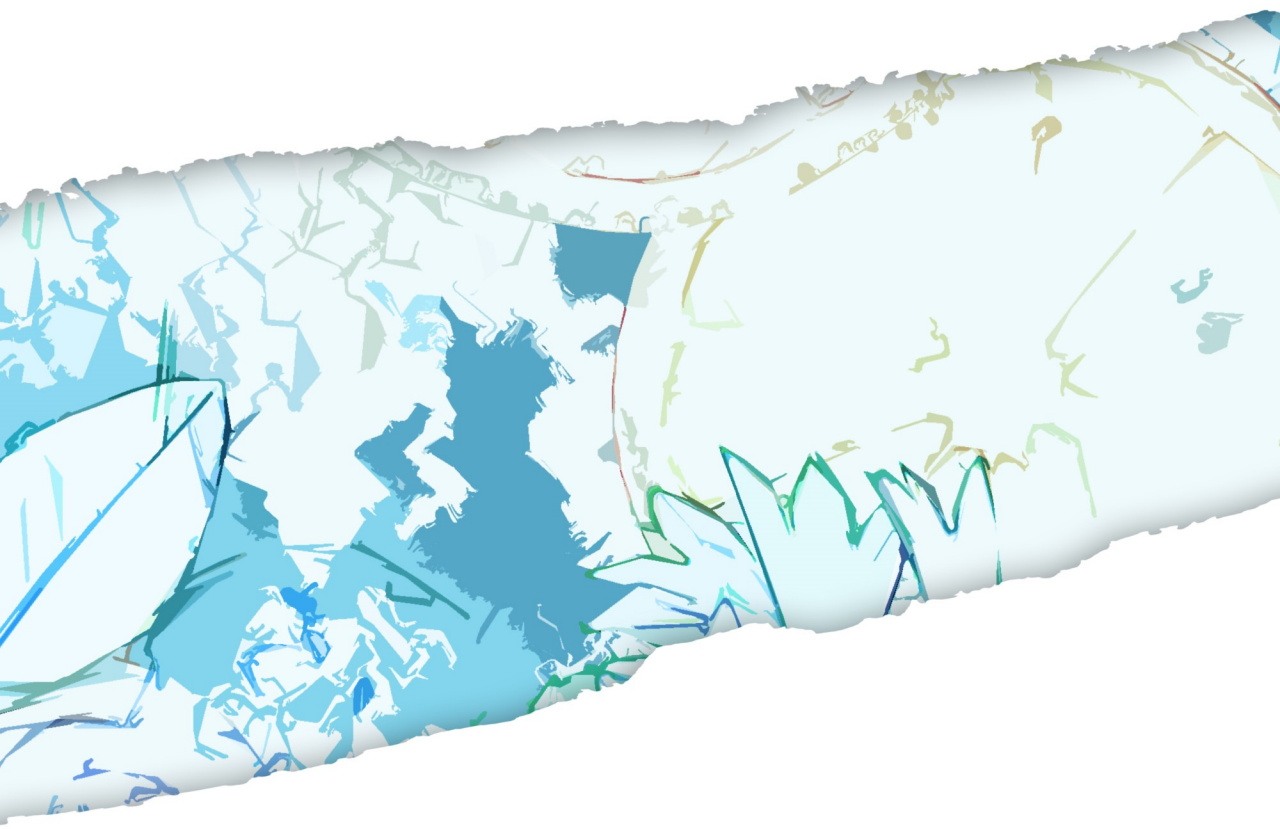
The influence of phase II conjugation on the biological activity of flavonoids



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The influence of phase II conjugation on the biological activity of flavonoids

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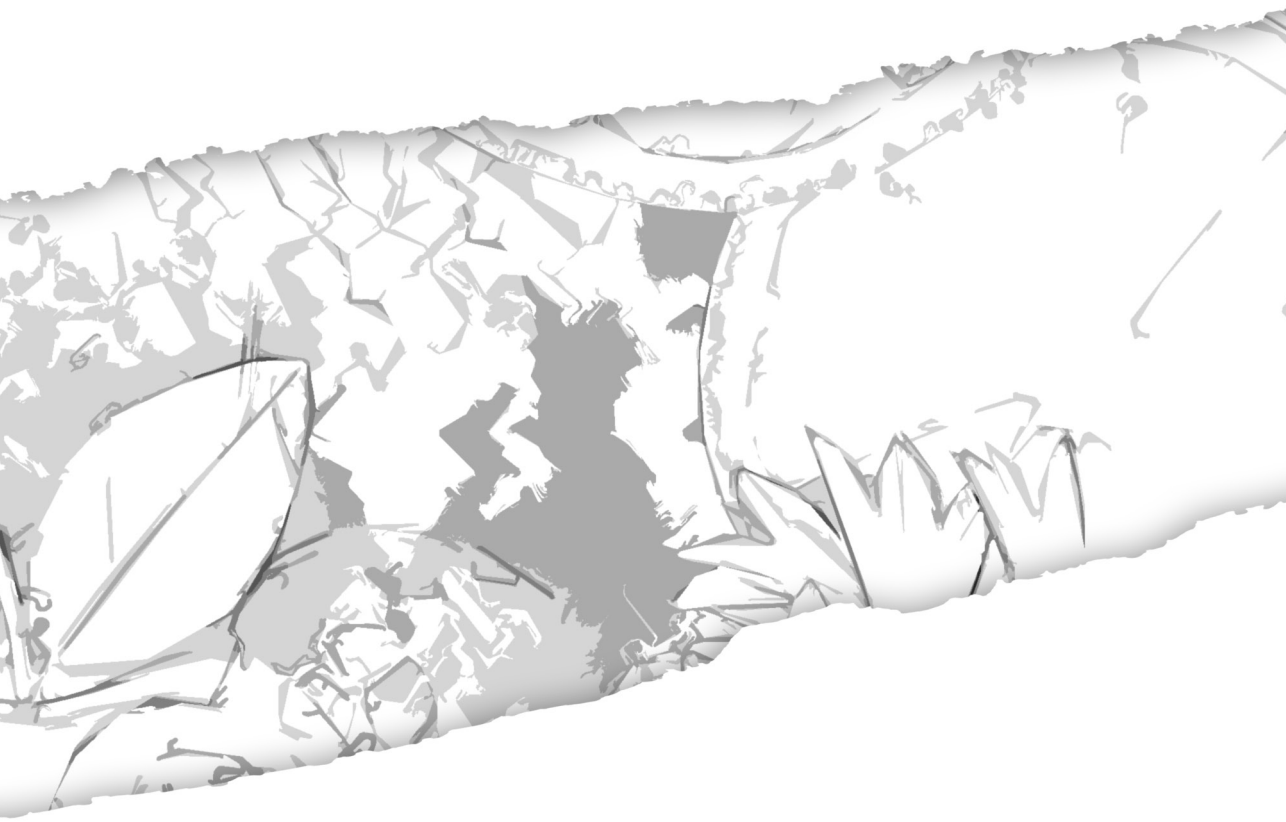
Chapter 1

General introduction, aim and outline of the thesis



General introduction

Flavonoids are a class of polyphenolic chemicals ubiquitously present in the plant kingdom where they occur as secondary metabolites fulfilling a range of functions such as pigmentation and UV protection [1]. In food, flavonoids contribute to the coloring and taste [2]. The most important dietary sources of flavonoids are fruits, vegetables, tea, wine and cocoa. Flavonoids attract attention as supposedly bioactive food constituents - consumption of a diet high in flavonoids is correlated to a wide range of health effects, such as the prevention of cardiovascular diseases [3, 4], neurodegenerative diseases [5] and diabetes [6, 7]. A vast amount of scientific publications on the supposed mechanisms of action of flavonoids report flavonoids to act as ligands for receptors, inhibitors of kinases, of other enzymes, and/or of transport proteins [8-12].

Flavonoids commonly consist of two aromatic rings (i.e. A- and B-ring) which are linked by the C-ring consisting of an additional three carbons and one oxygen (Figure 1.1).

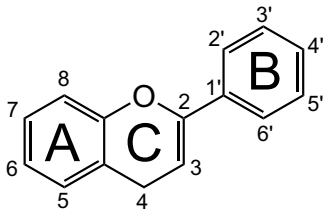


Figure 1.1 Basic flavonoid skeleton structure with conventional numbering of carbons and naming of rings.

More than 5,000 different flavonoids have been identified [13] and they are divided into the following six main subclasses of flavonoids: flavonols, isoflavones, flavanols, flavanones, flavones, and anthocyanidins [14, 15]. These subclasses show distinct saturation and oxygenation of the C-ring, as well as positioning of the B-ring. Most flavonoid subclasses are diversely distributed throughout the plant kingdom and flavonoids often occur in several different food sources (see Table 1.1).

Bioavailability

Recent studies estimate the mean dietary intake of flavonoids to be 370– 428 mg/day in Europe [16, 17] and 190 to 345 mg/day in the USA [18, 19]. The intestinal uptake of flavonoids is low and the systemic bioavailability for most types of flavonoids is reported to be only a few percent of the ingested dose ranging from not detectable to 30% [20, 21]. Flavonoids occur nearly exclusively as conjugated metabolites in plasma [22-25], and maximum concentrations of these flavonoid metabolites in plasma from normal dietary intake usually do not exceed the low micromolar range [20]. The plasma half-life of flavonoid conjugates is reported to be mostly around a few hours, ranging from 1h to 28 h depending on the flavonoid type (see Table 1.1 for details).

Absorption, distribution, metabolism and excretion of flavonoids

The low systemic bioavailability and high rate of metabolism are generally considered to oppose the biological activity of flavonoids. In the following sections, the absorption, distribution, metabolism and excretion of flavonoids are described; Figure 1.2 depicts a compartmentalized overview of the pharmacokinetics of flavonoids. The study of the pharmacokinetics and pharmacodynamics of microbial metabolites and their conjugates is outside the scope of this thesis and will not be described in further detail.

