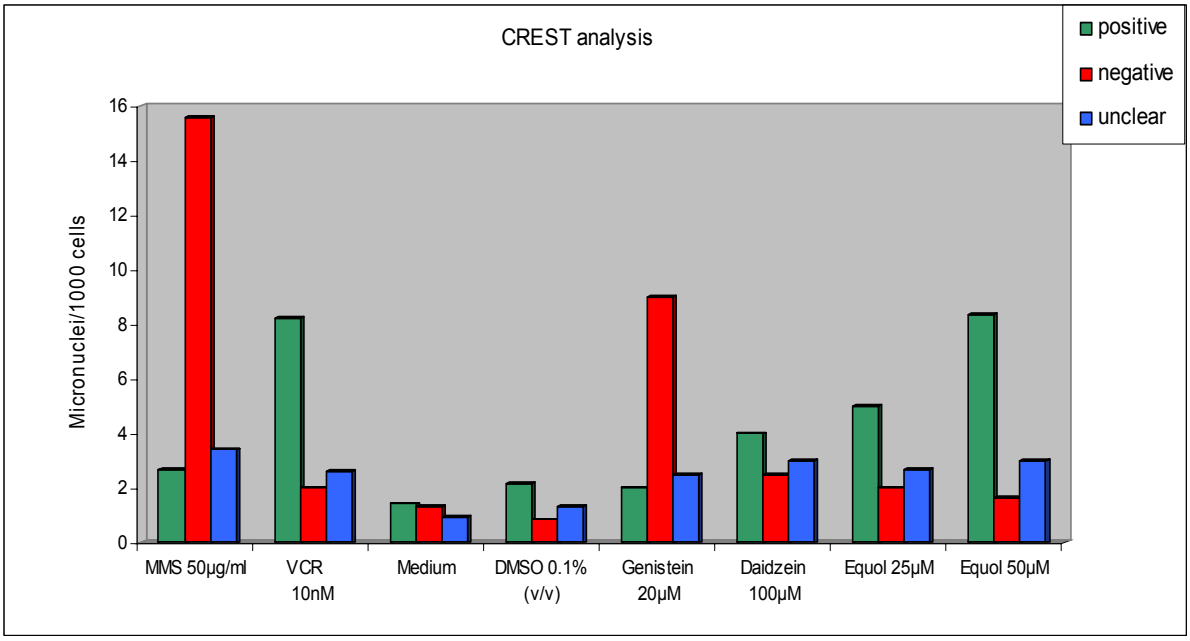


Fig. 40. CREST analysis of micronuclei (MN). V79 cells were treated for 18 h with 20 µM genistein, 100 µM daidzein, 25 µM and 50 µM equol. The presence of kinetochor-protein in micronuclei (MN) was analysed with CREST antibodies and immunofluorescence dyes. MN were classified as CREST positive, negative or unclear as described in section 3.1.5.5.

### *3.2.2.5. Induction of DNA damage assessed by the COMET assay*

The micronucleus assay reveals certain types of (chromosomal) damage, such as clastogenic and aneugenic effects. The COMET assay covers a different spectrum of DNA damage: in the "neutral" version, mostly double strand breaks, in the "alkaline" version also single strand breaks and alkali labile sites become apparent. Under alkaline conditions, DNA loops containing breaks loose supercoiling, unwind and are released from the nucleus and form a "comet-tail" after gel electrophoresis. DNA strand breaks are thus visualised by the COMET assay and can be quantified by analysing the average length of the cellular comet tails in each experiment.

The COMET assay with daidzein (up to 100 µM) and genistein (up to 50 µM) was carried out for 3, 12 and 18 hours and with equol (up to 50 µM) for 3 h in the first set of experiments. In the second one, the three compounds were studied in the concentration range from 50 to 200 µM for 3 h. In both cases, the DNA migration was evaluated through the tail length. Figure 41 shows the results of the first set of experiments, Fig. 41 A shows the outcome for daidzein:

100 cells (2 slides) were analysed for each substance concentration, each column length represents the mean, and the bars the standard deviation. There is a weakly positive trend in the tail length with increasing concentrations of daidzein up to 75 and 100 μM, compared with the controls for 12 and 3 hour treatment. It can be also observed that the mean tail length obtained after 18 hours of daidzein exposure (up to 75 μM) is smaller than that obtained after 12 h, indicating that there might be DNA repair after 12 h, although the standard deviations are large. Giannotti et al. (2002) suggested that strand breaks are too short-lived to allow detection after long-term treatment, due to preferential repaired. It should be noted that daidzein is a relatively lipophilic compound with an octanol-water partition coefficient of 2.51 (Rothwell et al., 2005). It can therefore quickly enter the cell, resulting in possible DNA damage, which could then be repaired. Nevertheless, misrepairing of the initial lesions, as strand breaks, may lead to the chromosome aberrations observed in the in vitro micronucleus test (Pfeiffer et al., 2000).

Figure 41 B shows the results for genistein; in this case there is no clear increase in tail length in any case. The result for equol is plotted in Fig. 41 C, where a positive but faint tendency can be observed.

In the second set of experiments, the compounds were analysed up to higher concentrations (200 μM), and the period treatment was 3 h. Figure 42 shows the outcome of this assay for the phytoestrogens tested. Only genistein displays a weakly positive tendency in the tail length up to 200 μM. Thus, in comparison to the micronucleus assay, the COMET assay seems to be less sensitive in identifying isoflavone-induced damage.

Overall, only little DNA damage has been detected in the COMET assay under the conditions described (single and double strand breaks).

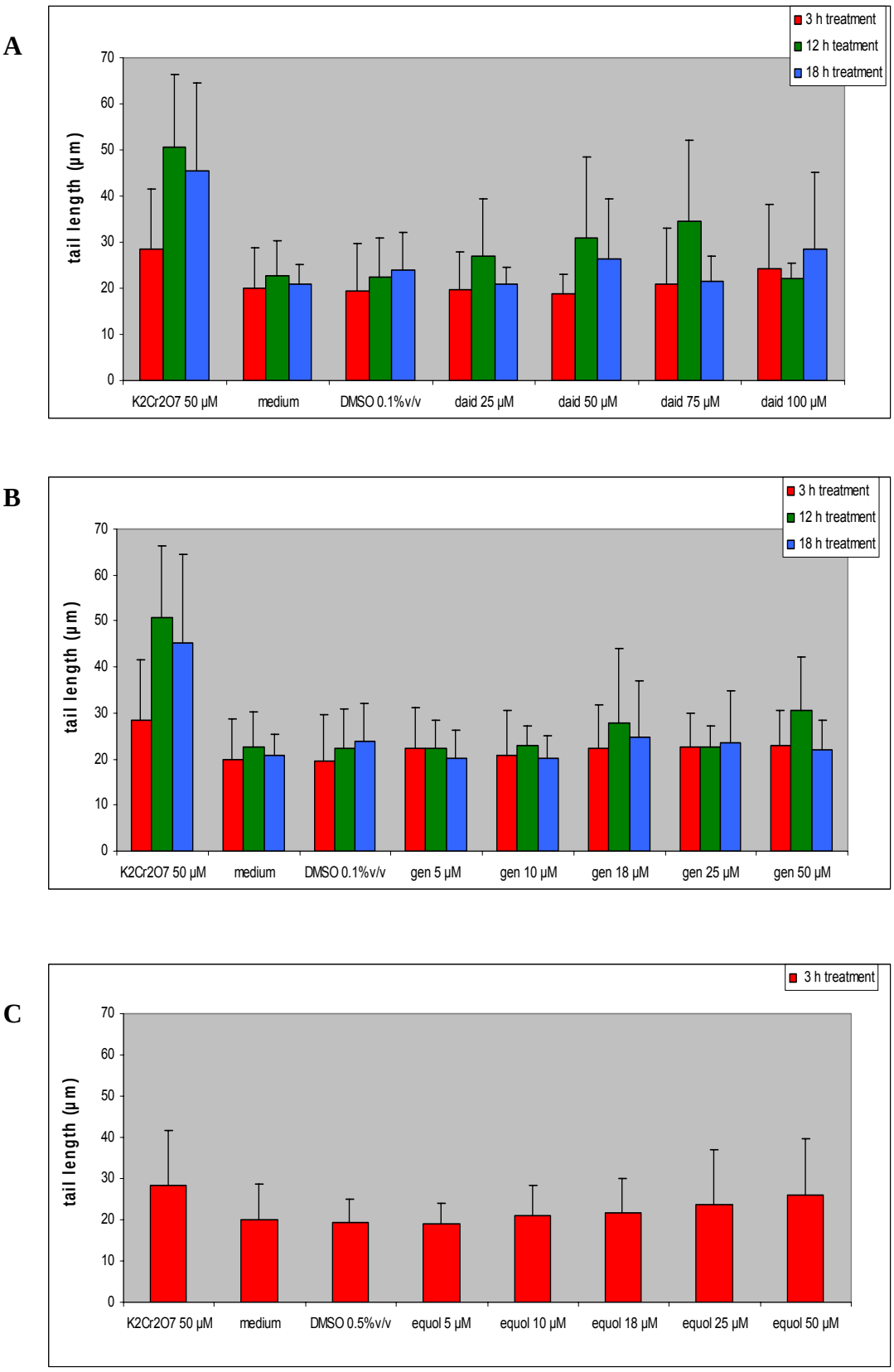


Fig. 41. Induction of DNA damage (strand breaks) by daidzein (A), genistein (B) and equol (C). V79 cells were incubated with different concentrations of the isoflavones for 3, 12 and 18 hfor daidzein and genistein, and 3 h for equol, then the DNA strand breaks were analysed by single cell electrophoresis (COMET assay).