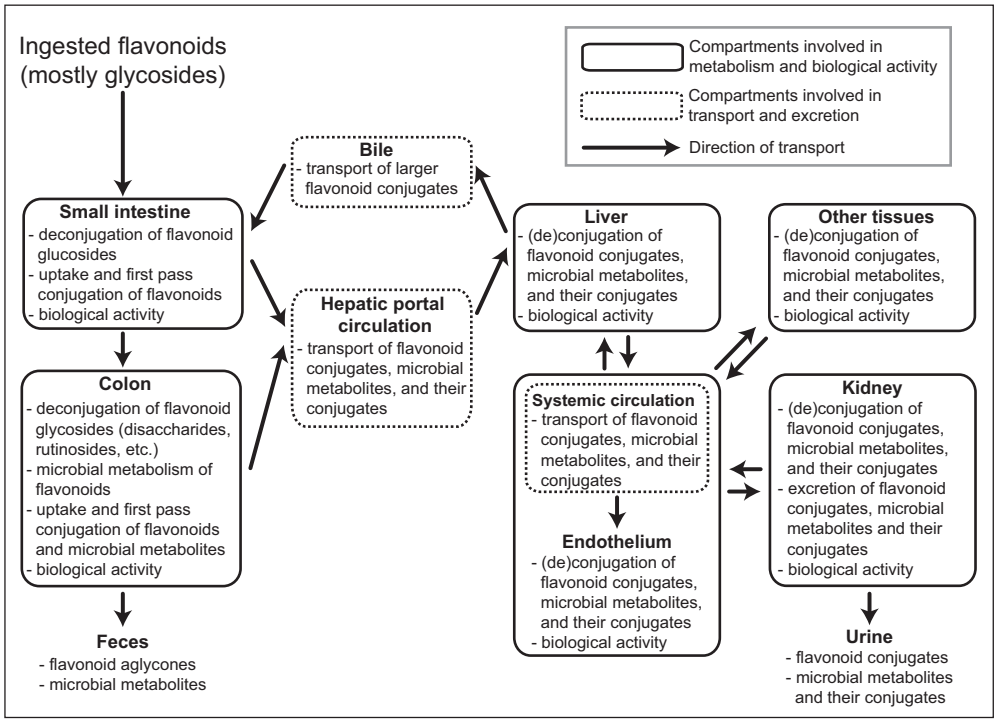


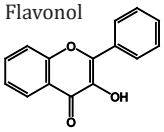
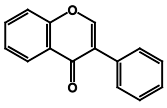
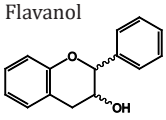
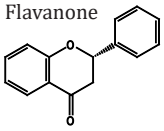
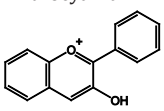
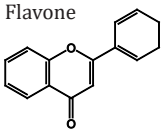
Figure 1.2 Schematic representation of major routes of absorption, distribution, metabolism and excretion of flavonoids.

Absorption

In plants, flavonoids occur as monomers, oligomers or polymers and are mostly present as glycosides (i.e. conjugated to sugar molecules); flavanols constitute an exception as they mostly occur in nonglycosylated form [26], as well as leaf surface flavonoids of certain herbs [27]. The glycosidic bonds of the majority of flavonoid glycosides resist food processing; however, during microbial fermentation of soy-based foods isoflavone aglycones (i.e. the unconjugated form) are released [28-32]. Therefore, apart from the exceptions named above, flavonoids are usually ingested as glycosides.

Due to their size and hydrophilicity, flavonoid glycosides are not readily absorbed in the gastrointestinal tract; the glycosides need to be deconjugated to their respective aglycones before they can be further metabolized and become systemically available.

Table 1.1 Chemical structures of flavonoid subclasses, common flavonoid members, main dietary sources and plasma kinetics [14, 15, 33-35]

Flavonoid subclass and chemical structure	Common flavonoids in subclass	Main dietary sources	Plasma kinetics
Flavonol 	Quercetin Kaempferol Myricetin	Vegetables (e.g. onion, kale, broccoli), fruits (e.g. apple), tea	T_{max} 0.5-5.5 h $T_{1/2}$ 2-11h [36-39] (one study reporting $T_{1/2}$ up to 28h [40])
Isoflavone 	Daidzein Genistein Glycitein	Legumes (especially soy bean)	T_{max} 4.5-6 h $T_{1/2}$ 3-8.5 h [41-44]
Flavanol 	Catechin Epicatechin	Cocoa, tea, fruits (e.g. apricot, apple)	T_{max} 0.5-4 h $T_{1/2}$ 1-6 h [45-49]
Flavanone 	Hesperetin Naringenin Eriodictyol	Citrus fruits (e.g. orange, grapefruit, lemon)	T_{max} 4.5 h $T_{1/2}$ 3.6-3.8 h [50]
Anthocyanidin 	Cyanidin Pelargonidin Peonidin Delphinidin Malvidin	Berries (e.g. blackberry, black currant), fruits (e.g. cherry, black grape), vegetables (e.g. aubergine)	T_{max} 1 h $T_{1/2}$ 2 h [51, 52]
Flavone 	Apigenin Luteolin	Leaf vegetables (e.g. celery), herbs (e.g. parsley)	No data available

The type of glycosylation determines at which site in the gastrointestinal tract the flavonoid glycosides can be deconjugated to the respective aglycone. Especially flavonoid glucosides (i.e. flavonoids conjugated to glucose) are subject to enzymatic deglycosilation in the small intestine by two major pathways. The glucosides can be deglycosylated by the brush-border enzyme lactase-phlorizin hydrolase (LPH) before the aglycone produced can diffuse passively into the enterocytes. Another route of uptake is through transport of the glycoside into the enterocytes by glucose transporters

and subsequent deglycosylation to the aglycone by cytosolic β-glucosidase (CBG). In the enterocytes, first pass conjugation leads to the formation of flavonoid metabolites which will be further transported into the portal vein to reach the liver, or back into the lumen of the gastrointestinal tract [53-55]. Other glycosides that are no substrates for LPH or CBG, for example rutinosides or disaccharides, reach the colon where they interact with colonic microbiota. For certain flavonoids it is suggested that they can be deglycosylated by colonic microorganisms and subsequently taken up via the colon [56], while many flavonoids are reported to be metabolized to smaller phenolic acids by colonic microorganisms [33, 57, 58]. The time to reach maximal plasma concentrations (T_{max}) is indicative of the place of absorption in the intestinal tract: short times (around 1h) indicate that the flavonoids are absorbed in the small intestine, while longer times (several hours) indicate uptake in the colon (see Table 1.1).

Distribution and Metabolism

During uptake and before entering the systemic circulation the flavonoid aglycones are extensively metabolized to glucuronidated, methylated and/or sulfated conjugates in intestinal tissue and the liver [20, 22, 59]. These conjugation reactions are catalyzed by distinct enzymes which have different tissue-distribution and substrate-specificities. UDP glucuronosyl transferases (UGTs) are present in the endoplasmic reticulum and especially active in the intestine, liver and kidney. UGTs catalyze the conjugation of polyphenols to glucuronic acid donated by uridine diphosphate glucuronic acid to form conjugates that are more hydrophilic than the parent compound. Catechol-O-methyltransferases (COMTs) are present in a wide range of tissues and catalyze the O-methylation of catechol-containing polyphenols to form metabolites that are slightly less polar than the parent compound [20, 54]. The cofactor donating the methyl group is S-adenosyl methionine. Sulfotransferases (SULTs) are present in a wide range of tissues and catalyze the conjugation of flavonoids to sulfate leading to the formation of conjugates that are more hydrophilic than the parent compound. The most common cofactor for this reaction donating the sulfo group is 3'-phosphoadenosine-5'-phosphosulfate. Interestingly, SULT activity can be inhibited by certain flavonoids [60-62].

Flavonoid metabolites that are formed in intestinal tissues and the liver are distributed throughout the body via the systemic circulation. In the systemic circulation, flavonoids bind to serum albumin which is likely to increase their plasma half-life [63] but at the same time leads to a decrease in their biological activity and availability to tissues [64, 65]; the extent of albumin binding is affected by conjugation [63, 66, 67].

There is only limited information available on the tissue distribution of flavonoids in humans as this cannot be studied noninvasively. It is reported that isoflavones could be detected in breast tissue [68], and flavanols and isoflavones in prostate tissue [69, 70]. In animal studies flavonoids could be detected in a wide range of tissues, namely

endothelial cells, brain, lung, heart, kidney, spleen, pancreas, prostate, uterus, ovary, mammary gland, testes, bladder, bone, stomach, small intestine and skin [71-85].

It is presumed that flavonoid aglycones can enter cells through passive diffusion, while many flavonoid conjugates, especially glucuronides, have to rely on active transport into the cells [86, 87]. Active cellular uptake of flavonoid conjugates appears to be affected by the conjugation position [88] and it has been shown in vitro for certain cell types, e.g. hepatocytes and macrophages, that flavonoid glucuronides can be deglucuronidated and/or further conjugated intracellularly, usually followed by cellular export [87-89]. Flavonoid glucuronides can also be deglucuronidated extracellularly by β-glucuronidase released from neutrophils and macrophages, and the activity of extracellular β-glucuronidases is reported to be higher during inflammation, which leads to higher concentrations of flavonoid aglycones at sites of inflammation [86, 90, 91].

Excretion

The main routes of excretion of flavonoid conjugates, their microbial metabolites, and their conjugates are via urine and feces. Especially flavonoid conjugates with a higher molecular weight can be excreted via the bile into the duodenum and, after deconjugation, undergo enterohepatic circulation, or reach the colon for microbial breakdown, colonic reuptake or excretion via feces [22, 33]. Urinary excretion of flavonoid metabolites is reported to vary from 1% to 30% of the ingested dose [40, 92-94].

Flavonoids used in this thesis

In the studies described in this thesis the flavonols quercetin and kaempferol, as well as the isoflavones genistein and daidzein are used as model flavonoids. These compounds are important dietary flavonoids and an extensive body of research already exists on the biological effects of their aglycone forms.

Quercetin and kaempferol

Flavonols are the most ubiquitous class of flavonoids; quercetin and kaempferol (Figure 1.3) are the two most common flavonoids of this class. Glycosides of quercetin and kaempferol occur in a wide range of plants and are present in foods such as onions, apples, certain berries, wine and tea [22, 33].

The most frequently reported association of flavonol intake with human health is an inverse correlation with the occurrence of cardiovascular diseases and related risk factors [95-99]. A positive correlation of flavonol intake and cognitive performance in middle-aged adults is reported for language and verbal memory. However, the same study reported a negative association with executive functioning (using the Forward and Backward Digit Span test) [100]. Further, a negative correlation of flavonol intake

with the occurrence of colorectal cancer is reported [101]. A range of in vivo, ex vivo and in vitro studies report results supporting but also contradicting these findings for flavonols and their metabolites [102].

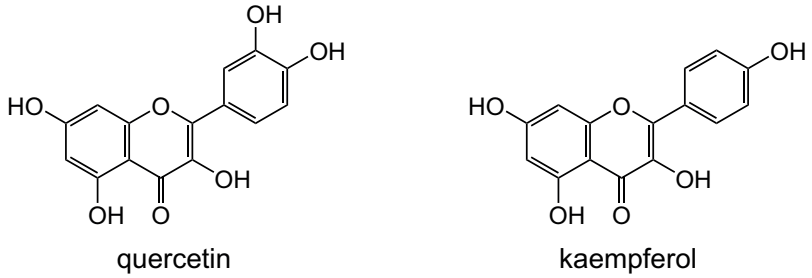


Figure 1.3 Chemical structures of quercetin and kaempferol.

After the consumption of foods rich in quercetin glycosides, the main metabolites in plasma are reported to be quercetin-3-*O*-glucuronide and quercetin-3'-*O*-sulphate; further glucuronidated, sulfated and/or methylated conjugates can be found at lower concentrations. Most conjugates detected in plasma are also reported to be present in urine [36, 37, 103-105]. After the consumption of endive, kaempferol-3-*O*-glucuronide is the only kaempferol conjugate reported to be present in plasma, in addition to small amounts of the aglycone. In urine, kaempferol-3-*O*-glucuronide is the major conjugate; additionally, a mono-sulfated and a di-sulfated conjugate are reported to be found [106].

Genistein and daidzein

Isoflavones are found nearly exclusively in leguminous plants, and the most important dietary sources are soy beans and soy-based foods. Genistein and daidzein (Figure 1.4) are the most common isoflavones [33, 107].

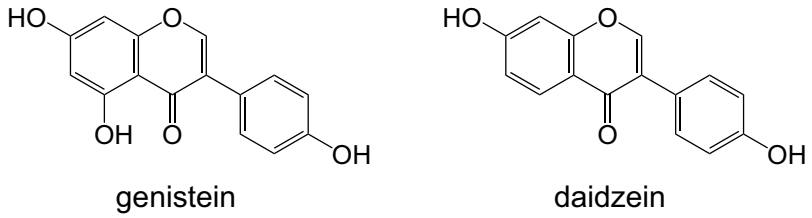


Figure 1.4 Chemical structures of genistein and daidzein.

Isoflavone intake was observed to be negatively correlated with systolic and diastolic blood pressure in hypertensive patients [108], and to improve arterial stiffness [109-114], which is a risk factor for cardiovascular disease. Further, improvement of menopausal symptoms [115] and bone health [116] is suggested to be correlated with isoflavone intake. Because of their phyto-estrogenic activity, isoflavones also carry the

potential to cause possible adverse health effects, for example those related to induction of proliferation of especially estrogen receptor-α (ERα) positive cells [117]. Some studies suggest an association between isoflavone intake and the reduction of cancer incidence at certain sites, while other studies do not find such an association [113]. A wide range of in vivo, ex vivo and in vitro studies report on biological effects of isoflavones [113].

After consumption of genistein and daidzein glycosides only marginal amounts of the respective aglycones are present in plasma and the most common conjugates of genistein and daidzein are glucuronides and/or sulfates conjugated at the 7-OH and the 4'-OH position [118-120].

The biological effects of the microbial metabolite equol are well-studied but are outside the scope of this thesis.

Aim and outline of the thesis

Despite the extensive knowledge available on the bioavailability and metabolism of flavonoids, only in a small fraction of the in vitro studies the metabolism of flavonoids is considered in the experimental design. The majority of these in vitro experiments are conducted with flavonoid aglycones or glycosides and generally no attention is paid to the conjugated metabolites that are present in biological fluids after uptake.

The aim of this thesis was to study the effect of conjugation on the biological activity of selected flavonoids towards selected endpoints. To this end, conjugation with glucuronic acid was taken as the model type of conjugation because it is considered to be the main metabolic conjugation reaction for flavonoids in both animal and man [33]. As glucuronidation of flavonoids alters their size, polarity and solubility, it was hypothesized that glucuronidation also affects their biological activity. It was shown that conjugation can affect the biological activity of flavonoids, and that the effect that conjugation has on the biological activity depends on the flavonoid used, the type and position of conjugation, as well as the assay system used [121].

Chapter 1, the present chapter, presents an introduction to the thesis as well as its aims and a general overview of its contents. Chapter 2 presents an overview of scientific literature on the influence of metabolic conjugation on the biological activity of flavonoids that was available at the beginning of the studies described in this thesis [121].

The often low commercial availability of flavonoid conjugates for in vitro use and as reference standards for their identification can hamper research on biologically relevant flavonoid metabolites. For certain flavonoid conjugates procedures for their chemical synthesis are described; however, these are usually very complex and specific to one conjugate of one flavonoid only. In Chapter 3, a versatile approach to biosynthesize flavonoid conjugates is described and a convenient semi-automated strategy for the

identification of flavonoid conjugates based on their elemental composition and ^{1}H-NMR spectra is presented.

In Chapters 4-6, the effect of glucuronidation on the biological activity of the chosen model flavonoids for different endpoints is characterized. Chapter 4 describes the effect of quercetin, kaempferol, and their 3-O-glucuronidated conjugates on endpoints related to peroxisome-proliferator activated receptor-gamma (PPAR-γ) activation. PPAR-γ is a ligand-activated nuclear receptor that plays a role in the regulation of fatty acid storage and energy metabolism; PPAR-γ is of pharmacological relevance as a target for the treatment of type-II diabetes [122]. To elucidate the consequences of flavonoid conjugation on PPAR-y activity several in vitro models for detection of PPAR-y mediated activation of gene expression were applied. A stably transfected reporter gene assay for PPAR-γ activation was employed, and next to reporter gene expression also the effect on PPAR-γ receptor mRNA expression was determined by qPCR. Furthermore, the intrinsic activity to activate the PPAR-γ ligand binding domain (LBD) was tested using the cell free Microarray Assay for Real-time Coregulator-Nuclear Receptor Interaction (MARCoNI) assay to study ligand induced LBD – nuclear receptor coregulator interactions. The MARCoNI assay allows to study the intrinsic potential of the flavonoids and their glucuronide conjugates for PPAR-γ activation in a cell free system, thus eliminating possible interference of differential cellular uptake between the aglycones and their glucuronide metabolites.

In Chapter 5, the intrinsic activity of genistein, daidzein, and their 7-*O*-glucuronidated conjugates to induce ERα and ERβ LBD – coregulator interactions was studied and compared using the MARCoNI assay. ERs are the main targets of estrogenic compounds, and upon their activation different transcriptional responses with opposite effects on cell proliferation and apoptosis are elicited; ERα activation stimulates cell proliferation, while ERβ activation causes apoptosis and reduces ERα mediated induction of cell proliferation [123].

Chapter 6 compares the inhibitory activity of kaempferol and its 3-*O*-glucuronidated conjugate on serine/threonine protein kinases. Protein kinases are involved in a wide range of physiological (both cellular and extracellular) processes by controlling signaling cascades and regulating protein functions. The inhibitory effect of the tested compounds on the activity of recombinant protein kinase A and cell lysate prepared from HepG2 cells using a microarray platform containing 141 peptides carrying putative phosphorylation sites was studied.

Chapter 7 provides a general discussion of the results presented in this thesis. Further, the advances in the knowledge on the effect of conjugation on the biological activity of flavonoids since the beginning of the studies described in this thesis are presented.

The thesis concludes with a summary of the results in Chapter 8.

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Chapter 2

A state-of-the-art overview of the effect of metabolic conjugation on the biological activity of flavonoids

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Abstract

Diets rich in flavonoids are associated with various positive health effects. Most in vitro research conducted to elucidate the modes of action of flavonoids uses flavonoid aglycones, but not their circulating conjugated metabolites. Conjugation alters the physico-chemical properties of flavonoids and it is widely assumed that this can affect their biological activity. This article gives a state-of-the-art overview of scientific literature reporting on the effect of metabolic conjugation on the biological activity of flavonoids. The biological activity of flavonoid aglycones is compared to that of their conjugates for a broad range of endpoints. Even though there is only limited literature available, it is shown that contrary to common belief, conjugation does not always decrease the biological activity of flavonoids. There are also endpoints which are unaffected by conjugation, and endpoints on which the conjugates have a higher or inverse activity when compared to the aglycone. The effects of conjugation can differ depending on the type and position of conjugation, the flavonoid concentration, the endpoint studied and the assay system used so that no general rules can be deducted. It is concluded that further studies on the effects of conjugation have to be done on a case-by-case basis, and a characterization of the stability and metabolic fate of the flavonoids in the assay system under consideration is needed to avoid false positive or false negative outcomes.

Introduction

Flavonoids are ubiquitously present in plants as secondary metabolites. Important dietary sources of flavonoids are fruits, vegetables and their juices, as well as tea, wine, and cocoa-derived products [1, 2]. In a varied diet, flavonoids are consumed on a daily basis. Diets rich in flavonoids are associated with the prevention of a variety of degenerative diseases, most importantly of cardiovascular diseases [2-5]. Flavonoids are known for their antioxidant activities *in vitro*. Apart from this antioxidant activity, flavonoids may induce their biological effects amongst others by acting as ligands for receptors, kinases, enzymes, and/or transport proteins [6-10].

Most flavonoids are present in foods as glycosides. Upon ingestion these glycosides undergo deconjugation to the corresponding aglycones. During uptake the aglycones are extensively metabolized to sulfated, methylated and/or glucuronidated conjugates in intestinal tissue or the liver before they enter the systemic circulation. Therefore, under physiological conditions flavonoids usually do not occur in the same form in biological fluids as they occur in plants and foods and it is widely assumed that conjugation and deconjugation can significantly influence the biological activity of flavonoids [11]. In spite of this, the majority of *in vitro* research identifying the modes of action and health effects of dietary flavonoids has been conducted with flavonoids in their aglycone forms or as they appear in plants and not with the conjugates actually present in the body.

A previously published overview [11] of scientific literature on the biological activities of some flavonoid conjugates presented the biological activities of quercetin metabolites but a direct comparison between the activity of the aglycone and that of the respective metabolites was not made.