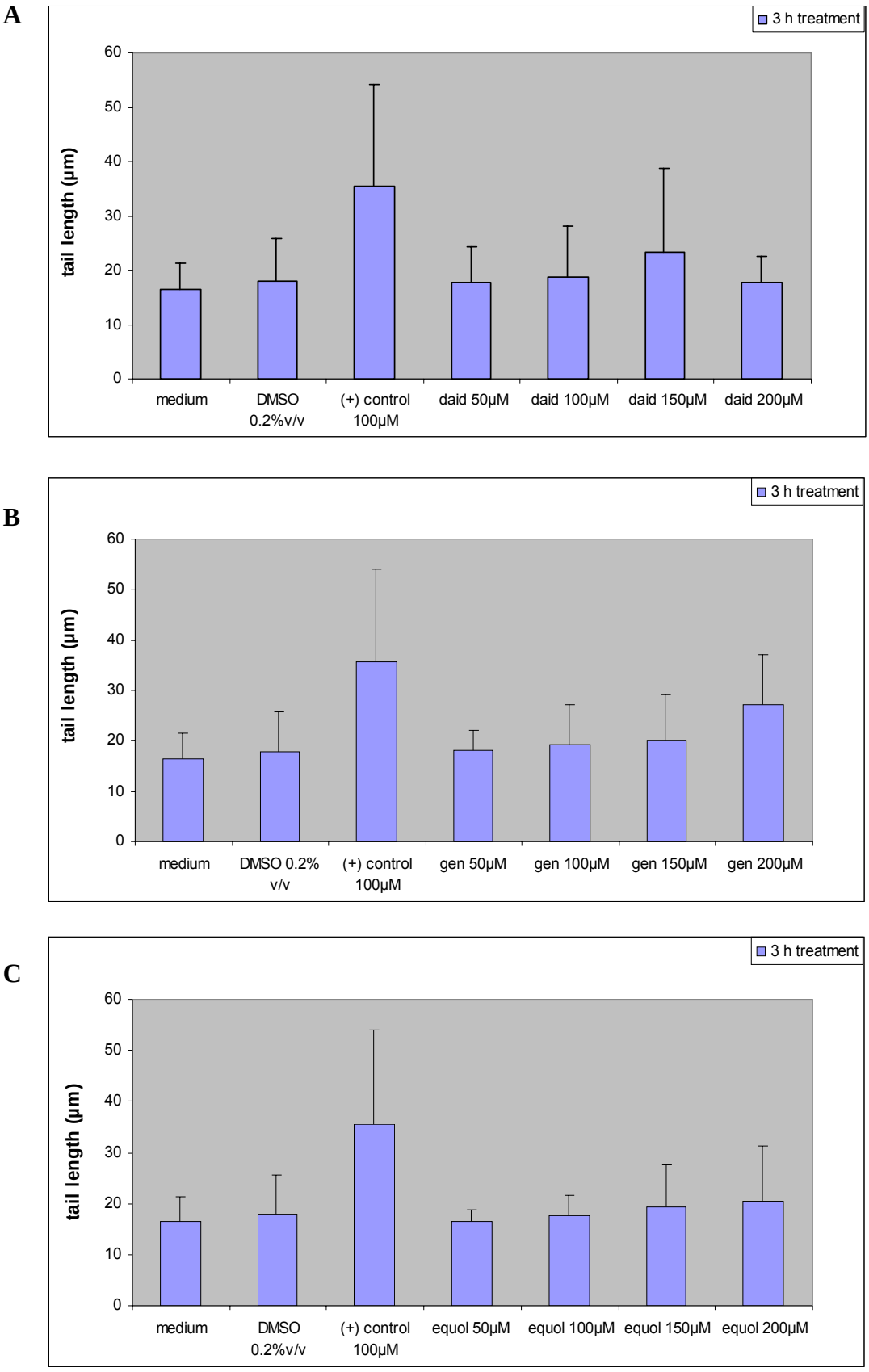


Fig. 42. Induction of DNA damage (strand breaks) by daidzein (A), genistein (B) and equol (C). V79 cells were incubated with different concentrations of the isoflavones for 3 h, then the DNA strand breaks were analysed by single cell electrophoresis (COMET assay).

In support, a series of COMET assays performed in Prof. Kahl´s laboratory in Düsseldorf showed that genistein displays a clear clastogenic action, detectable by the COMET assay, only at 250 µM while at 100 µM no comets were observed (Fig. 43). The DNA damage detected at very high concentrations of genistein (above 100 µM) may be due to secondary events, related to overt cytotoxicity, and present a possibility to explain the bell-shaped dose response curve of genistein in the micronucleus assay as explained in section 3.2.2.2. In fact, the primary disadvantage of the COMET assay is its low specificity that leads to a large number of false-positives (Lee et al., 2003). This low specificity of COMET assay may be associated with cytotoxicity.

Another possibility to explain the bell-shaped dose-response curve in the MN assay relates to antioxidant properties of genistein and related isoflavonoids (Arora et al.,1998; Mitchell et al., 1998). If genistein acts as an antioxidant, it may protect cells (partly) against its own genotoxicity or against oxidative damage induced by other substances. To address this question, the COMET assay was also performed to study whether genistein provides protection against hydrogen peroxide-induced DNA damage. As shown in Fig. 44, genistein at 50 µM did not confer measurable antioxidative protection against hydrogen peroxide.

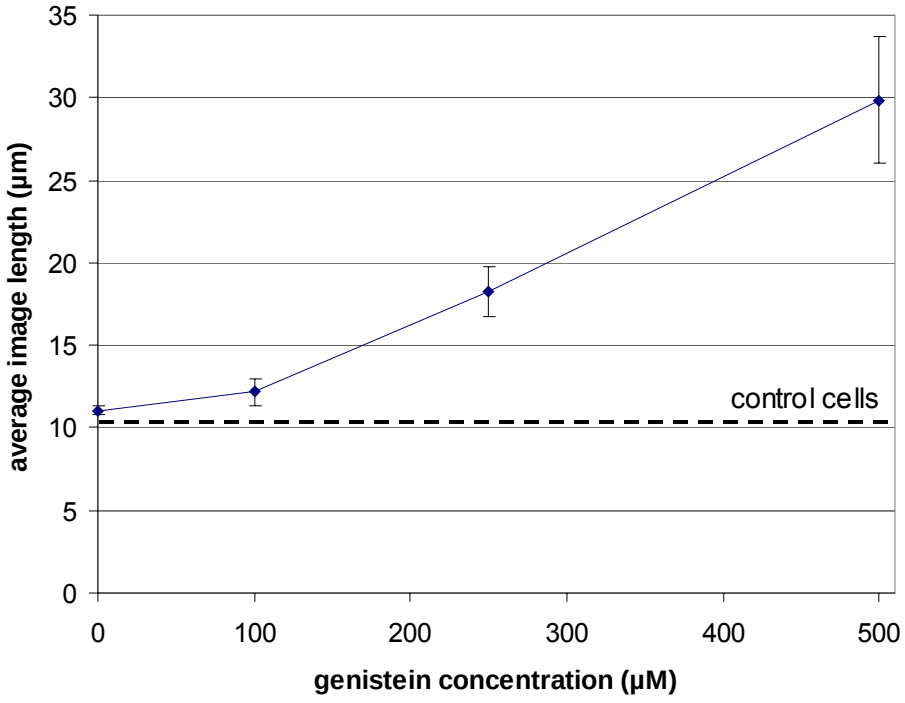


Fig. 43. Induction of DNA damage (strand breaks) by genistein. V79 cells were incubated with different concentrations of the isoflavones for 3 h, then the DNA strand breaks were analysed by single cell electrophoresis (COMET assay). The average image length of 50 cells in µm ± SD is shown representing the extent of genistein-induced DNA damage.

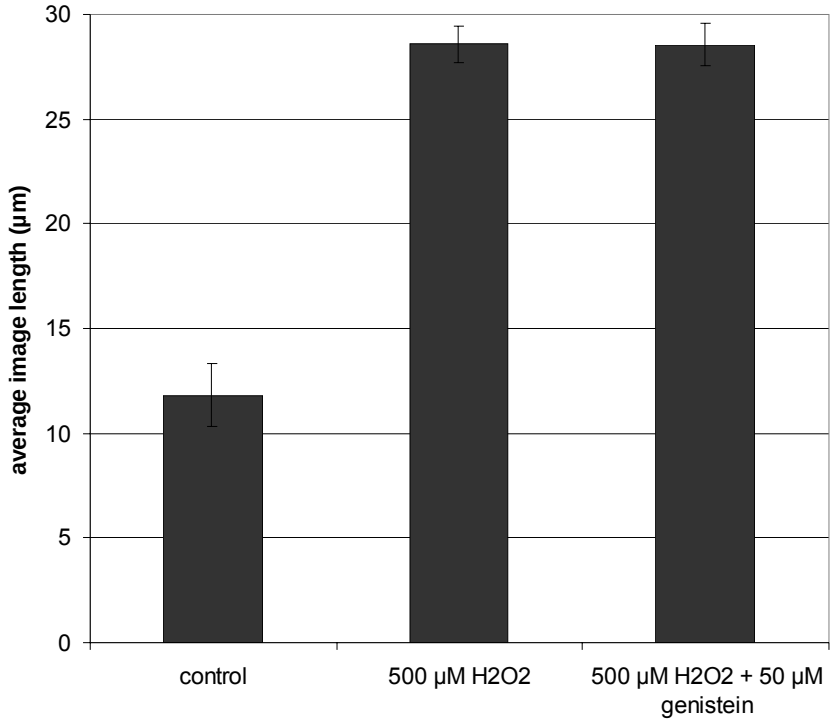


Fig. 44. COMET assay analysis of DNA damage induced by hydrogen peroxide in the absence or presence of genistein. V79 cells were incubated with $H_2O_2$ for 2 h with or without 1 h pre-incubation of genistein (50 µM). DNA strand break formation was assessed with the COMET assay.

## 3.3. Discussion

### *3.3.1. Analytical procedures*

#### *3.3.1.1. Analytical criteria for isoflavones*

The aim of the present thesis work was to set into perspective the contents of the isoflavones daidzein, genistein and the daidzein metabolite, equol, in human diet with the toxicity of these compounds. In order to experimentally study the coherence of dietary exposure with resulting blood concentrations, female Wistar rats were chosen as an animal model. Analysis of the isoflavones daidzein and genistein by microbore HPLC was performed in dietary rodent chow and in rodent plasma.

After suitable preparation of the samples, total isoflavone analysis in rodent diet and in rodent plasma samples was performed with a microbore HPLC system with ultraviolet (UV) detection. This was based on the method of Franke et al. (1994, 1995 and 1998), which was slightly modified for the microbore scale to optimise the solvents used as mobile phase, the flow rate and the gradient with regard to (i) selectivity, (ii) limits of detection, (iii) reproducibility and (iv) recovery rates.

(i) The isoflavones daidzein and genistein in plasma and samples of rodent diet were identified by comparison of the retention times with the respective standards and by spiking with authentic compounds. Figures 17 and 18 show a HPLC chromatograms of rodent diet and plasma samples, respectively, demonstrating adequate separation of the compounds from both matrixes (see retention times in section 3.2.1.1.).

The calibration curves for the compounds analysed from plasma were linear over the concentration range expected for this matrix (25-1500 ng/ml) as well as the calibration curves of rodent diet samples (50-350 ng/ml).

(ii) The limits of detection for daidzein and genistein in both matrixes were previously established using the same methods as presented here and published by Degen et al., 2002a. For dietary analysis, the detection limit was 10 µg isoflavone/g feed and 20 ng isoflavone per ml plasma.

(iii) The overall reproducibility of measurements of daidzein and genistein in plasma and rodent diet using the HPLC method ranged from 2.1- 21.8 % (coefficient of variation for three samples measured on three separate occasions). When genistein was analyzed, the mean CV%

resulted in 11.4% in rodent diet and 10.1% in plasma. Daidzein presented a mean CV% of 9.2% and 6.7%, respectively (see Tables 8-11).
As expected, the magnitude of the variation depended on the concentrations of the individual isoflavones in the samples. In general, lower coefficients of variation were found at higher concentrations of the compounds.
(iv) Experiments conducted to study recovery rates of genistein and daidzein revealed that, even under the best conditions of plasma extration (Coward et al., 1996: 150 mM ammonium acetate, pH 7 and 500 mM TEAS), there was incomplete recovery of isoflavones, but the mean recovery rates were in general above 80%. This was considered acceptable. The recovery rates were not dependent upon the concentration of the analytes in both plama and rodent chow.

These methods have some disadvantages because of the time-consuming hydrolysis and solid-phase extractions of plasma samples and the relative long HPLC runs (45 min for rodent diet and 50 min for plasma samples). However, the analytical procedures employed in this work were well applicable for the determination of isoflavones in biological samples.