Therefore, the objective of the present paper was to compare the biological activities of flavonoid aglycones to the activities of their respective metabolites for a broad range of flavonoids and endpoints, where possible at physiological or near physiological concentrations.

Activities of flavonoid aglycones and their conjugates have been reported for a large number of biological endpoints and assays. Most studies have focused on biological activities that can be related to the antioxidant and/or radical scavenging activity of flavonoid (conjugates) or to their potential to prevent formation of reactive oxygen species (ROS) or the adverse effects of ROS. Other endpoints have been studied as well, including for example effects on cyclooxygenase-2 activity, on adhesion molecules and the process of cell adhesion, on transport proteins, or on vasorelaxation-related endpoints. In the following sections the effects of flavonoid aglycones and their conjugates on these different endpoints are discussed in more detail providing an overview of the effects of (de)conjugation on the biological activity of flavonoids.

In most articles reporting on differences between the biological activity of flavonoid aglycones and their respective conjugates, no statistical significance testing was included. Therefore, the data presented in this overview are based on a critical assessment of the data presented and the authors' conclusions on the activity of the conjugates as compared to that of the aglycone.

Effects on oxidative stress and free radicals

Effects on formation of reactive oxygen species and other free radicals

An overview of the effects of flavonoids and their different conjugates (G= glucuronidated; M=methylated; S=sulfated) in assays detecting formation of reactive oxygen species (ROS) and other free radicals is provided in Table 2.1. From this overview it appears that most research on the effects of flavonoids and their conjugates on production and scavenging of free radicals and/or ROS was conducted with quercetin and its conjugates, and most predominantly with its glucuronidated and methylated conjugates (Table 2.1). The overview also reveals that results can differ between different assays, each using a different methodology to detect production and/or elimination of ROS and other radicals.

In spite of these apparent inconsistencies between different assays, the overview presented in Table 2.1 clearly reveals that most flavonoid conjugates inhibit ROS generating enzymes as effectively as or less effectively than the aglycone, with the actual effect depending on the conjugation position (Table 2.1); conjugation of hydroxyl moieties in the A- or C-ring with a glucuronide, methyl or sulfate moiety generally reduces the activity in the different assays to a higher extent than conjugation of hydroxyl moieties in the B-ring. An exception is nicotinamide adenine dinucleotide phosphate (NADPH) oxidase, a superoxide-generating enzyme, which is not inhibited by the aglycones but which can be inhibited by glucuronidated and methylated conjugates pointing at an increased instead of a decreased or unmodified activity of the flavonoid upon conjugation [13]. The extent to which the activity of the flavonoids is affected can further depend on the type of conjugation. For example sulfation of quercetin at the 3 position decreases its activity to a lesser extent than glucuronidation at the same position [16].

Also for free radical scavenging, the position of conjugation can influence the biological activity. In most cases the activity of the conjugates is lower than that of the aglycone, with only some exceptions where the conjugates are equally or more active [12, 15, 19, 23-26]. In some cases there is a difference depending on which chemical was used to induce radical formation, e.g. interleukin-1β or H_2O_2 [12].

Table 2.1 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) in assays detecting formation of ROS and other free radicals.

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(+)-Catechin					
Inhibition of ROS formation / scavenging		=/↑		A mixture of unidentified conjugates extracted from plasma of (+)-catechin dosed rats was used. The aglycone only inhibited IL-1β induced ROS, whereas the plasma extract inhibited both IL-1β and H_2O_2 induced ROS.	[12]
(-)-Epicatechin					
Inhibition of NADPH oxidase (ROS generation).	↑	↑	-	The aglycone does not inhibit NADPH oxidase.	[13]
$O_2^{\cdot-}$ scavenging.	↓/=/↑	↓	-	(G + M)[b]: ↓ Different assay systems were used and the results depend on the assay and conjugation position (3'M-EC > 4'M-EC [13]; EC-7G > EC-3'G [14])	[13, 14]
Kaempferol					
Influence on superoxide generation by phenazine methosulphate / NADH	↓	-	-	Kaempferol aglycone increases superoxide generation, whereas kaempferol-3-*O*-glucuronide inhibits the formation.	[15]
Luteolin					
Inhibition of NADPH oxidase.	-	↑	-	The aglycone does not inhibit NADPH oxidase.	[13]
$O_2^{\cdot-}$ scavenging (xanthine oxidase dependent generation)	-	↓	-	The methylated conjugates do not scavenge $O_2^{\cdot-}$.	[13]
Quercetin					
Xanthine oxidase, lipoxygenase and myeloperoxidase inhibition.	↓/=	↓/=	↓	(G + M)[b]: ↓ The results depend on the assay and conjugation position. Xanthine oxidase inhibition Q-4'G > Q-3'G > Q-7G > Q-3G [16]. Lipoxygenase inhibition: Q-7G > Q-3'G > Q-4'G > Q-3G [16].	[16-18]
NADPH oxidase inhibition.	↑	↑	-	The aglycone does not inhibit NADPH oxidase.	[13]
$O_2^{\cdot-}$ scavenging (xanthine oxidase dependent generation)	↑	↓/↑	-	(G + M)[b]: ↑ The results depend on the conjugation position (3'M-Q > 4'M-Q).	[13]
$O_2^{\cdot-}$ scavenging (NADPH oxidase dependent generation)	↓	-	=	(G + M)[b]: ↓	[19]
Antioxidant activity	↓[c]	↓[c]	↓[c]	(M + S)[b]: ↓[c] Determined by ABTS/persulphate and FRAP assay	[20]

Table 2.1 *continued*

DPPH radical scavenging	↓	↓	-		[17, 21, 22]
Inhibition of 13-hydroperoxyoctadeca-dienoic acid induced ROS production	↓	-	-		[22]
Inhibition of H_2O_2 induced ROS production	↓[c]/↑[c]	↓/=	-	Differences in activity of methylated quercetin depends on concentration and conjugation position (4'M-Q > 3'M-Q)[23]. During co-incubation the glucuronide is less active than the aglycone; after pre-incubation, the aglycone is not active [24].	[23, 24]
Inhibition of H_2O_2 induced dityrosine formation	↓/=	-	-	Only at very low concentrations (0.1 μM) the glucuronide was less active than the aglycone.	[25]
Superoxide generation by phenazine methosulphate / NADH	↓	-	↓/=	Quercetin aglycone and quercetin-3'-*O*-sulfate increase superoxide generation, whereas quercetin-3-*O*-glucuronide inhibits the formation.	[15]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available; A > B conjugate A is more active than conjugate B. [b] the conjugate is conjugated at two positions to different moieties. [c] Statistical analysis was carried out to assess differences between conjugates and aglycone ($p < 0.05$).

It is noteworthy that the flavonoids can exert effects on the oxidative stress related-endpoints already at low concentrations (e.g. 0.1 μM [25]). Another factor that can influence the activity of the flavonoids and their conjugates in the *in vitro* assays is whether the cell cultures are pre- or co-incubated with the flavonoids. Shirai *et al.* [24] report for example that after pre-incubation, quercetin aglycone does not reduce H_2O_2 induced ROS production, whereas glucuronidated quercetin does. In contrast, during co-incubation the aglycone reduces H_2O_2 induced ROS production more effectively than the glucuronidated conjugate. These observations may in part be related to differences in the stability of the flavonoid and its glucuronidated conjugate during the (pre) incubation conditions, as is discussed in further detail in section 8.

Effects on adverse effects of oxidative stress

Most research on the effect of flavonoid (de)conjugation on adverse effects of oxidative stress (Table 2.2) is reported for methylated conjugates. Methylated conjugates of (-)-epicatechin are as active as the aglycone [27-29], whereas methylated conjugates of quercetin are in most cases less active than the aglycone [17, 18, 23, 30]. In some instances, methylated quercetin is reported to be equally active [18] or more active [17] than the aglycone, depending on the assay and the position of methylation.

There is only little information reported for the effect of glucuronidation on these endpoints and no consistent pattern emerges for the effect on the biological activity. Quercetin glucuronides have been reported to be less active than the aglycone at 20 μM [31], while others report that at lower concentrations (1 μM) the glucuronides are less active than the aglycone but that at higher concentrations (10 μM) the glucuronides are as active as the aglycone [18]. In contrast, glucuronidated quercetin has been reported to protect human serum albumin (HSA) from peroxynitrite induced oxidation more actively than the aglycone [32]. For (-)-epicatechin it has been shown that the position of glucuronidation can influence the biological activity in assays detecting the adverse effects of oxidative stress [14].

Table 2.2 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) in assays detecting adverse effects of oxidative stress

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(+)-Catechin					
Protection of cells from oxidative damage	↓	-	-		[33]
(-)-Epicatechin					
Protection (of cells) from oxidative damage	↓	=	-	(G + M)[b]: ↓ There are differences in activity depending on the glucuronidation position (EC-7G > EC-3'G [14]).	[14, 27, 28, 33]
Quercetin					
Inhibition of lipoprotein chlorination	↓/=	↓	↓	(G + M)[b]: ↓ The glucuronidated and the methylated conjugate were only slightly less active than the aglycone	[18]
Protection of cells from oxidative damage	↓	↓/↑	-	The results differ with different assays. Different endpoints were assayed.	[17, 23, 33]
Protection of HSA from peroxynitrite induced oxidation	↑	-	↑		[32]
Protection from DNA / chromosomal damage	-	↓	↓	The flavonoids protected cells from hydrogen peroxide induced chromosomal damage, but at higher concentrations the aglycone also induced chromosomal damage	[23, 30]
Induction of EpRE mediated gene transcription	↓	↓	-		[10]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available; A > B conjugate A is more active than conjugate B. [b] the conjugate is conjugated at two positions to different moieties.

Sulfated metabolites of quercetin are reported to be less active than the aglycone, with the exception of the protection of HSA from peroxynitrite induced oxidation where the sulfated metabolite was more active than the aglycone [32].

Effects on low-density lipoprotein oxidation

Low-density lipoprotein (LDL) oxidation increases the risk for cardiovascular diseases like atherosclerosis and thrombosis [34]. Flavonoids can protect LDL from oxidation (Table 2.3). With respect to a possible effect of flavonoid conjugation on their potential to inhibit LDL oxidation, most information is available for glucuronidated conjugates for which it has been shown that the position of conjugation can determine whether the conjugate is less, equally or more active than the aglycone. In all reported cases conjugation of a hydroxyl group at the 3' and 4' position of the B-ring reduces the activity [14, 21, 35-37]. Conjugation of a hydroxyl moiety at the C-ring is reported to affect the biological activity only to a low extent [21, 37] and conjugation the biological activity only to a low extent [21, 37] and conjugation of hydroxyl groups in the A-ring on the 7 position can increase, reduce, or not affect the activity, depending on the assay used [14, 36, 37].

Table 2.3 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) on LDL oxidation

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
Daidzein					
Protection of LDL from oxidation	↓	-	↓	The glucuronidated conjugate was more active than the sulfated compound.	[36]
(-)-Epicatechin					
Protection of LDL from oxidation	↓/=	-	-	(G + M)[b]: ↓ Glucuronidation of the A-ring retained protective effects, whereas glucuronidation of the B-ring nearly completely abolished its activity.	[14]
Quercetin					
Protection of LDL from oxidation	↓[c]/=/↑	↓	↓[c]	(G + M)[b]: ↓ The activity of the glucuronides depends on the position of conjugation (Q-7G > Q-3G > Q-4'G [37]) and the assay system used.	[17, 18, 21, 31, 35, 37, 38]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available; A > B conjugate A is more active than conjugate B. [b] the conjugate is conjugated at two positions to different moieties. [c] Statistical analysis was carried out to assess differences between conjugates and aglycone ($p < 0.05$).

Effects on cyclooxygenase-2 gene transcription and enzyme activity

Cyclooxygenase-2 (COX-2), also known as prostaglandin endoperoxide synthase 2, converts arachidonic acid to prostaglandin. COX-2, unlike COX-1, is inducible and upregulated *inter alia* in rheumatoid arthritis and cancer. The effects of flavonoids and their conjugates on COX-2 gene transcription and enzyme activity (Table 2.4) appear to greatly depend on the concentration of the flavonoid. Especially at low concentrations (0.1 μM) nearly all conjugates showed an inverse activity compared to that of the aglycone [39]; quercetin aglycone inhibits COX-2 gene transcription and enzyme activity while the conjugates increase transcription and activity. At increasing concentrations the differences become less pronounced; at 10 μM gene transcription is inhibited by the conjugates more actively than by the aglycone [39].

Table 2.4 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) on assays detecting COX-2 activity and gene transcription

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
Quercetin					
COX-2 mRNA transcription	↓/↑	-	↓/↑	(G + M)[b]: ↓/↑ Depending on the concentrations, the conjugates had an inverse, lower, or higher activity than the aglycone. At low concentrations (i.e. 0.1 μM) the conjugates increased gene transcription; at higher concentrations (i.e. 10 μM) the conjugates decreased gene transcription. During co-exposure with IL-1β the glucuronidated and the sulfated metabolites were more actively decreasing COX-2 expression than the aglycone or than the conjugates that are both glucuronidated and methylated.	[39]
Inhibition of COX-2 activity	↓	↑	↓	(G + M)[b]: ↓ Depending on the concentrations and experimental conditions, the sulfated and glucuronidated conjugates had an inverse or lower activity than the aglycone.	[39, 40]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available. [b] the conjugate is conjugated at two positions to different moieties.

These effects on COX-2 gene transcription and enzyme activity are a striking example of the profound differences that can be caused by flavonoid conjugation. The observed dose-dependent differences for the conjugates underline the complexity of the effects flavonoid conjugation can have.

Effects on cell adhesion

The adhesion of monocytes to vascular endothelial cells is a key event in inflammation and atherosclerosis [41] and works through cross-linking of adhesion molecules on the cells [42]. It has been suggested that inhibition of platelet aggregation contributes to the prevention of cardiovascular diseases by flavonoids [43].

Quercetin, (+)-catechin and their metabolites are reported to reduce cell adhesion, adhesion molecule expression and gene transcription (Table 2.5). The effect of the flavonoids and their conjugates is concentration-dependent and interestingly, some metabolites are more active at lower concentrations than at higher concentrations. In a few cases, at low concentrations the methylated or glucuronidated conjugates are as active or even more active than the aglycone [44] but in most cases, glucuronidation and sulfation reduce the activity. Methylation is reported to affect the activity of quercetin in assays testing the influence on cell adhesion only marginally [45].

Table 2.5 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) on cell adhesion

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(+)-Catechin					
Reduction of cell adhesion		↑[c]		A mixture of unidentified conjugates extracted from plasma of (+)-catechin dosed rats was used.	[12]
Quercetin					
Reduction of cell adhesion		↓[c]		A mixture of unidentified conjugates extracted from plasma of quercetin dosed rats was used.	[12]
Reduction of ICAM-1, VCAM-1, E-selectin and MCP-1 expression	↓/=/↑	↓/=	↓/=/↑	(G + M)[b]: ↓/↑ Depending on the experimental conditions the glucuronidated and methylated conjugates had a lower or equal activity.	[44-47]
Reduction of ICAM-1, VCAM-1 and MCP-1 gene transcription	↓	-	↓	(G + M)[b]: ↓	[44, 47]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available. [b] the conjugate is conjugated at two positions to different moieties. [c] Statistical analysis was carried out to assess differences between conjugates and aglycone ($p < 0.05$).

(+)-Catechin aglycone is reported to not inhibit cell adhesion while plasma extract from rats containing (+)-catechin metabolites are reported to reduce cell adhesion [12]. The opposite was observed for quercetin; the aglycone inhibited cell adhesion, while the rat plasma extract containing metabolites did not. Plasma extracts were prepared from rats dosed intragastrically with (+)-catechin or quercetin 1h after administration of the flavonoids; the extracts contained predominantly unidentified metabolites.

The data reveal that conjugation can significantly affect the activity of flavonoids and their conjugates on cell adhesion, both reducing and increasing the effects. The observation of negative dose-response relationships shows that some conjugates can be active at low concentrations that are in the range of physiological plasma levels.

Effects on transport proteins

The human organic anion transporters 1 and 3 (OAT1 and OAT3, respectively) are involved in the transport of many metabolites of drugs in the kidney into the proximal tubules as well as in the intestine [48, 49]. Flavonoids are reported to be able to inhibit these transporters which can lead not only to serious food-drug interactions but can also influence physiological processes in the human body [49-51]. OAT1 and OAT3 selectively transport flavonoid conjugates, but are also inhibited by the flavonoids. OAT1 transports sulfated but not glucuronidated genistein and quercetin; OAT3 transports glucuronidated but not sulfated genistein and quercetin (Table 2.6). The aglycones are not actively transported but could pass cellular membranes by passive diffusion [49].

Genistein and quercetin are reported to inhibit OAT1 and OAT3; the sulfated conjugates are more actively inhibiting the transporters than the glucuronidated conjugates. The effect of quercetin glucuronidation on the inhibitory activity depends on the conjugation position [49]. The selectivity of the transporters for certain conjugates shows that some metabolites have to rely on active transport to pass cell membranes which can limit the availability of the metabolites to certain tissues.

Multidrug resistance protein (MRP) 1 and 2 are *ATP-binding cassette* transporter efflux proteins. Quercetin and 3'-methyl-quercetin equally inhibit MRP1 and 2 while 4'-methyl-quercetin has a lower inhibitory activity on MRP1 and no inhibitory effect on MRP2 [52]. A mixture of glucuronidated quercetin (around 85% quercetin-7-*O*-glucuronide) formed during incubation with H4IIE cells inhibited MRP1 and 2 activities more effectively than the aglycone.

Effects on angiogenesis and vasorelaxation

The most prominent health outcomes linked to the intake of polyphenols is the prevention of cardiovascular diseases [5]. Quercetin and its metabolites influenced several *in vitro* and *ex vivo* endpoints related to vascular function, such as proliferation, chemotaxis and signaling kinases in coronary venular endothelial cells (CVEC) [53] and vasodilation of aortic rings [19, 54] (Table 2.7). *In vivo*, (-)-epicatechin and its metabolites influence flow mediated vasodilation (FMD) [55].

Table 2.6 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) on transport proteins

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
Genistein					
Inhibition of OAT1	↓	-	=/↑	The differences are concentration-dependent	[49]
Inhibition of OAT3	↓	-	↓/=	The differences are concentration-dependent	[49]
OAT1 mediated uptake	=	-	↑	The aglycone and glucuronide are no substrate for the transporter; the aglycone could pass through passive diffusion	[49]
OAT3 mediated uptake	↑	-	=	The aglycone and sulfated conjugate are no substrate for the transporter; the aglycone could pass through passive diffusion	[49]
Quercetin					
Inhibition of OAT1	↓	-	↑		[49]
Inhibition of OAT3	↓/=/↑	-	=	The differences in the glucuronides' activities depend on the conjugation position (Q-3'G > Q-7G > Q-3G)	[49]
OAT1 mediated uptake	=	-	↑	The aglycone and glucuronide are no substrate for the transporter; the aglycone could pass through passive diffusion	[49]
OAT3 mediated uptake	↑	-	=	The aglycone and sulfated conjugate are no substrate for the transporter; the aglycone could pass through passive diffusion	[49]
Inhibition of MRP 1 and 2	-	↓[b]/=	-	Methylation at the 3' position does not affect MRP activity, while methylation at the 4' position reduces MRP activity.[b]	[52]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available; A > B conjugate A is more active than conjugate B. [b] Statistical analysis was carried out to assess differences between conjugates and aglycone ($p < 0.05$).

Differences in the biological activity of quercetin and its metabolites on endpoints affecting angiogenesis and vasorelaxation are reported. Depending on the endpoint studied and the assay used, increased, equal and reduced activities are reported [19, 53, 54]. Interestingly, quercetin-3'-*O*-sulfate has an opposite pattern of activity compared to the aglycone and glucuronide regarding most assays related to angiogenesis [53]. Unlike quercetin aglycone and glucuronide, the sulfated quercetin conjugate does not inhibit the activity of vascular endothelial growth factor (VEGF); in contrast, the sulfated conjugate promotes endothelial cell proliferation and angiogenesis on its own, while the aglycone and glucuronide do not [53].

Ingestion of flavanol-rich cocoa or pure (-)-epicatechin improved vascular function in healthy volunteers [55]. The predictive value of the circulating flavonoids in plasma of volunteers for the observed cardiovascular effects was statistically analyzed. In a

multivariate regression analysis, only (-)-epicatechin-7-*O*-glucuronide and (-)-epicatechin aglycone were predictive for the magnitude of FMD.