*3.3.1.2. Isoflavone concentrations in experimental rodent diets*

In the present experiment, female Wistar rats were fed with 2 different types of rodent diet: (i) an isoflavone-low diet (<10 µg (daidzein + genistein)/g feed), or (ii) an isoflavone-rich diet (mean of 472 µg total daidzein plus genistein/g feed) that resulted in a daily average oral intake of 26.8 mg (total daidzein plus genistein)/kg b.w. The phytoestrogen concentrations analysed in the feed samples agree with published data (Degen et al., 2002a), where the total isoflavone (daidzein plus genistein) concentrations ranged between 100-540 µg/g feed (Table 21).

| Group studied | Diet consumed/Intake* | Plasma concentration | | References |
|---|---|---|---|---|
| | | Daidzein | Genistein | |
| DA/Han rats | 100-540 µg/g feed | 105 ng/ml (0.41 µM) | 99 ng/ml (0.37 µM) | Degen et al., 2002a |
| Wistar rats | phytoestrogen-free diet | < 20 ng/ml | < 20 ng/ml | Degen et al., 2002a |
| Sprague-Dawley rats | 750 µg genistein/g feed | - | 590 ng/ml (2.2 µM) | Santell et al., 1997 |
| Female Sprague-Dawley rats | 60 µg/g | 64 ng/ml (0.25 µM) | 167 ng/ml (0.62 µM) | Doerge et al., 2000 |
| 4 month-old infants | 4.5-8 mg/kg b.w. | 295 ng/ml (1.2 µM) | 684 (2.5µM) | Setchell et al., 1997 |
| Men | Traditional Japanese | 27.2 ng/ml | 74.7 ng/ml | Adlercreutz et al., 1993b |
| Men | Traditional Japanese | total isoflavone 20-600 ng/ml (0.08-2.4 µM) | | Setchell, 1998 |
| Men | Traditional Japanese | 15-230 ng/ml | 24-320 ng/ml | Adlercreutz et al., 1994 |
| Finnish women | Vegetarian | 4.6 ng/ml | 4.6 ng/ml | Adlercreutz et al., 1994 |
| Finnish women | Omnivore | 1 ng/ml | 1.3 ng/ml | Adlercreutz et al., 1994 |
| Men | Omnivore | 1.6 ng/ml | 1.7 ng/ml | Adlercreutz et al., 1993b |
| Premenopausal women | Vegetarian | 15.1 ng/ml | 12.1 ng/ml | Adlercreutz et al., 1993a |
| Premenopausal women | Omnivore | 1.6 ng/ml | 2.5 ng/ml | Adlercreutz et al., 1993a |
| Women | Omnivore (0.436 mg/day) | 2 ng/ml | 4.1 ng/ml | Grace et al., 2004 |
| Prostate cancer patients | 600 mg genistein/day | - | 9.2-10.7 µM | Miltyk et al., 2003 |

Table 21. Compilation of data on isoflavone content in food and the resulted plasma levels, as it is now available. * Intake of total daidzein plus genistein except when it is detailed.

### *3.3.1.3. Isoflavone plasma levels in experimental animals*

In our study animals kept on the "isoflavone-rich" diet with a mean content of daidzein plus genistein of 472 µg/g feed showed mean daidzein and genistein plasma levels of 93.6 ng/ml and 100.3 ng/ml, respectively, after 6 weeks on this diet. By contrast, animals fed with the isoflavone-low diet (<10 µg daidzein + genistein/g feed) showed daidzein and genistein plasma levels below the detection limit of 20 ng/ml.

An analysis of isoflavone plasma concentrations in DA/Han rats on the standard commercial rodent chow Ssniff R/M H, as well as rats which had received daidzein by gavage at a dose of 1 mg/kg b.w., resulted in very similar mean plasma isoflavone levels of 105 ng/ml daidzein and 99 ng/ml genistein (Degen et al., 2002a). In another experiment, Wistar rats, kept on phytoestrogen-free diet, displayed plasma levels of genistein or daidzein below the detection limit of < 20 ng/ml (Degen et al., 2002a, Table 21).

A study on Sprague-Dawley rats on a diet with 750 µg genistein/g feed, presented higher genistein plasma levels of 590 ng/ml (Santell et al., 1997). Another study by Doerge et al. (2000) showed that when female Sprague-Dawley rats were fed with a "soy-free" rodent

diets, containing 60 µg total isoflavones/g feed, the plasma levels in female rats were 64 ng/ml daidzein and 167 ng/ml genistein (Table 21).
Taking into account possible differences in toxicokinetics between different rat stains, the present data are consistent with each other, confirming the coherence between dietary exposure to soy isoflavones and the resulting isoflavone plasma levels.

### *3.3.2. Coherence of isoflavone exposure and plasma levels between experimental animals and humans*

In humans, exposure scenarios to daidzein and genistein present a general pattern similar to that observed in experimental rodents, varying with dietary habits.
The nutritional uptake of these compounds is high in Japan and other East Asian countries where traditionally a soy-rich diet is consumed, as well as in vegetarians in Western countries where the uptake has been estimated to be 50-100 mg/day (Barnes et al., 1990, Adlercreutz et al., 1991, Coward et al., 1993). In Japanese adults or in Westeners on a soy-supplemented diet, resulting plasma levels of total isoflavones (daidzein plus genistein) were found between 40-500 ng/ml (0.15- 1.9 µM; Adlercreutz et al., 1993b; Adlercreutz et al., 1994; Cassidy 1996) or even higher, 20-600 ng/ml (Setchell, 1998, see Table 21).
By contrast, the intake of isoflavones is consistently lower in Westeners on an "omnivorous" diet. In the UK, the intake of isoflavones has been estimated to be < 1 mg/day (Jones et al., 1989), reflected by low isoflavone plasma levels of < 6 ng/ml (< 0.02 µM) (Grace et al., 2004) as well as in omnivorous Finnish women (Adlercreutz et al., 1993a).
Other studies were performed in infants receiving soy-based formulas (the concentration of isoflavones in such "soy milk" ranges between 32-47 µg/ml; Setchell et al., 1997). Exposure to isoflavones of these babies appeared even higher than that found in Asian populations, at a range of 4.5-8 mg/kg b.w. per day, with mean plasma concentrations of 295 ng/ml daidzein and 684 ng/ml genistein.
The question of how human exposure scenarios fit to the dietary exposure data of isoflavones in experimental rodents may be answered as follows. The total isoflavone intake per day for the presently investigated isoflavone-rich rodent diet with mean contents of daidzein of 232 µg/g and of genistein of 240 µg/g is estimated at a daily feed consumption by rats of 20 g of chow. Hence, the calculated daily intake per animal on the isoflavone-rich diet (IRD) was

4.6 mg daidzein and 4.8 mg genistein, or 9.4 mg of total isoflavones. Animals on this isoflavone-rich diet had a mean body weight of 350 g, resulting in a daily intake of 26.8 mg total isoflavone/kg b.w. This dietary isoflavone exposure for rats was even higher than that of humans consuming Asian-style food, and was also higher than that of infants on a soy-based dietary formula ("soy milk").

There is sufficient information on the isoflavone contents of human foods. In the UK, Margaret Ritchie has established a database of isoflavone contents of more than 600 types of foods, and she found that the dietary intakes were clearly reflected by the resulting isoflavone concentrations in the plasma (Ritchie et al., 2004).

According to Ritchie et al. (2004), the correlation between dietary isoflavone intake (x [mg], using duplicate diet analysis) and plasma concentrations (y [ng/ml]) was highly significant: $y = 9.08 x + 13.53$; $r = 0.92$; $P < 0.001$. This was valid within a wide range of intakes, from 0 – 44 mg isoflavone per day.

When this relationship, based on data in humans, is used to predict the plasma concentrations in the rats of the present study (i.e. an exposure level of 26.8 mg isoflavone/kg b.w. or 9.4 mg per day), it results in an expected isoflavone plasma level of 99.2 ng/ml, while the measured total isoflavone plasma concentrations were 193.9 and 142.2 ng/ml after 6 and 12 weeks of dietary exposure, respectively. Considering the biological differences between the species, this demonstrates a similar range of resulting plasma concentrations across the species, which in turn confirmes the validity of animal experiments concerning the toxicokinetics of dietary isoflavones.

### *3.3.3. Genotoxicity*

#### *3.3.3.1. Genotoxic vs. non-genotoxic mechanisms of chemical carcinogenicity*

Chemical carcinogens are substances that cause tumours in humans and/or experimental animals. Part of the chemical carcinogens are "non-genotoxic". In this case, there is no direct interaction of the compound or its metabolites with the genetic material (DNA), and the tumor induction is triggered by other mechanisms, e.g. cell proliferation or receptor-mediated mechanisms. On the other hand, tumours are often induced by genotoxic mechanisms, which can be divided into three main categories:

(i) Substances that produced single point mutations. Example of this type of genotoxic damage are base-pair substitutions, addition or deletion of bases.
(ii) Substances that produce structural chromosomal aberrations. Major changes in structure of chromosomes which are visible under the light microscope. Compounds eliciting such effects are called *clastogens*. Such aberrations arise from breaks, deletions, exchange and rearragement of the chromosomal material during the cell cycle.
(iii) Substances that cause aneuploidy. This term comprises abnormalities in the number of chromosomes. Chemicals which cause aneuploidy are called *aneugens*. Aneuploidy may be the result of the loss of a chromosome during cell division, or of an irregular distribution ("non-disjuntion") of the chromatids over two daughter cells. Potential targets for compounds causing aneuploidy are spindle microtubules, centromeres, centrioles and centrosomes.

The evaluation of the nature of genotoxicity is important for the European classification and labelling of chemicals as carcinogens and as mutagens. The present system of categorization of carcinogens of the European Union used for the labelling of the chemicals implies 3 categories: category 1 comprises compounds of which the carcinogenicity has been proven by *human* epidemiology, in category 2 compounds are listed of which carcinogenicity is based on positive *animal* experimentation, and category 3 includes compounds with a *suspicion* of carcinogenicity.
This system is presently being debated, and a proposal has been made by the Deutsche Forschungsgemeinschaft (Neumann et al., 1998) for modifications, which gives more consideration to modes of action of carcinogenicity.
For matters of standard setting, e.g. for residues of chemicals in food, it is important to know whether a genotoxic/carcinogenic response of a chemical has a threshold or not. This has been discussed very recently on several occasions (Hengstler et al. 2003, Bolt 2003).
In general, genotoxicity that is mediated by direct interaction of the chemical (or metabolites), which is also associated with mutagenicity, is considered non-threshold, and a linear extrapolation towards low doses is commonly applied. By contrast, genotoxic effects on chromosomal aberrations or aneuploidy, due to chemical-protein interactions, may be associated with a threshold. In this case, allowable safe doses may be derived from experimental non-effects levels, by introducing safety margins.
There is almost general agreement that a distinction between genotoxic and non-genotoxic compounds should be made when conducting assessment of cancer risk to humans.

Non-genotoxic carcinogens are characterized by a conventional dose-response that allows derivation of a no-observed-adverse-effect level (NOAEL). Insertion of an uncertainty (or safety) factor permits the derivation of permissible exposure levels at which no relevant human cancer risks are anticipated. The risk assessment approach for non-genotoxic chemicals is generally similar among different regulatory bodies worldwide (Seeley et al., 2001).