Table 2.7 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) on angiogenesis and vasorelaxation

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(-)-Epicatechin					
Correlation with flow-mediated vasodilation *in vivo* (univariate)	↑	↑	-	(G + M)[b]: ↑ The aglycone is not predictive for FMD	[55]
Correlation with flow-mediated vasodilation *in vivo* (multivariate)	=	↓	-	(G + M)[b]: ↓	[55]
Quercetin					
Effects on angiogenesis	↓/=/↑	-	↓/=/↑	The results depend on the experimental conditions; the aglycone did not affect several endpoints.	[53]
Vasorelaxation and vasodilation	↓/=	-	↓/=	(G + M)[b]: ↓ The results depend on the experimental conditions	[19, 54]
Nitric oxide scavenging	↓	-	-		[26]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available.
[b] the conjugate is conjugated at two positions to different moieties.

Altogether, the consequences of flavonoid conjugation for the effect on the prevention of cardiovascular disease is difficult to predict from the *in vitro* studies performed so far, as different models show variable effects of the different conjugates as compared to the aglycone. What emerges, however, is that for some *in vitro* surrogate endpoints for vascular disease the conjugates may be equally or more effective as the aglycone.

Effects on various other endpoints

Glucuronidation can decrease the biological activity of the tested flavonoids on various other endpoints, i.e. cell viability [56], estrogenic activity [57], silent information regulator two ortholog 1 (SIRT1) deacetylase activity [58], monoamine oxidase A (MAO-A) activity [59], osteoclast formation [60] and erythrocyte deformability [61] (Table 2.8). SIRT1 deacetylase activity is increased by quercetin aglycone but reduced by the glucuronide [58].

The effects of (+)-catechin and quercetin methylation on platelet function [62] and cellular protection from UV damage [63], respectively, depend on the experimental setups and can be higher, equal or lower than the aglycone. Methylation of quercetin decreases the covalent binding to DNA of HepG2 cells [64].

General factors affecting the studies on the effects of flavonoid conjugation on biological activities

There are several general factors that need to be considered when studying the effects of flavonoid conjugation on the biological activity of flavonoids. Flavonoid conjugation can affect polarity and size of the molecules which in turn can affect protein and lipid binding [31, 37, 46, 65, 66], cellular uptake [23, 27, 67], as well as stability against oxidative degradation [26, 30] (Tables 2.9 and 2.10). These factors can affect the availability of the flavonoids to cells and tissues.

Table 2.8 Effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated) on various further endpoints

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(+)-Catechin					
Effects on platelet function	-	↓/=/↑	-	The effects of (-)-epicatechin and its metabolite are not reported on here as these were tested at different concentrations. Depending on the specific endpoints there were differences in conjugate activity depending on position of methylation (4'M-CAT >/= 3'M-CAT)	[62]
Daidzein					
Estrogenic activity	↓/=	-	-	The results differ depending on the experimental setup	[57]
(-)-Epicatechin					
Protection from UV induced cell damage	-	↓[b]/=	-	The results depend on the experimental setup.	[63]
Genistein					
Estrogenic activity	↓	-	-		[57]
Quercetin					
Decrease in cell viability of cortical neurons (MTT assay)	↓	↓	-	Akt phosphorylation was reduced.	[56]
MAO-A activity	↓	-	-		[59]
RANKL induced osteoclast formation in RAW264.7 cells	↓	-	-		[60]
Improvement of erythrocyte deformability	↓[b]	-	-		[61]
SIRT1 deacetylase activity	↓	-	-	The aglycone increases SIRT01 deacetylase activity while the glucuronide reduces it.	[58]
Covalent binding to DNA of HepG2 cells	-	↓	-		[64]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available; A >/= B conjugate A is more active than or equally active as conjugate B. [b] Statistical analysis was carried out to assess differences between conjugates and aglycone ($p < 0.05$).

Instability, most importantly through auto-oxidation, has been reported and discussed to occur with flavonoids *in vitro* [23, 56, 68-70] and may be influenced by conjugation. Lodi *et al.* [26] show that especially glucuronidation protects quercetin from auto-oxidative degradation. Protection from auto-oxidation can increase the half- life of the compounds thereby increasing their apparent biological activity, whereas instability in in vitro experiments can lead to false negative results, or false positive results in the case that reactive metabolites are formed. Additionally, radicals can be generated through auto-oxidation and therefore flavonoids can also act as pro-oxidants.

Furthermore, flavonoids have been reported to be taken up and further metabolized by various cell types during incubation [10, 24, 71, 72]. Given that conjugation may affect the biological effects of flavonoids those changes may influence the results and need to be taken into consideration. Unfortunately, most reports do not touch upon this subject and do not characterize the fate of the flavonoids tested by analyzing samples at the end of the incubation period.

Table 2.9 Effects of flavonoids and their different conjugates (G= glucuronidated; M=methylated; S=sulfated) in assays detecting pro-oxidant activity

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
Quercetin					
H_2O_2 generation in WIL2-NS cell cultures	-	↓	↓		[30]
Superoxide anion radical generation through auto-oxidation at pH 9	↓	=/↑	↓	(G + M)[b]: ↓ The results depend on the assay system.	[26]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available. [b] the conjugate is conjugated at two positions to different moieties.

Another issue that influences the outcome of studies on the effects of conjugation on biological effects of flavonoids is that due to their size and polarity, especially glucuronidated metabolites of flavonoids have to rely on active transport or deconjugation to be able to pass cell membranes and exert certain biological activities within cells [10, 71]. Some evidence is presented that the biological activity of some conjugates on specific endpoints depends on deglucuronidation of glucuronidated metabolites [10]. It has been reported that glucuronidated quercetin needs to be deglucuronidated before uptake into human umbilical vein endothelial cells (HUVEC) [71]; for HepG2 cells it has been shown that quercetin glucuronides can be taken up without deconjugation [72]. Such differences may explain why the effect of conjugation on a biological activity is different in different model systems. Additionally, in sites of inflammation there is a higher expression of β-glucuronidases [73] and therefore potentially a higher availability of the deglucuronidated flavonoids.

Table 2.10 Effects of flavonoids and their different conjugates (G= glucuronidated; M=methylated; S=sulfated) in assays detecting protein binding and cellular uptake

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(+)-Catechin					
Uptake by brain endothelial cells	=	↑	-	The aglycone and glucuronide were not taken up	[67]
(-)-Epicatechin					
Association with dermal fibroblasts	↓	↑	-		[27]
Uptake / association with cortical neurons	↓	↑	-		[27]
Hesperetin					
Uptake by brain endothelial cells	↓	-	-	After incubation with the glucuronidated conjugate also hesperetin aglycone was detected in the cells	[67]
Transfer across *in vitro* blood-brain barrier	↓	-	-		[67]
Naringenin					
Uptake by brain endothelial cells	↓	-	-	After incubation with the glucuronidated conjugate also naringenin aglycone was detected in the cells	[67]
Transfer across *in vitro* blood-brain barrier	↓	-	-		[67]
Quercetin					
Human serum albumin binding	↓[b]/=	↑	↓/=/↑	The activity of the sulfated metabolites depends on conjugation position (Q-7-G > Q-3-G > Q-4'G [b] [37] Q-7S > Q-3S = Q-4',7S [65, 66])	[37, 65, 66]
Bovine serum albumin binding	-	↓	↓/=	The activity of the sulfated metabolites depends on conjugation position (Q-7S > Q-3S > Q-4',7S [65, 66])	[65, 66]
Incorporation into liposomes	↓	-	↓		[46]
Binding to phospholipid membranes of LUV	↓[b]	-	-		[31]
Cellular uptake / association	↓	↑	-	Glucuronidated quercetin was tested with PC12 cells [22]. Methylated quercetin was tested with H9c2 cardiomyoblasts [23].	[22, 23]

[a] ↓ the respective conjugate(s) have a lower activity than the aglycone; = the respective conjugates have an equal activity to the aglycone; ↑ the respective conjugates have a higher activity than the aglycone; - no data available; A >/= B conjugate A is more active than or equally active as conjugate B. [b] Statistical analysis was carried out to assess differences between conjugates and aglycone ($p < 0.05$).

Conjugation can also cause changes in the polarity of the flavonoids which in turn can change the partitioning and thus distribution in the body or test system, thereby affecting the ultimate biological effect. Binding to serum albumin can reduce the activity and availability of circulating flavonoids [13, 71, 74] but it might also increase their half-life [66]. Albumin binding differs between the aglycone and different metabolites [37, 65, 66]. Glucuronidation, especially of the hydroxyl moiety at the 4' position of the B-ring, strongly reduces quercetin binding to HSA; glucuronidation of the hydroxyl moieties in the A-ring has the least effects on HSA binding [37]. Sulfation at different positions only marginally reduces binding, while disulfation at the hydroxyl groups at both the 4' and 7 positions strongly reduces binding [37, 65, 66]. Methylation can increase binding to HSA [66], Quercetin binding to bovine serum albumin (BSA) in contrast is reduced by sulfation and methylation [65, 66].

Discussion and conclusions

This review clearly shows that conjugation and deconjugation can affect the biological activity of flavonoids. It is apparent that the predominant view that conjugation always reduces flavonoid activity does not hold true. The effects of conjugation can differ greatly depending on the type and position of conjugation, the flavonoid concentration, the endpoint studied and the assay system used so that no general rule can be deducted. In a majority of the cases the conjugates are less active or equally active to the aglycone, but in some cases the conjugates were observed to be more active than the aglycone or even showed an inverse activity. This shows that the effects of conjugation of flavonoid aglycones on their biological effect has to be taken into account when studying the biological activity of flavonoids in *in vitro* models and that preferably in such studies relevant flavonoid conjugates should be included. Furthermore, given that in tissues *in vivo* deconjugation may occur [10, 75] studying the aglycone is still relevant also because comparison of its activity to that of its conjugates provides insight in the effect of conjugation on the biological activity. The effect of flavonoid glucuronidation is more extensively studied than the consequences of methylation and sulfation, the latter being the least extensively investigated. This is at least in part due to the often limited availability of sufficient quantities of the conjugated compounds. (Bio)synthesis is usually laborious and time consuming and only yields small quantities [76-80]. Fortunately, an increasing amount of flavonoid conjugates becomes commercially available or their synthesis is described in scientific literature.