For genotoxic carcinogens, Streffer et al. (2004) have suggested several possibilities for assessing carcinogenic risk. Positive data of chromosomal effects only, e.g., aneugenicity or clastogenicity, in the absence of mutagenicity, may support the characterization of a compound that produces carcinogenic effects only at high, toxic doses (Schoeny, 1996). For instance, non-DNA-reactive genotoxicans, such as topoisomerase inhibitors (Lynch et al., 2003) or inhibitors of the spindle apparatus or associated motor proteins (Decodier et al., 2002), are considered in this respect. In these cases, relevant arguments have been put forward in favour of the existence of practical thresholds (Crebelli, 2000; Parry et al., 2000). Sometimes, a practical threshold may be quite low compared to existing environmental or occupational exposures (Thier et al., 2003).

Moreover, genotoxicity (especially when of a local nature) may be relevant only under conditions of sustained local tissue damage and associated increased cell proliferation. Formaldahyde (Morgan, 1997) and vinyl acetate (Bogdanffy and Valentine, 2003) have been noted as examples. In any case, the derivation of practical thresholds and, in consequence, of health-based exposure limits, must be sufficiently justified.

*3.3.3.2. Differentiation of carcinogens by mode of action, for the purpose of risk assessment and standard setting*

These ideas have been taken together and a practical distinction of carcinogens has been proposed, with the following examples (Streffer et al., 2004, as summarized by Bolt et al., 2004; see Fig. 45):

1. *Non-threshold genotoxic carcinogens*; for risk low-dose assessment, the linear non-threshold (LNT) model appears appropiate. Regulations may be based on the principle of as low as reasonably achievable (ALARA), technical feasibility, and other socio-

political considerations. Recent examples include ionisation radiation, vinyl chloride, and diethylnitrosamine (Bolt el al., 2004).

2. *Genotoxic carcinogens, for which the existence of a threshold cannot be sufficiently supported*. In these cases, the LNT model is used as a default assumption, based on the scientific uncertainty and generally backed by the precautionary principle. Recent examples include acrylamide (Dybing and Sanner, 2003), acrylonitrile, and arsenic.
3. *Genotoxic carcinogens for which a practical threshold is supported* by studies on mechanisms and/or toxicokinetics; health-based exposure limits may be based on an established NOAEL. Recent examples include formaldehyde (Morgan, 1997) and vinyl acetate (Hengstler et al., 2003).
4. *Nongenotoxic carcinogens and non-DNA-reactive carcinogens*; for these compounds a true (or "perfect", according to Hengstler et al., 2003) threshold is associated with a NOAEL, and health-based exposure limits are to be derived. Recent examples include tumor promoters, spindle poisons, topoisomerase II poisons, and hormones.

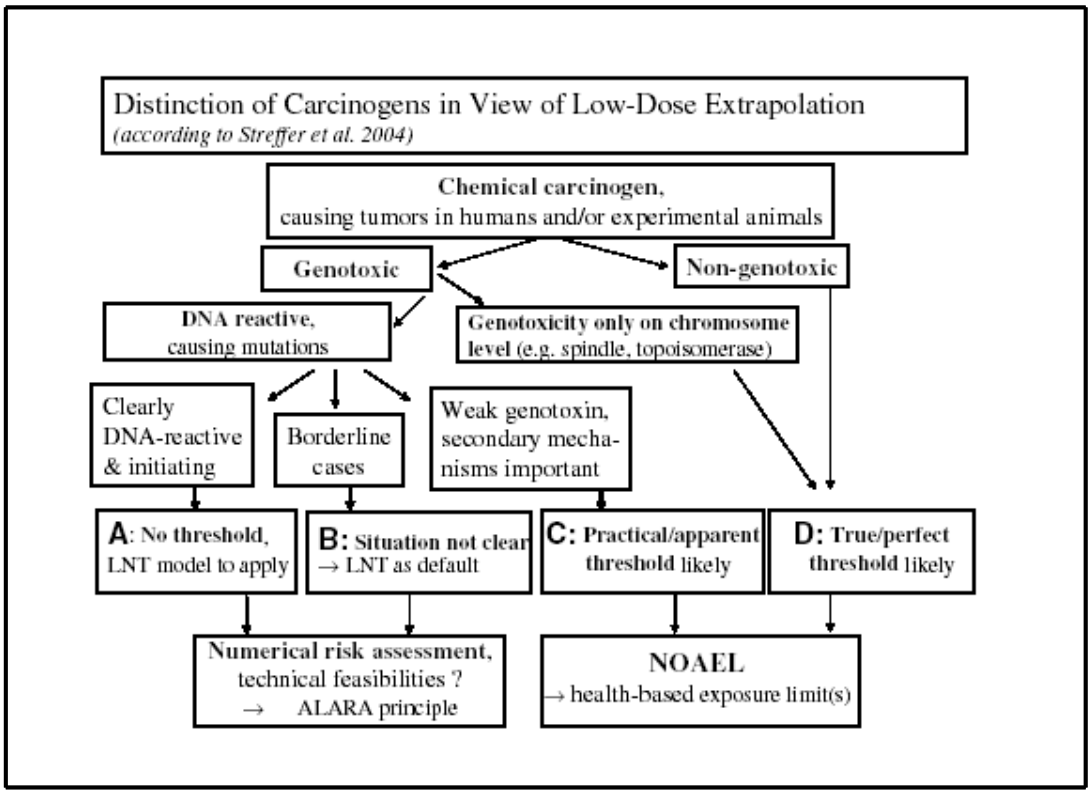


Fig. 45. Proposal of a flow scheme toward risk assessment and standard setting for chemical carcinogens, according to discussions by Streffer et al. (2004).

For phytoestrogens, carcinogenic effects could be due to either genotoxicity, or to hormone-mediated effects on target tissues. According to Streffer et al. (2004), to hormone-mediated effects a clear threshold can be assigned. The same is true for topoisomerase II poisons. Lynch et al. (2003) have discussed the genotoxicity of genistein and have ascribed this to inhibition of topoisomerase II. Inhibition of topoisomerase II could result in DNA double-strand breaks, which is compatible with the present results on clastogenicity of this compound.

As a result of a discussion of this mode of action, Lynch et al. (2003) have proposed a "pragmatic threshold" for clastogenicity of genistein (in L5178Y mouse lymphoma cells) in the order of 1 µg/ml (3.7 µM).

# 4. RISK ASSESSMENT PART

In the assessment of the toxicological risks of chemicals to humans, relevant processes are hazard identification, exposure assessment and risk assessment. For the regulatory handling, also aspects of communication of risks of chemicals with the public are of importance.
For the phytoestrogens, both hormonal and genotoxic effects must be considered, as mentioned in section 3.3.3.2. It has been discussed that thresholds for hormonal effects can clearly be defined, and that thresholds for genotoxicity may be defined under certain conditions.
In the following, this will be further elaborated, focussing on the compounds examined in this thesis.

## 4.1. Hormonal activity of isoflavones

The typical hormonal effects of isoflavones are estrogenic effects at different target organs. Tissue-specific estrogenic responses and underlying molecular mechanisms have been discussed at large by Diel (2002). These receptor-mediated responses, imply that the existence of a threshold, below which no such effects can occur.
Based on the uterotropic response (increase of the weight of the uterus in immature or castrated animals) in different strains of rats, Diel et al. (2000) reported daily doses of daidzein of 10 mg/kg b.w. and below to be without a hormonal effect. In most experimental systems in vivo and in vitro, genistein and daidzein are about equipotent. A sensitive target tissue regarding estrogenic effects is the epithelium of the vagina of castrated (ovariectomized) rats; Fig. 46 shows an example based on the data of Diel et al. (2004). In three different rat strains tested (DA/Han, Sprague-Dawley and Wistar), genistein caused a clear dose-response in the thickness of the vaginal epithelial layer, at daily doses between 25 and 100 mg/kg b.w. A non-hormonal effect dose of 10 mg genistein/kg b.w. daily is compatible with these data. According to the data of the present thesis, this non-hormonal effect dose, when given repeatedly, is related to total genistein blood concentrations (free plus conjugated combined) of about 100 ng/ml plasma, corresponding to a concentration of 0.37 µM.

A worst-case food uptake scenario, presented by the "Senatskommission zur Beurteilung der gesundheitlichen Unbedenklichkeit von Lebensmitteln (SKLM)" of the Deutsche Forschungsgemeinschaft, (1998) was 1 mg/kg b.w. daily, related to total human isoflavone dietary uptake (SKLM, 1998). Consistent with this, plasma levels (total genistein, free plus conjugated) reported in humans on typical European/Western diets were below 10 ng/ml plasma; for East Asia such levels were up to 320 ng/ml (Adlercreutz et al., 1994, Table 21). From these comparisons it appears that hormonal (estrogenic) effects, due to phytoestrogen uptake in the human diet, are not to be expected under European/Western dietary conditions. Both, the uptake and the resulting plasma levels remain about one order of magnitude below the non-hormonal effect level (safety factor for hormonal effects = 10). Only under conditions of very high consumption of soy, as prevailing in the Far East, the non-hormonal effect may in some cases be reached or even slightly exceeded.
When hormonal effects due to dietary intake of isoflavones are avoided, also hormone-mediated carcinogenic effects can consequently be excluded.

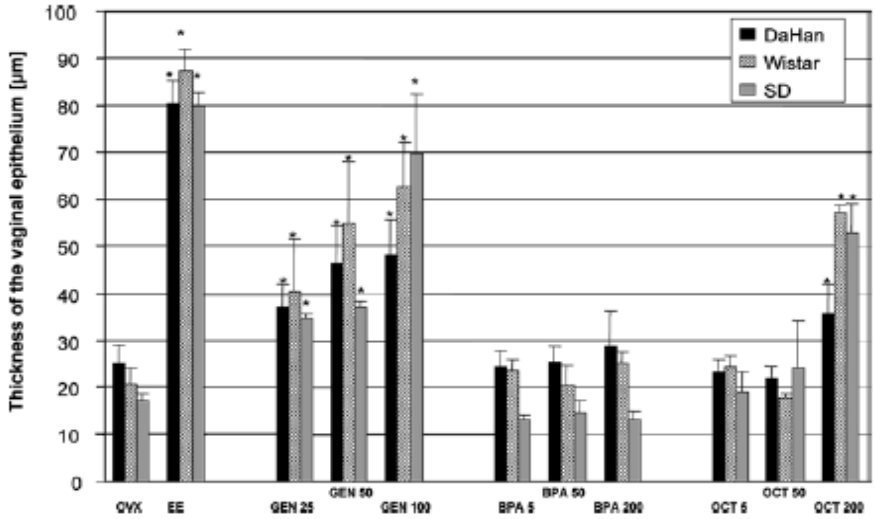


Fig. 46. Semiquantitative evaluation of vaginal epithelial thickness of DA/Han, Wistar and Sprague-Dawley (SD) ovariectomized (OVX) rats by morphometry (means ± SEM). Rats were treated by gavage with different doses of genistein (GEN, 25, 50 and 100 mg/kg b.w. per day), bisphenol A (BPA, 5, 50 and 200 mg/kg b.w. per day), p-ter-octylphenol (OCT, 5, 50 and 200 mg/kg b.w.) or ethinylestradiol (EE, 100 µg/kg per day). * $P \leq 0.05$, significantly different from the control group (OVX), Mann-Whitney U-test. Data from Diel et al. (2004).

### 4.2. Genotoxicity of isoflavones

Based on a set of preliminary data, Kulling and Metzler (1997) were the first to postulate genotoxic properties of the phytoestrogen genistein. This was the reason to presently study genistein, daidzein and the daidzein metabolite, equol, in vitro in V79 cells, for various endpoints of chromosomal genotoxicity.