Some general factors like protein binding or stability to auto-oxidation can affect the availability of the compounds during the experimental procedures and thus affect the results. Some of these general factors might be part of an underlying mechanism of the observed different activities of the conjugates. Especially through auto-oxidation

the availability of the compounds in the assay system can be limited, and through the resulting introduction of oxidative stress also further unwanted alterations in the assay system can be caused. This stresses the need to define the stability and (metabolic) fate of the compounds in the assay system to be able to draw solid conclusions.

Altogether this review reveals that the effects of flavonoid conjugation on the biological activity can differ greatly between different assay systems and the endpoints studied. It appears that conjugation can do more than just reduce the biological activity of the flavonoids. Research on the consequences of flavonoid conjugation on the biological activity has to be done on a case-by-case basis to fully understand the effects of flavonoid conjugation and the mechanisms behind it. It is crucial that relevant conjugates, concentrations and incubation times are chosen and that the stability and the metabolic fate of the flavonoids in the assay system under consideration is adequately characterized to avoid false positive or false negative outcomes.

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Chapter 3

Flavonoid conjugate biosynthesis and identification using the Metabolite Identification Database (MetIDB)

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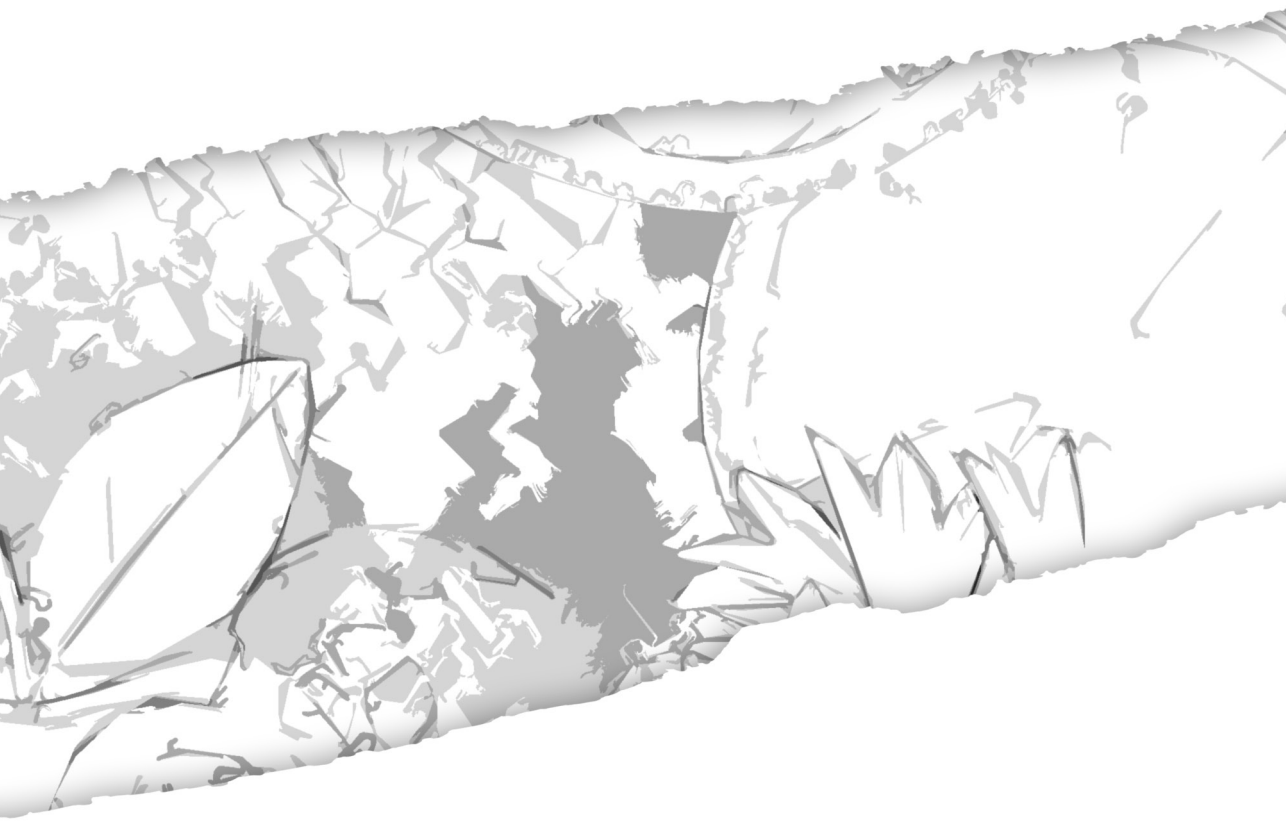

In preparation

Abstract

The consumption of flavonoids is linked to various beneficial health effects. While flavonoids are extensively metabolized during uptake and occur nearly exclusively as conjugated metabolites in the systemic circulation, most in vitro studies to date study the biological effects of the aglycone forms instead of the effects of the conjugated metabolites. This is in part due the low commercial availability of the conjugated metabolites. To facilitate the use of relevant metabolites in in vitro research, a simple but versatile method for the biosynthesis of metabolically relevant flavonoid conjugates using Hepa-1c1c7 cells as a model cell line, and recombinant UGTs as model enzymes is presented. A range of different types of flavonoids was used as model substrates (i.e. hesperetin, naringenin, kaempferol, quercetin, glycitein, genistein, daidzein, and dalbergin). Subsequently, the conjugates were characterized and identified based on their LC-MS and ^{1}H-NMR characteristics using the Metabolite Identification Database (MetIDB), a publicly accessible database of predicted and experimental ^{1}H-NMR spectra of flavonoids. Using the described method of biosynthesis, sufficient amounts of relevant flavonoid conjugates for in vitro bioassays can be generated and the use of MetIDB proved to be a useful tool for the quick reliable identification of even small amounts of flavonoid conjugates.

Introduction

Flavonoids are ubiquitously present in plants as secondary metabolites. Important dietary sources of flavonoids are fruits, vegetables and their juices, as well as tea, coffee, wine, and cocoa derived products [1, 2]. Flavonoids are generally consumed on a daily basis. Diets rich in flavonoids are associated with the prevention of a variety of degenerative diseases, such as cardiovascular diseases [1, 3], neurodegenerative diseases [4] and diabetes [5, 6]. Most flavonoids are present in foods as glycosides. Upon ingestion these glycosides undergo deconjugation to the corresponding aglycones. During uptake these aglycones are extensively metabolized to methylated, glucuronidated and/or sulfated conjugates in intestinal tissue or the liver before they enter the systemic circulation. Therefore, under physiological conditions flavonoids usually do not occur in the same form in biological fluids and tissues as they occur in plants and foods, and it is widely accepted that conjugation or deconjugation can significantly influence the biological activity of flavonoids [7, 8]. In spite of this, the majority of in vitro research studying the modes of action and health effects of dietary flavonoids has been conducted with flavonoids in their aglycone forms and not with the conjugates actually present in the body. One of the reasons hampering the research with relevant flavonoid conjugates is that most conjugates are not commercially available and have to be custom synthesized. For a limited number of flavonoid conjugates chemical synthesis pathways are described in literature [9-17], and some studies report the biosynthesis of flavonoid conjugates using (genetically engineered) bacteria [18, 19]. An alternative method of generating flavonoid conjugates is through biosynthesis using microsomes, recombinant metabolic enzymes, S9 fractions or cell cultures [20-36]. The biosynthesis of flavonoid conjugates, as opposed to chemical synthesis, offers great flexibility regarding the use of different flavonoid substrates. In addition, the use of metabolically active cells or (sub-)cellular fractions to conjugate flavonoid aglycones can yield methylated, glucuronidated and/or sulfated flavonoid conjugates, depending on the cell type and flavonoid used.

A challenge when synthesizing flavonoid conjugates is the subsequent identification of the molecules formed, as authentic reference standards are often lacking. In this article we describe a convenient and efficient way of flavonoid conjugate biosynthesis using Hepa-1c1c7 cells or recombinant uridine 5'-diphospho-glucuronosyltransferases (UGTs) to generate relevant flavonoid conjugates in quantities sufficient for bioassays (in the lower milligram range), as well as their quick and reliable identification based on LC-MS and ^{1}H-NMR spectra using MetIDB [37], a publicly accessible database of predicted and experimental ^{1}H-NMR spectra of flavonoids. To illustrate this principle, in this article the biosynthesis as well as MetIDB based identification of conjugates of the relevant dietary flavonoids hesperetin, naringenin, kaempferol, quercetin, glycitein, genistein, daidzein, and dalbergin are described.

Materials & Methods

Materials

Hesperetin (CAS no: 520-33-2), naringenin (CAS no: 480-41-1), kaempferol (CAS no: 520-18-3), quercetin (CAS no: 117-39-5), daidzein (CAS no: 486-66-8), L-ascorbic acid (CAS no: 50-81-7), and uridine 5′-diphosphoglucuronic acid trisodium salt (UDPGA; CAS no: 63700-19-6) were purchased from Sigma Aldrich (Missouri, USA). Glycitein (CAS no: 40957-83-3), genistein (CAS no: 446-72-0), and dalbergin (CAS no: 482-83-7) were purchased from Extrasynthese (Genay Cedex, France). The aforementioned chemicals were dissolved in dimethylsulphoxide (DMSO, 99.9%) from Acros (Geel, Belgium) and stored at –20 °C. Human UGT1A1, UGT1A9 and UGT2B7 supersomes were purchased from BD Biosciences (Franklin Lakes, NJ, USA). Acetic acid was purchased from VWR International (Darmstadt, Germany). Methanol (HPLC supra-gradient) and acetonitrile were purchased from Biosolve BV (Valkenswaard, the Netherlands). Minimum Essential Medium α (αMEM) and trypsin were purchased from Gibco Invitrogen Corporation (Breda, The Netherlands). Fetal bovine serum (FBS) was purchased from PAA (Pasching, Austria). Dextran-coated charcoal-stripped fetal calf serum (DCC-FCS) was purchased from Thermo Scientific (Waltham, Missouri, USA). Nanopure water was prepared with a Barnstead Nanopure Type I ultrapure water system.

Cell culture

The murine liver hepatoma cell line Hepa-1c1c7 was a kind gift from Dr. M.S. Denison, (University of California, Davis, CA, USA). The cells were cultured in αMEM supplemented with 10% FBS. The cells were maintained at 37 °C in a humidified atmosphere with 5% CO2.