Both isoflavones, genistein and daidzein, were negative in the induction of hypoxanthine-guanine-phosphoribosyltransferase (HPRT) point mutations (Kulling and Metzler, 1997). Also the substances did not affect the cytoplasmic microtubule complex or the mitotic spindle (Kulling and Metzler, 1997). However, genistein but not daidzein induced DNA strand breaks, indicated by use of the "alkaline filter elution" technique; the induction of micronuclei was also reported (Kulling and Metzler, 1997). The isoflavones were also studied in cultured human peripheral blood lymphocytes (Kulling et al., 1999). Only genistein caused structural chromosomal aberrations, at a concentration of 25 µM.

In accordance with these results, the data presented here show that both phytoestrogens, genistein and daidzein, as well as the daidzein metabolite equol, display genotoxic properties in vitro. The studies were carried out in estrogen receptor-negative Chinese hamster lung fibroblasts (V79 cells), in order to avoid receptor-mediated (hormonal) effects (see section 3.1.5.1).

The genotoxic properties of the isoflavones genistein, daidzein and equol appear now well characterised by a positive dose-dependent response in the micronucleus assay (see Fig. 33, 35 and 36). However, the quantities of these responses are different: at low concentrations (25 µM or less), the effects of genistein and, to a lesser effect, of equol are much more pronounced than those of daidzein. The compounds also differ in the shape of their dose-response curves; the genotoxicity of genistein in the micronucleus assay starts already at 5 µM and has a maximum at 25 µM. Higher concentrations (above 25 µM) of genistein lead to decreasing genotoxicity in the micronucleus assay, in parallel with observed cytotoxicity, which becomes more pronounced within this dose range.

It is generally accepted that estrogens stimulate cell proliferation in target tissues by receptor-mediated mechanisms and may thereby act as tumour promoters. Earlier studies in Syrian hamster embryo cells have shown that steroidal estrogens may lead to aneuploidy (Tsutsui et al., 1983, 1987). In order to further study the mechanisms of micronuclei formation, several experiments were conducted in the framework of the present thesis. The gliding velocity of microtubules along immobilized kinesin molecules was investigated with daidzein and

genistein at 100 and 500 µM to study effects on the cytoskeleton and on motor protein functions. But none of these isoflavones showed an effect in this direction. A lack of aneuploidogenic potential was supported by the observation that no CREST-positive micronuclei (i.e. micronuclei containing chromosomes/chromatids with kinetochores) were induced by genistein. Daidzein showed a mixed response and the majority of the micronuclei induced by the daidzein metabolite equol proved to be CREST-positive, pointing to a predominantly aneugenic effect (see Fig. 40). This shows that differential modes of actions should be operative, as far as the micronuclei induction by genistein and daidzein are concerned.

The more recently developed COMET assay (single-cell electrophoresis) is an useful additional technique to study cellular DNA damage. Single and double-strand breaks can be observed under alkaline conditions. Clastogenic substances are supposed to induce breaks of chromosomal material that might be detectable by the comet formation. However, only at higher, cytotoxic concentrations (> 250 µM), genistein induced a damage detectable by this technique. This may be due to a lower sensitivity of the COMET compared to the micronucleus assay, in the detection of specific clastogenic effects.

In essence, the present data basically confirm the clastogenicity of genistein that has first been postulated by Kulling and Metzler (1997). Furthermore, they show a quantitative difference in potency between genistein and daidzein, and they also clearly indicate a genotoxic potential for the isoflavone metabolite equol, a metabolite of daidzein that is found both in experimental rodents (Setchell et al., 1984) and in humans (Rowland et al., 2000; Maubach et al., 2003).
For matters of assessment of genotoxic risk, it appears from the present study that the phytoestrogens in question must be seen differently; in the micronucleus assay, key results were:

- Daidzein: statistically positive response between 50 and 100 µM
- Equol: statistically positive response between 20 and 25 µM
- Genistein: statistically positive response at 5 µM and higher (maximum at 25 µM)

As in soy-derived food genistein and daidzein are mostly present at about equal amounts, it appears that genistein is the leading compound for assessment of the associated genotoxic risk, and for studying the underlying mode of action.

The biochemical mechanism underlying to the clastogenicity of genistein has been characterised in part. This effect is independent of its hormonal (receptor-mediated) activity. Genistein is a non-intercalative topoisomerase II inhibitor, at concentrations of 10-100 μM in vitro (Markovits et al., 1989; Okura et al., 1988; Yamashita et al., 1990). DNA topoisomerase-II is a nuclear enzyme that catalyses a transient double-strand break in DNA, allowing DNA strands or double helices to pass through each other (Toonen and Hande, 2001; Wang, 2002). By this action, topoisomerase-II solves topological problems in DNA replication, transcription and recombination. Topoisomerase-II inhibitors can interfere by forming a stable drug-topoisomerase II-DNA complex, leaving the DNA with a permanent double-strand break.
Genistein has also been postulated to be a potent inhibitor of tyrosine protein kinase (Akiyama et al., 1987). Inhibition of tyrosine kinases may inactivate the checkpoint by which damaged cells are prevented from entering mitosis (Kaufman 1998; Baguley and Ferguson 1998). If such damaged cells survive, cells with permanent genetic alterations could be a result.

### *4.2.1. Mode of action of the clastogenicity of genistein as a topoisomerase-II inhibitor*

Lynch et al. (2003) have characterised and discussed the action of genistein as a topoisomerase II inhibitor in detail. They also arrived at the conclusion of an existence of thresholds for genotoxic compounds with this type of mechanism.
As mentioned above, topoisomerases regulate the topological state of DNA during replication, transcription and repair. DNA topoisomerases fall into two major classes: type I enzymes that induced single-stranded cuts in DNA, and type II enzymes that cut and pass double-stranded DNA.
During the topoisomerase-II catalytic cycle, the enzyme covalently binds to DNA and produces a temporary double-strand break, thus creating a transient gate through which another DNA double strand can pass. After the strand passage, the break is again ligated, and

the DNA structure is restored. The mechanism of action of topoisomerase-II can be dissected into a series of steps initiated by the binding of DNA to both subunits (S1 and S2) of the enzyme (Fig. 47 A, Hengstler et al., 2002). Topoisomerase II cleaves the DNA, forming a phosphotyrosine linkage between each DNA single-strand break and the catalytic tyrosines of both topoisomerases subunits (Fig. 47 B). In a next ATP-dependent step, topoisomerase II traps a second DNA double strand (Fig. 47 C), termed trans- or "T-strand". This will pass the gap of the first DNA double strand (termed gap- or "G-strand", Fig. 47 D). As soon as the T-strand has passed the G-strand, the carboxyl terminal portion of the enzyme undergoes a confirmational change (Fig. 47 E) that allows the exit of the T-strand (Fig. 47 F). In the next step, topoisomerase II reverses the cleavage reaction of the G-strand (Fig. 47 G), and hydrolysis of ATP allows dissociation of the G-strand (Fig. 47 H).

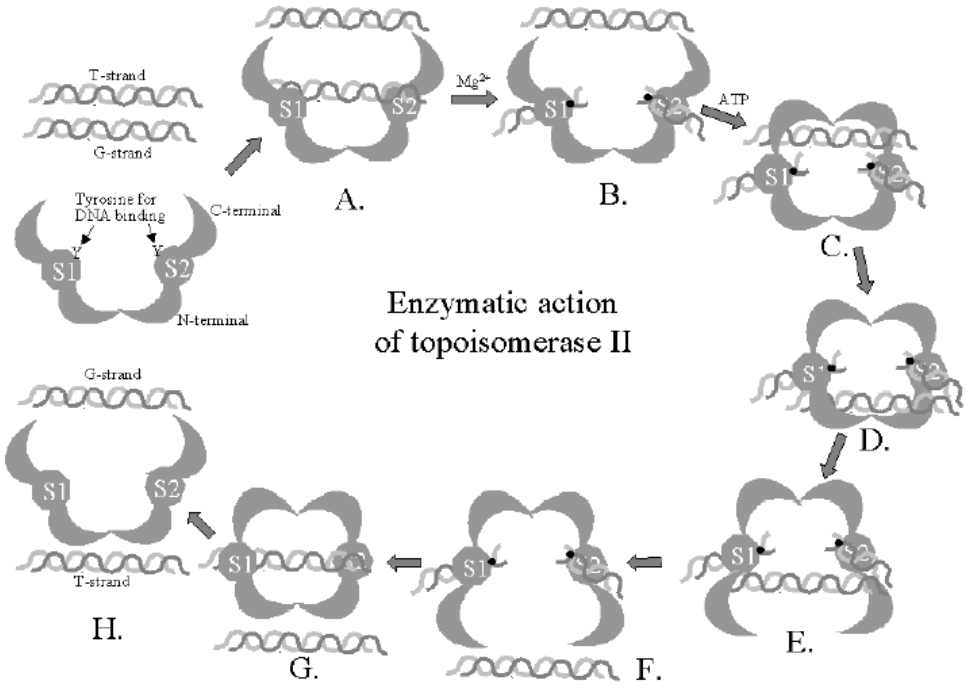


Fig. 47. Enzymatic action of DNA topoisomerase-II. The poisomerase II-poisons act by trapping the G-strand-enzyme intermediate (step F), thus, blocking religation and enzyme release, leaving the DNA with a permanent double-strand break (Hengstler et al., 2002).

Numerous compounds are known to disrupt the DNA breakage-reunion cycle of mammalian topoisomerase-II (Burden and Osheroff, 1998). They may either disrupt the catalytic cycle (catalytic inhibitors) or stabilize the normally transient cleavage complex formed between the enzyme and DNA (topoisimerase-II poisons). Whereas catalytic inhibitors disrupt the enzyme physiology, topoisomerase-II poisons transform the enzyme into a potent cellular toxin through the formation of stabilized cleavage complexes (SCCs) in which the enzyme remains covalently bound to both strands of the DNA double strand break, leaving the DNA with a permanent double strand break, that may lead to clastogenicity. Genistein induces, in a dose-dependent manner, the formation of SCCs in mouse lymphoma cells, studied by the TARDIS assay ("trapped in agarose DNA immunostaining"; Lynch et al., 2003).
An important implication of such an indirect (topoisomerase II-mediated) effect on DNA is the concept of a threshold for clastogenicity; a "pragmatic" threshold can be considered as a concentration below which any effect is considered biologically unimportant (Lutz, 1998). This may be defined with the help of statistical tests (Lovell, 2000). A concentration-response curve was determined for genistein using micronucleus induction as a measure of clastogenicity (Lynch et al., 2003). A combination of hypothesis testing and estimation (mathematical modelling) was used to resolve a "pragmatic" threshold for clastogenicity for genistein, which resulted in 1 µg/ml (3.7 µM). In the present study, 5 µM genistein produced the first statistically significant positive micronucleus response.

### 4.3. Genistein: threshold and associate margin of safety

According to Lynch et al. (2003) and supported by the present data, a pragmatic threshold of genistein for clastogenicity would be at 1 µg/ml (3.7 µM). This is about one order of magnitude higher than the blood levels of rats on isoflavone-rich diets in our investigation, or of human populations in the Far East with a dietary preference of soy products. It is about two orders of magnitude higher than plasma levels of European/Western populations under worst-case conditions (generally < 10 ng/ml).
For the European population, this would imply a margin of safety of about 100 between worst-case plasma levels and the possible beginning of a chromosomal genotoxic response. This is usually considered sufficient.

# 5. CONCLUSIONS

There is a variety of existing data on potential uptake of isoflavones in the human diet and in animal feed. Genistein and daidzein are the compounds that must be considered from a quantitative point of view; both compounds are about equipotent considering their hormonal (estrogenic) activities.

The available data on daily uptake of isoflavones with the human food have been evaluated by the Deutsche Forschungsgemeinschaft (SKLM, 1998). For a European/Western-type diet, a worst case uptake of 1 mg isoflavones (genistein plus daidzein) per kg body weight has been presented. On the basis of these figures, the resulting plasma levels of genistein and daidzein, and the toxicological effects discussed in this thesis, the following margins of safety (MOS) are deduced:

- For hormonal (estrogenic) effects: MOS = 10
- For chromosomal genotoxicity (micronucleus test): MOS = 100

For both types of toxic effects a threshold can reasonably be deduced.

Under these conditions, there appears to be sufficient safety, given the usual patterns of human food consumption in Europe.

## 6. SUMMARY

Hormonally active chemicals in the human diet, such as man-made estrogenic compounds or plant-derived substances (phytoestrogens), have become a matter of public concern. A significant part of human exposure to phytoestrogens is attributable to soy isoflavones. The phytoestrogens genistein and daidzein are naturally occurring isoflavones, present in a number of plants, especially in soybeans and soy-derived products.
In whole-animal models and in experimental systems in vitro, phytoestrogens appear capable of acting both as partial estrogen agonists and as antagonists, the primary effects of which are mediated via interaction with the estrogen receptor. Besides their estrogenic properties, soy isoflavones also exert genotoxic actions. The aim of the present thesis work was to characterize the genotoxicity risk of the isoflavones daidzein, genistein and the daidzein metabolite, equol. Thus, an assessment of the human exposure was performed, based on literature data and on an own investigation of the toxicokinetics. For this purpose, a study was performed in rats to measure the blood levels of isoflavones, in relation to the dose that was given orally. This relation was crucial for the calculation of effective blood concentrations in relation to food intake. All the animals kept on a Ssniff Standard diet containing ~ 400 µg total isoflavone/g chow showed isoflavone mean plasma levels of 202 and 221 ng/ml for daidzein and genistein, respectively. The animals kept on an isoflavone-low diet (ILD, < 10 µg isoflavone/g feed) after 6 weeks showed a mean isoflavone plasma levels of 23.1 ng/ml daidzein and non-detectable levels of genistein (< 20 ng/ml). After12 weeks, the ILD animals presented non-detectable levels for both isoflavones analysed. The animals fed with an isoflavone-rich diet (IRD, 472 µg total isoflavone/g chow) showed isoflavone plasma concentrations of 100.3 ng/ml genistein and 93.6 ng/ml daidzein after 6 weeks and 78.4 ng/ml genistein and 63.8 ng/ml daidzein after 12 weeks on this diet.

On the other hand, a hazard identification was done: a qualitative/quantitative description of the possible effects of the substances. Again, the information on the known hormonal effects of the compounds was taken from the literature, whereas experimental data on the dose-response of genotoxicity and a characterization of the mode of action were generated as part of this thesis. For this purpose, the micronucleus (MN) assay in V79 cells was used to study chromosomal genotoxicity. Genistein caused a clear dose-related induction of MN within the

range of 5–25 µM; MN rates were declining at higher genistein concentrations. This was probably due to cytotoxicity of genistein. Daidzein induced a comparatively shallow increase in the number of MN between 25 and 100 µM. In contrast, the daidzein metabolite equol caused an increase in the number of MN up to 25 µM with no further increase at higher concentrations. In order to further study the mechanisms of micronuclei formation, several experiments were conducted in the framework of the present thesis. The gliding velocity of microtubules along immobilized kinesin molecules was investigated for daidzein and genistein at 100 and 500 µM to study effects on the cytoskeleton and on motor protein functions. But none of these isoflavones showed an effect in this direction. A lack of aneuploidogenic potential was supported by the observation that no CREST-positive micronuclei (i.e. micronuclei containing chromosomes/chromatids with kinetochores) were induced by genistein. Daidzein showed a mixed response and the majority of the micronuclei induced by the daidzein metabolite equol proved to be CREST-positive, pointing to a predominantly aneugenic effect.

Clastogenic substances are supposed to induce breakages of chromosomal material that might be detectable by the comet formation (COMET assay). However, only at higher, cytotoxic concentrations (> 250 µM), genistein induced a damage detectable by this technique. This may be due to a lower sensitivity of the COMET compared to the micronucleus assay, in the detection of specific clastogenic effects.

In essence, the present data basically confirm the clastogenicity of genistein that had first been postulated by Kulling and Metzler (1997). Furthermore, they show a quantitative difference in potency between genistein and daidzein, and they also clearly indicate a genotoxic potential for the isoflavone metabolite equol.

Finally, both aspects, possible human exposure and compound-related effects were put into a perspective to arrive at a characterisation of the possible risk of genotoxicity for humans due to phytoestrogen contents of human diet.

Based on uterotropic response, a non-hormonal daily effect dose of 10 mg/kg b.w. was established. This non-effect dose is related to a total genistein plasma level of about 100 ng/ml. Both, the uptake and the resulting plasma levels remain about one order of magnitude below the non-hormonal effect level (safety factor for hormonal effects = 10).

The biochemical mechanism underlying to the clastogenicity of genistein has been characterised in part. Genistein is a non-intercalative topoisomerase II inhibitor, at

concentrations of 10-100 µM in vitro. Lynch et al. (2003) arrived at the conclusion of a possible existence of thresholds for genotoxic compounds with this type of mechanism. A "pragmatic" threshold can be considered as a concentration below which any effect is considered biologically unimportant. For genistein a pragmatic threshold for clastogenicity resulted in 1 µg/ml (3.7 µM). This is about one order of magnitude higher than the blood levels of rats on isoflavone-rich diets in our investigation, or of human populations in the Far East with a dietary preference of soy products. It is about two orders of magnitude higher than plasma levels of European/Western populations under worst-case conditions (generally < 10 ng/ml). For the European population, this would imply a margin of safety of about 100 between worst-case plasma levels and the possible beginning of a chromosomal genotoxic response. Such a margin of safety is usually considered sufficient.

## 7. REFERENCES

Adlercreutz H., Fotsis T., Bannwart C., Wähäla K., Mäkela T., Brunow G., Hase T. (1986). Determination of urinary lignans and phytoestrogens metabolites, potential antiestrogens and anticarcinogens, in urine of women on various habitual diets. J. Steroid Biochem. 25, 791-797.

Adlercreutz H., Honjo H., Higashi A., Fotsis T., Hamalainen E., Hasegawa T., Okada H. (1991). Urinary excretion of lignans and isoflavonoid phytoestrogens in Japanese men and women consuming a traditional Japanese diet. Am. J. Clin. Nutr. 54, 1093-1100.

Adlercreutz H.A., Fotsis T., Lampe J., Wähäla K., Brunow G., Hase T. (1993a). Quantitative determination of lignans and isoflavonoids in plasma of omnivorous and vegetarian women by isotope dilution gas chromatography-mass spectrometry. Scand. J. Clin. Lab. Invest. (Suppl. 215) 53, 5-18.

Adlercreutz H.A., Makkanen H., Watanabe S. (1993b). Plasma concentration of Phytoestrogens in Japanese men. Lancet 342, 1209-1210.

Adlercreutz H.A., Fotsis T., Watanabe S., Lampe J., Wähäla K., Mäkela T., Hase T. (1994). Determination of lignans and isoflavonoids in plasma by isotope dilution gas chromatography-mass spectrometry. Cancer Detect.Prev.18(4), 259-271.

Adlercreutz H., Goldin B.R., Gorbach S.L., Höckerstedt K.A.V., Watanabe S. et al. (1995). Soybean phytoestrogen intake and cancer risk. J. Nutr. 125, 757-770S.

Akiyama T., Ishida J., Nakagava S., Ogawara S., Watanabe S., Itoh N., Shiyba M., Fukami Y. (1987). Genistein, a specific inhibitor of tyrosine-specific protein kinases. J. Biol. Chem. 262, 5592-5595.

Andlauer W., Kolb J., Furst P. (2000). Absorption and metabolism of genistein in the isolated rat small intestine. FEBS Letters 475, 127.

Arora A., Nair M.G., Straburg G.M. (1998). Antioxidant activities of isoflavones and their biological metabolites in a liposomal system. Arch. Biochem. Biophys. 356, 133-141.

Axelson M.and Setchell K.D.R. (1981). The excretion of lignans in rats: evidence for an intestinal bacterial source of this new group of compounds. FEBS Lett. 123, 337-342.

Back D.J., Bolt H.M., Breckenridge A.M., Crawford F.E., Orme M.L.E., Rowe P.H., Schindler A.E. (1980). The pharmacokinetics of a large (3 mg) oral dose of ethinylestradiol in women. Contraception 21, 145-153.

Baguley B.C. and Ferguson L.R. (1998). Mutagenic properties of topoisomerase-targeted drugs. Biochem. Biophys. Acta 1400, 213-222.

Barnes S., Grubbs C., Setchell K.D.R., Carlson J. (1990). Soyabeans inhibit mammary tumor growth in breast cancer. Mutagens and Carcinogens in the Diet, pp 239-253. New York: Wiley-Liss.

Block S.M., Goldstein L.S., Schnapp B.J. (1990). Bead movement by single kinesin molecules studied with optical tweezers. Nature 348: 348-352.

Bogdanffy M.S. and Valentine R. (2003). Differentiating between local cytotoxicity, mitogenesis, and genotoxicity in carcinogen risk assessment : The case of vinyl acetate. Toxicol. Lett. 140/141, 83-98.

Böhm K.J., Stracke R., Unger E., (2000). Speeding up kinesin-driven microtubule gliding in vitro by variation of cofactor composition and physicochemical parameters. Cell Biol. Int. 24, 335-341.

Bolt H.M. (2003). Genotoxicity-threshold or not? Introduction of cases of industrial chemicals. Toxicology Letters. 140-141, 43-51.

Bolt H.M. and Degen G. (1996/97). Wenn Fremdstoffe wie Hormone wirken. Chemie heute pp. 84-89.

Bolt H.M. and Degen G. (2000). Hormoneffekte von Chemikalien in Nahrung und Umwelt. Zur Diskussion von Wirkungen östrogener Stoffe als « endokrine Disruptoren ». Chemie in unserer Zeit 34, 30-37.

Bolt H.M., Janning P., Michna H., Degen G.H. (2001). Comparative assessment of endocrine modulators with oestrogenic activity : I. Definition of a hygiene-based margin of safety (HBMOS) for xeno-oestrogens against the background of European developments. Arch. Toxicol. 74, 649-662.

Bolt H.M., Foth H., Hengstler J.G., Degen G. H. (2004). Carcinogenicity categorization of chemicals : New aspects to be considered in a European perspective. Toxicol. Lett. 151, 29-41.

Borenfreund E.and Borrero O. (1984). In vitro cytotoxicity assays. Potential alternatives to the Draize ocular allergy test. Cell Biol. Toxicol. Oct ;1 (1), 55-65.

Burden D.A. and Osheroff N. (1998). Mechanism of action of eukariotic topoisomerase II and drugs targeted to the enzyme. Biochim. Biophys. Acta 1400, 3-18.

Cassidy A. (1996). Physiological effects of phytoestrogens in relation to cancer and other human health risk. Proceed. Nutr. Soc. 55, 399-417.

Coldham N.G., Darby C., Hows M., King L.J., Zhang A.Q., Sauer M.J. (2002). Comparative metabolism of genistein by human and rat gut microflora: detection and identification of end-products of metabolism. Xenobiotica 32, 45-67.

Coward L., Barnes N.C., Setchell K., Barnes S. (1993). Genistein, daidzein, and their ß-glycoside conjugates: antitumor isoflavones in soybean foods from American and Asian diets. J. Agric. Food Chem. 41,1961-1967.

Coward L., Kirk M., Albin N., Barnes S. (1996). Analysis of plasma isoflavones by reversed-phase HPLC-multiple reaction ion monitoring-mass spectrometry. Clinica Chimica Acta 247, 121-142.

Crebelli R. (2000). Threshold-mediated mechanisms in mutagenesis: Implications in the classification and regulation of chemical mutagens. Mutat. Res. 464, 129-135.

Day A.J., DuPont M.S., Saxon Ridley S. (1998). Deglycosylation of flavonoid and isoflavonoid glycosides by human small intestine and liver and beta glucosidase activity. FEBS Letters 436, 71-75.

Decodier I., Dillen L., Cundari E., Kirsch-Volders M. (2002). Elimination of micronucleated cells by apoptosis after treatment with inhibitors of microtubules. Mutagenesis 17, 337-344.

Degen G. H. (1990). Interactions of phytoestrogens and environmental estrogens with prostaglandin synthase: an in vitro investigation. J. Steroid. Biochem. 35, 473-479.

Degen G. H., Janning P., Diel P., Bolt H.M. (2002a). Estrogenic isoflavones in rodent diets. Toxicology Letters 128, 145-157.

Degen G. H., Janning P., Diel P., Michna H., Bolt H.M. (2002b). Transplacental transfer of the phytoestrogen daidzein in DA/Han rats. Arch. Toxicol.. 76, 23-29.

Derendorf H.and Garett E.R. (1987). Pharmakokinetik: Einführung in die Theorie und Relevanz für die Arzneimitteltherapie. Wissenschaftliche Verlagsgesellschaft, Stuttgart.

Deutsche Forschungsgemeinschaft (1998). Hormonally active agents in food. In: Eisenbrand G., Daniel H., Dayan A.D., Elias P.S., Grunow W., Kemper F.H., Löser E., Metzler M., Schlatter J. (eds) Symposium. Wiley-VCH, Weinheim.

Diel P. (2002). Tissue-specific estrogenic response and molecular mechanisms. Toxicology Letters 127, 217-224.

Diel P., Schulz T., Smolnikar K., Strunck E., Vollmer G., Michna H. (2000). Ability of xeno-and phytoestrogens to modulate expression of estrogen-sensitive genes in rat uterus: estrogenicity profiles and uterotropic activity. J. Steroid Biochem. Molec. Biol. 73, 1-10.

Diel P., Schmidt S., Vollmer G., Janning P., Upmeier A., Michna H., Bolt H.M., Degen G.H. (2004). Comparative responses of three rat strains (DA/Han, Sprague-Dawley and Wistar) to treatment with environmental estrogens. Arch. Toxicol. 78, 183-193.

Doerge D.R., Churchwell M., Delclos K.B. (2000). On-line sample preparation using restricted-access media in the analysis of the soy isoflavones, genistein and daidzein, in rat serum using liquid chromatography electrospray mass spectrometry. Rapid Commun. Mass Spectr. 14, 673-678.

Dybing E. and Sanner T. (2003). Risk assessment of acrylamide in foods. Toxicol. Sci. 75, 7-15.

Ferguson L.R.and Pearson A.E. (1996). The clinical use of mutagenic anticancer drugs. Mutat. Res. 355, 1-12.

Fischer W.H., Keiwan A., Schmitt E., Stopper H. (2001). Increased formation of micronuclei after hormonal stimulation of cell proliferation in human breast cancer cells. Mutagenesis 16, 209-212.

Franke A.A., Custer L.J., Cerna C.M., Narala K.K. (1994). Quantitation of phytoestrogens in legumes by HPLC. J. Agric. Food. Chem. 42, 1905-1913.

Franke A.A., Custer L.J., Cerna C.M., Narala K.K. (1995). Rapid HPLC analysis of dietary phytoestrogens from legumes and from human urine. Proceedings of the Society for Experimental Biology and Medicine 208, 18-26.

Franke A.A., Custer L.J., Wang W., Shi C.Y. (1998). HPLC analysis of isoflavonoids and other phenolic agents from food and from human fluids. Proc. Soc. Exp. Biol. Med. 217, 263-273.

Gamache P.H. and Acworth I.N. (1998). Analysis of isoflavoids and other phenolic agents in plasma, tissue, and urine using HPLC with coulometric array detection. Proc. Soc. Exp. Biol. Med. 217: 274-280.

Giannotti E., Vandin L., Repeto P., Comelli R. (2002). A comparison of the in vitro Comet assay with the in vitro chromosome aberration assay using whole human blood or Chinese hamster lung cells: validation study using a range of novel pharmaceuticals. Mutagenesis 17(2), 163-170.

Grace P.B., Taylor J.I., Low Y.L., Luben R.N., Mulligan A.A., Botting N.P., Dowsett M., Welch A.A., Khaw K.T., Wareham N.J., Day N.E., Bingham S.A. (2004). Phytoestrogen concentrations in serum and spot urine as biomarkers for dietary phytoestrogen intake and their relation to breast cancer risk in European Prospective Investigation of Cancer and Nutrition-Norfolk. Cancer Epidemiol. Biomarkers Prev. 13(5), 698-708.

Heinonen S.M., Hoikkala A., Wähälä K., Adlercreutz H. (2003). Metabolism of the soy isoflavones daidzein, genistein and glycitein in human subjects. Identification of new metabolites having an intact isoflavonoid skeleton. J. Steroid Biochem. Mol. Biol. 87, 285-299.

Heinonen S.M., Wähälä K., Liukkonen K.H., Aura A.M., Poutanen K., Adlercreutz H. (2004). Studies of the in vitro intestinal metabolism of isoflavones aid in the identification of their urinary metabolites. J.Agric. Food Chem. 52, 2640-2646.

Hengstler J.G., Heimerdinger C.K., Schiffer I.B., Gebhard S., Sagemueller J., Tanner B., Bolt H.M., Oesch F. (2002). Dietary topoisomerase II-poisons : contribution of soy products to infant leukemia? EXCLI Journal 1, 8-14.

Hengstler J.G., Bogdanffy M.S., Bolt H.M., Oesch F. (2003). Challenging dogma: thresholds for genotoxic carcinogens? The case of vinyl acetate. Annu. Rev. Pharmacol. Toxicol. 43, 485-520.

Hsu J., Jean T., Chan M., Ying C. (1999). Differential display screening for specific gene expression induced by dietary nonsteroidal estrogen. Mol. Reprod. Dev. 52, 141-148.

Janning P., Schuhmacher U.S., Upmeir A., Diel P., Michna H., Degen G.H., Bolt H.M. (2000). Toxicokinetics of the phytoestrogen daidzein in female DA/Han rats after single i.v. and oral application. Arch. Toxicol. 74, 421-430.

Joannou G.E., Kelly G.E., Reeder A.Y., Waring M.A., Nelson C. (1995). A urinary profile study of dietary phytoestrogens. The identification and mode of metabolism of new isoflavonoids. J. Steroid Biochem. Mol. Biol. 54, 167-184.

Jones A.E., Price K.R., Fenwick G.R. (1989). Development and application of a high-performance liquid chromatography method for the analysis of phytoestrogens. J. Sci Food Agric. 46, 357-364.

Kaufmann W.K. (1998). Human topoisomerase II function, tyrosine phosphorylation and cell cycle checkpoints. Proc. Soc. Exp. Biol. Med. 217, 327-334

Kelly G.E., Joannou G.E., Reeder A.Y., Nelson C., Waring M. A. (1995). The variable metabolic response in humans. Proc. Soc. Exp. Biol. Med. 208, 40-43.

Kreja L.and Seidel H.J. (2002). On the cytotoxicity of some microbial volatile organic compounds as studied in the human lung cell line A549. Chemosphere 49 (1), 105-110.

Kulling S.E and Metzler M. (1997). Induction of Micronuclei, DNA strand breaks and HPRT mutations in cultured Chinese hamster V79 cells by the phytoestrogen coumoestrol. Food and Chem. Toxicol. 35, 605-613.

Kulling S.E., Rosenberg B., Jacobs E., Metzler M. (1999). The phytoestrogens coumestrol and genistein induce structural chromosomal aberrations in cultured human peripheral blood lymphocytes. Arch. Toxicol. 73, 50-54.

Kulling S.E., Honig D.M., Simat T.J., Metzler M. (2000). Oxidative in vitro metabolism of the soy phytoestrogens daidzein and genistein. J. Agric. Food Chem. 48, 4963-4972.

Kulling S.E., Honig D.M., Metzler M. (2001). Oxidative metabolism of the soy isoflavones daidzein and genistein in humans in vitro and in vivo. J. Agric. Food Chem. 49, 3024-3033.

Kulling S.E., Lehmann L., Metzler M. (2002). Oxidative metabolism and genotoxic potential of major isoflavone phytoestrogens. J. Chromatog. B Anal Technol. Biomed. Life Sci. 777, 211-218.

Kurzer M.S. and Xu X. (1997). Dietary phytoestrogens. Annu. Rev. Nutr., 17, 353-81.

Kuznetsov S.A.and Gelfand V.I. (1986). Bovine brain kinesin is a microtubule-activated ATPase. Proc. Natl. Acad. Sci. USA 83: 8530-8534.

Lampe J.W., Karr S.C., Hutchins A.M., Slavin J.L. (1998). Urinary equol excretion with soy challenge: influence of habitual diet. Proc. Soc. Exp.Biol. Med. 217, 335-339.

Lee M., Kwon J., Chung M. K. (2003). Enhanced prediction of potential rodent carcinogenicity by comet assay and apoptotic assay in combination. Mut. Res. 541, 9-19.

Löffler S.and Bolt H.M. (1980). Untersuchungen zum enterohepatischen Kreislauf von Östradiol und Äthinylöstradiol bei der Ratte. Arzneimittelforschung 30, 810-813.

Lovell D.P. (2000). Dose-response and threshold-mediated mechanisms in mutagenesis: statistical models and study design. Mutat. Res.464, 87-95.

Lutz W. (1998). Dose-response relationship in chemical carcinogenesis ; superposition of different mechanisms of action, resulting in linear non-linear curves, practical thresholds, J-shapes. Mutat. Res. 405, 117-124.

Lynch A., Harvey J., Aylott M., Nicholas E., Burman M., Siddiqui A., Walker S., Rees R. (2003). Investigations into the concept of a threshold for topoisomerase inhibitor-induced clastogenicity. Mutagenesis 18, 345-353.

Markovits J., Linassier C., Fosse P., Couprie J., Pierre J., Jacquemin-Sablon A., Saucier J.-M., Le Pecq J.-B., Larson A.K. (1989). Inhibitory effects of the tyrosine kinase inhibitor genistein on mammalian DNA topoisomerase II. Cancer Research 49, 5111-5117.

Maubach J., Bracke M.E., Heyerick A., Depypere H.T., Serreyn R.E., Mareel M.M., De Keukeleire D. (2003). Quantitation of soy-derived phytoestrogens in human breast tissue and biological fluids by high-performance liquid chromatography. Journal of Chromatography B 784, 137-144.

Meek D.W. (2000). The role of p53 in the response to mitotic spindle damage. Pathol. Biol. 48, 246-54.

Miller B.M.and Adler I.D. (1989). Suspect spindle poisons: analysis of c-mitotic effects in mouse bone marrow cells. Mutagenesis 4, 208-215.

Miltyk W., Craciunescu C.N., Fischer L., Jeffcoat R.A., Koch M.A., Lopaczynski W., Mahoney C., Jeffcoat R.A., Crowell J., Paglieri J., Zeisel S. (2003). Lack of significant genotoxicity of purified soy isoflavones (genistein, daidzein and glycitein) in 20 patients with prostate cancer. Am. J. Clin. Nutr. 77, 875-882.

Mitchell J.H., Gardner P.T., McPhail D.B., Morrice P.C., Collins A.R., Duthie G.G. (1998). Antioxidant efficacy of phytoestrogens in chemical and biological model systems. Arch. Biochem. Biophys. 360, 142-148.

Morgan K.T. (1997). A brief review of formaldehyde carcinogenesis in ralation to rat nasal pathology and human risk assessment. Toxicol. Pathol. 25, 291-307.

Neumann H.G., Vamvakas S., Thielmann H.W., Gelbke H.P., Filser J.G., Reuter U., Greim H., Kappus H., Norpoth K.H., Wardenbach P., Wichmann H.E. (1998). Changes in the classification of carcinogenic chemicals in the work area. Int. Arch. Occup. Environ. Health 71, 566-574.

Okura A., Arakawa H., Oka H., Yoshinari T., Monden Y. (1988). Effects of genistein on topoisomerase activity and on the growth of [VAL 12]Ha-ras-transformed NIH 3T3 cells. Biochem. Biophys. Res. Com. 157, 183-189.

Parry J.M., Jenkins G.J.S., Haddad F., Bourner R., Parry E.M. (2000). In vitro and in vivo extrapolations of genotoxic exposures: Consideration of factors which influence dose-response thresholds. Mutat. Res. 464, 53-63.

Pfeiffer P., Goedecke W., Obe G. (2000). Mechanisms of DNA double-strand break repair and their potential to induce chromosomal aberrations. Mutagenesis 15 (4), 289-302.

Riddell R. J., Clothier R. H. and Balls M. (1986). An evaluation of three in vitro cytotoxicity assays. Food. Chem. Toxic. 24, 469-471.

Ritchie M. R., Morton M.S., Deighton N., Blake A., Cummings J.H. (2004). Plasma and urinary phytoestrogens as biomarkers of intake: validation by duplicate diet analysis. British Journal of Nutrition 91, 447-457.

Rowland I.R., Wiseman H., Sanders T.A.B., Adlercreutz H., Bowey E.A. (2000). Interindividual variation in metabolism of soy isoflavones and lignans: influence of habitual diet on equol production by the gut microflora. Nutr. Cancer 36, 27-32.

Rowland I., Faughnan M., Hoey L., Wähälä K., Williamson G., Cassidy A. (2003). Bioavailability of phytoestrogens. British J. Nutr. 89 (suppl 1), S45-S58.

Rothwell J.A., Day A.J., Morgan M.R. (2005). Experimental determination of octanol-water partition coefficients of quercetin and related flavonoids. J. Agric. Food Chem. Jun 1, 53 (11), 4355-60.

Santell R.C., Chang Y.C., Nair M.G., Helferich W.C. (1997). Dietary genistein exerts estrogenic effects upon the uterus, mammary gland and the hypothalamic/pituitary axis in rats. J. Nutr. 127, 263-269.

Sathyamoorthy N.and Wang T.T.Y. (1997). Differential effects of dietary phytoestrogens daidzein and equol on human breast cancer MCF-7 cells. Eur. J. Cancer 33, 2384-2389.

Schempp W. and Vogel W. (1979). Difference between diploid and aneuploid Chinese hamster cells in replication at mid-S-phase. Chromosoma 73 (1), 109-115.

Schmitt E., Dekant W., Stopper H. (2001). Assaying the estrogenicity of phytoestrogens in cells of different estrogen sensitive tissues. Toxicology in vitro. 15, 433-439.

Schnapp B.J., Crise B., Sheetz M.P., Reese T.S., Khan S., (1990). Delayed start-up of kinesin-driven microtubule gliding following inhibition by adenosine 5´-[beta, gamma-imido] triphosphate. Proc. Natl. Acad.Sci. USA 87, 10,053-10,057.

Schoeny R. (1996). Use of genetic toxicology data in U.S. EPA risk assessment: The mercury study report as an example. Environ. Health Perspect. 104 (Suppl. 3), 663-673.

Seeley M.R., Tonner-Navarro L.E., Beck B. D., Deskin R., Feron V.J., Johanson G., Bolt H.M. (2001). Procedures of health risk assessment in Europe. Regul. Toxicol. Pharmacol. 34, 153-169.

Setchell K.D.R. (1998). Phytoestrogens: the biochemistry, physiology, and implications for human health of soy isoflavones. Am. J. Clin. Nutr. 68 (suppl), 1333S-46S.

Setchell K.D.R. and Adlercreutz H. (1988). Mammalian lignans and phytoestrogens. Recent studies on their formation, metabolism and biological role in health and disease. In

Role of the Gut Flora in Toxicity and Cancer, ed. IR Rowland, pp. 315-45. London: Academic Press.

Setchell K.D.R. and Cassidy A. (1999). Dietary isoflavones: biological effects and relevance to human health. J. Nutr. 129, 758S-767S.

Setchell K.D.R., Lawson A.M., Borriello S.P., Harkness H., Gordon H., Morgan D.M.L., Hirk D.N., Adlercreutz H. (1981). Lignan formation in man-microbial involvement and possible roles in relation to cancer. Lancet 2, 4-8.

Setchell K.D.R., Borriello S.P., Hulme P., Kirk D.N., Axelson M. (1984). Non-esteroidal estrogens of dietary origin: possible roles in hormone-dependent disease. Am. J. Clin. Nutr. 40, 569-78.

Setchell K.D.R., Zimmer-Nechemias L., Cai J., Heubi J.E. (1997). Exposure of infants to phytoestrogens from soy-based infant formula. Lancet 350, 23-27.

Setchell K.D.R., Brown N.M., Desai P., Zimmer-Nechemias L., Wolfe B.E., Brashear W.T., Kischner A.S., Cassidy A., Heubi J.E. (2001). Bioavailability of pure isoflavones in healthy humans and analysis of commercial soy isoflavone supplements. J. Nutr. 131, 1362S-1375S.

Setchell K.D.R., Brown N.M., Zimmer-Nechemias L., Brashear W.T., Wolfe B.E., Kirschner A.S., Heubi J.E. (2002). Evidence for lack of absorption of soy isoflavone glycosides in humans, supporting the crucial role of intestinal metabolism for bioavailability. Am. J. Clin. Nutr. 76, 447-453.

Singh N.P., McCoy M.T., Tice R.R., Schneider E.L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. Experimental Cell Research 175, 184-191.

Sorger P.K., Dobles M., Tournebize R., Hyman A. (1997). Coupling cell division and cell death to microtubule dynamics. Curr. Opin. Cell Biol. 9, 807-14.

Speit G., Habermeier B., Helbig R. (1994). Differences in the response to mutagens between two V79 sublines. Mut. Res. 325 (2-3), 105-111.

Streffer C., Bolt H.M., Föllesdal D., Hall P., Hengstler J. G., Jacob P., Oughton D., Priess K., Rehbinder E., Swaton E. (2004). Environmental Standards-Dose-Effects Relations in the Low Dose Range and Risk Evaluation. Springer-Verlag, Berlin.

Supko J.G. and Malspeis L. (1995). Plasma pharmacokinetics of genistein in mice. Int. J. Oncol. 7, 847-854.

Tan E.M., Rodman G.P., Garcia L., Moroi Y., Fritzler M.J., Peebles C. (1980). Diversity of antinuclear antibodies in progressive systemic sclerosis. Arthritis Rheum. 23, 617-625.

Tao K.S., Urlando C., Heddle J.A. (1993). Mutagenicity of methyl methanesulfonate (MMS) in vivo at the Dlb-1 native locus and a lacI transgene. Environ. Mol. Mutagen. 22, 293-296.

Thier R., Bonacker D., Stoiber T., Böhm K.J., Wang M., Unger E., Bolt H.M., Degen G. H. (2003). Interaction of metal salts with cytoskeletal motor protein systems. Toxicol. Lett. 140/141, 75-81.

Toonen T.R. and Hande K.R.(2001). Topoisomerase II inhibitors. Cancer Chemother. Biol. Response Modif. 19, 129-147.

Tsutsui T., Maizumi H., McLachlan J.A., Barrett J.C.(1983). Aneuploidy induction and cell transformation by diethylstilbestrol: a possible chromosomal mechanism in carcinogenesis. Cancer Res. 43, 3814-3821.

Tsutsui T., Suzuki N., Fukuda S., Sato M., Maizumi H., McLachlan J.A., Barrett J.C.(1987). 17ß-Estradiol-induced cell transformation and aneuploidy of Syrian hamster embryo cells in culture. Carcinogenesis. 8, 1715-1719.

Tsutsui T., Tamura Y., Yagi E., Someya H., Hori I., Metzler M., Barrett J.C.(2003). Cell-transforming activity and mutagenicity of 5 phytoestrogens in cultured mammalian cells. Int. J. Cancer. 105, 312-20.

Vale R.D., Reese T.S., Sheetz M.P., (1985). Identification of a novel force-generating protein, kinesin, involved in microtubule-based motility. Cell 42, 39-50.

von Massow A., Mandelkow E.M., Mandelkow E., (1989). Interaction between kinesin, microtubules, and microtubule associated protein 2. Cell. Motil. Cytoskeleton 14, 562-571.

Wang J.C. (2002). Cellular roles of DNA topoisomerases: a molecular perspective. Nat. Rev. Mol. Cell Biol. 3, 430-440.

Wang H.J. and Murphy P.A. (1994). Isoflavone content in commercial soybean foods. J. Agric. Food Chem. 42, 1666-73.

Wang T.T.Y., Sathyamoorthy N., Phang J.M. (1996). Molecular effects of genistein on estrogen receptor mediated pathway. Carcinogenesis 17, 271-275.

Wei H., Bowen R., Cai Q., Barnes S., Wang Y. (1995). Antioxidant and antipromotional effects of the soybean isoflavone genistein. Proc. Soc. Exp. Biol. Med. 208, 124-130.

Willard S.T. and Frawley L.S. (1998). Phytoestrogens have agonistic and combinatorial effects on estrogen-responsive gene expression in MCF-7 human breast cancer cells. Endocrine 8, 117-121.

Xu X., Harris K.S., Wang H.J., Murphy P.A., Hendrich C.L. (1995). Bioavailability of soybean isoflavones depends upon gut microflora in women. J. Nutr. 125, 2307-2315.

Yamashita Y., Kawada S., Nakano H. (1990). Induction of mamalian topoisomerase II dependent DNA cleavage by non-intercalative flavonoids, genistein and orobol. Biochem. Pharmacol. 39, 737-744.