Biosynthesis of flavonoid conjugates using Hepa-1c1c7 cells

To produce conjugates of hesperetin, naringenin, kaempferol, quercetin, and glycitein, Hepa-1c1c7 cells forming a confluent monolayer in 150 cm^2 cell-culture flasks (Corning Inc., Corning, NY) were exposed to the flavonoid aglycones (40 μM, 0.5% DMSO) in αMEM (w/o phenol red, 20ml/flask) supplemented with 5% DCC-FCS at 37°C and 5% CO2 in a humidified atmosphere. To prevent auto-oxidation, quercetin was co-incubated with 750 μM ascorbic acid. Cell culture medium was collected after 24h of exposure. To precipitate proteins, 30% methanol was added to cell culture medium containing flavonoid conjugates (final concentration of methanol in samples: 23% v/v), the mixtures were left on ice for 20 minutes and subsequently centrifuged at 2,700 rcf for 40 minutes at 4°C. The resulting supernatant was subjected to solid phase extraction (SPE) as described below.

Biosynthesis of flavonoid conjugates using recombinant UGTs

To produce glucuronidated conjugates of genistein, daidzein, and dalbergin, incubation mixtures of 1ml containing 40 µM of flavonoids from a 200x concentrated stock in DMSO, 0.2 mg of individual UGTs in Tris-HCl buffer (pH 7.5) with 2 mM $MgCl_2$, and 2 mM UDPGA were incubated in a shaking water bath at 37°C. Dalbergin was incubated for 4h with UGT1A9, daidzein was incubated for 6h with UGT1A1 and genistein was incubated for 3h with UGT1A9. These isoenzymes were selected as they showed the highest rate of conjugate formation for the respective flavonoids when comparing the activities of UGT isoenzymes 1A1, 1A9, and 2B7. The incubation times were selected to achieve full substrate conversion. The reactions were terminated by the addition of 30% ice-cold methanol (final concentration of methanol in samples: 23% v/v) and the mixtures were left on ice for 15 minutes prior to centrifugation at 16,000 rcf for 10 minutes to precipitate proteins. The resulting supernatant was subjected to SPE as described below.

SPE clean-up of flavonoid conjugates

To decrease the concentration of methanol in the samples, 1 volume of water containing 0.5% acetic acid was added to the supernatants containing flavonoid conjugates and the mixture was subjected to SPE using Waters Oasis HLB 2cc (60mg) extraction cartridges. The cartridges were conditioned with 1 ml methanol and equilibrated with 1 ml 0.5% acetic acid before loading the samples. After loading of the samples, the columns were washed with 2.5 ml 0.5% acetic acid and dried subsequently. The flavonoid conjugates were eluted with 3 ml methanol. Methanol was evaporated under a stream of nitrogen and samples stored at -20 °C until further use.

UPLC analysis

After SPE, the dried conjugates produced in the incubations were resuspended in nanopure water and analyzed chromatographically using a Waters ACQUITY UPLC H-Class System with an ACQUITY UPLC BEH C18 1.7 µm (2.1 x 50 mm) column, connected to a Waters ACQUITY UPLC photodiode array detector. Detection was performed between 190 and 350 nm. UPLC chromatograms presented are based on detection at 280 nm. The mobile phases used were nanopure water (+0.1% acetic acid) and acetonitrile (+0.1% acetic acid). Subsequently, the samples were freeze-dried and those samples containing several conjugates (i.e. samples made using quercetin, kaempferol and hesperetin) were subjected to separation by HPLC as described below, while samples containing a single conjugate (i.e. samples made using naringenin, glycitein, genistein, daidzein, and dalbergin) were directly subjected to LC-MS and ^{1}H-NMR for identification as detailed below.

HPLC analysis and separation of flavonoid conjugates

The freeze-dried samples containing several flavonoid conjugates (i.e. those of hesperetin, quercetin, and kaempferol) were resuspended in nanopure water containing 0.5% DMSO and separated using a Waters HPLC system consisting of a Waters 600 pump and controller, Waters 717plus autosampler and a Waters 2996 diode array detector with an Alltima C18 5U column (4.6 mm × 150 mm; Alltech, Breda, The Netherlands). Detection was performed between 180 and 400 nm. Chromatograms presented are based on detection at 350 nm for quercetin and kaempferol and at 280 nm for hesperetin. Mobile phases were (A) nanopure water containing 0.1% acetic acid and (B) methanol (for hesperetin and kaempferol) or (B) acetonitrile (for quercetin).

Fractions containing the individual conjugates were collected at the outlet of the photodiode array detector. Methanol or acetonitrile was evaporated from the samples under a stream of liquid nitrogen and the remaining water removed by freeze-drying. The resulting conjugates were subjected to identification by LC-MS and ^{1}H-NMR as detailed below.

LC-MS experiments

The freeze-dried samples were resuspended in nanopure water and measured on an LC-MS system consisting of an Agilent 1200 quaternary solvent delivery pump, Agilent 1200 degasser, Agilent 1200 autosampler coupled to a Bruker Daltonics MicrOTOF ESI mass spectrometer. LC-MS analysis was performed using an Alltima HP column (Alltima, 4.6 × 150 mm i.d., particle size 3 µm) with a pre-column at a flow rate of 0.4 ml/min. The MicrOTOF ESI mass spectrometer was calibrated with a formic acid reference solution. The mass values observed were within 5 ppm precision. Based on the measured masses as well the isotopic pattern intensities, molecular formula calculations were performed with C, H, and O atoms included. Using the molecular formula's obtained, all possible flavonoid conjugates that fit the molecular formula deduced, were created as described below (see identification of flavonoid conjugates).

NMR experiments

NMR spectra were obtained as described before [38]. In brief, the purified and freeze-dried compounds were dissolved in 200 µl methanol-D4 and transferred to a 3 mm NMR tube (Bruker Match system). Measurements were performed at 300 K on an Avance III 600 MHz NMR spectrometer with 5 mm cryoprobe. A total of 3200 transients were acquired for each sample using a NOESY 1D pulse sequence. Spectra were aligned to the methanol resonance at 3.306 ppm.

Identification of flavonoid conjugates

The identification of the conjugates was based on the LC-MS results combined with a comparison of the predicted and experimental ^{1}H NMR chemical shifts of the molecules. First, the elemental composition of the prepared conjugates was derived based on their experimentally determined masses. By comparing the elemental composition of the substrates with that of the metabolites the type of conjugation could be determined. For each of the flavonoid aglycones used as substrate, all isobaric compounds (i.e. molecules with identical molecular formula) were selected from MetIDB. In a next step all possible glucuronidated conjugates of these compounds were created by in-silico biotransformations as described by Mihaleva et al. [37]. This resulted in a list of candidate molecules for which ^{1}H-NMR spectra were predicted as also described by Mihaleva et al. [37]. Subsequently, the splitting patterns resulting from the ^{1}H-^{1}H couplings of the predicted and the measured spectra were compared, and candidate compounds that were not matching the experimental data were excluded from the list of candidate molecules. This step can be done in automation using PERCH (PERCH Solutions Ltd., Kuopio, Finland) when samples extracted from natural sources are being identified. However, some chemically synthesized aglycones were used as substrates for the biosynthesis of the conjugates, which are racemic mixtures due to the presence of a chiral center (i.e. hesperetin and naringenin); in their natural counterparts one enantiomer is predominantly present. The resulting conjugates of these flavonoids were diastereoisomers and their spectra had to be assessed manually. Where the ^{1}H-resonances were split due to diastereoisomerism in the experimentally determined ^{1}H-NMR spectra, the average of the ^{1}H-resonances of the corresponding diastereoisomers were used for the comparison with the predicted spectra. Differences in ^{1}H-NMR resonances for the respective comparable protons in the diastereoisomers were within 0.1 ppm. Subsequently, the chemical shifts of the in-silico created molecules and the measured chemical shifts were compared using the same algorithm as described by Mihaleva et al. [37] and a scoring was prepared that ranked the remaining candidate compounds by similarity between measured and predicted spectra. For this ranking, the differences in predicted and measured chemical shifts were calculated in such a manner that all chemical shifts that showed a larger difference than 0.1 ppm between predicted and measured chemical shift were given a penalty score of 1 for each 0.1 ppm difference. Therefore, the lowest score indicates the best match of the predicted spectrum with the experimentally determined spectrum.

Results

Biosynthesis of flavonoid conjugates

Figure 3.1 shows the chromatograms of the products of all conjugation reactions. While the incubations with daidzein, dalbergin, genistein, glycitein and naringenin yielded each one conjugate, the incubations with hesperetin, kaempferol, and quercetin yielded each more than one conjugate. For the latter three flavonoids the different conjugates were separated by HPLC prior to identification. During the incubations with quercetin, genistein, and daidzein, very small amounts of additional conjugates were formed which were not purified and identified. It can further be seen that the described conditions for biosynthesis result in complete or near complete substrate conversion. The LC-MS data revealed that all conjugates were glucuronides.

Identification of flavonoid conjugates

For identification of the biosynthesized conjugates, their molecular masses and ^{1}H-NMR spectra were experimentally determined. A list of candidate molecules with matching elemental composition was derived from MetIDB. The candidate compounds were subjected to in-silico biotransformations and their ^{1}H-NMR spectra predicted. The predicted ^{1}H-^{1}H couplings and chemical shifts were compared to the experimentally determined ^{1}H-^{1}H couplings and chemical shifts of the biosynthesized conjugates, and the candidate compounds that were unlikely matches for the biosynthesized conjugates were excluded. The remaining candidate compounds were ranked based on the similarity between the predicted and the observed ^{1}H NMR chemical shift values. In the following section, this procedure for identification is described in detail using the two conjugates that were prepared of hesperetin as exemplary compounds.

Identification of hesperetin conjugates

As shown in Figure 3.1, 24h incubation of Hepa1c1c7 cells with hesperetin resulted in an almost complete conversion of the parent compound and the formation of two conjugates (retention times 12.5 and 14.3 minutes). After separation of these two conjugates, LC-MS analysis revealed that both conjugates are glucuronides. In MetIDB, 31 compounds with a matching chemical composition as the substrate hesperetin could be identified; these 31 compounds are distributed over 5 chemical classes: chalcones, dihydroaurones, flavanones, isoflavanones and pterocarpans. Based on the hydroxyl groups of these compounds available for conjugation, a total of 93 different mono-glucuronides could be formed. These 93 possible conjugates were created by in-silico biotransformations and their ^{1}H-NMR spectra were predicted. The ^{1}H-^{1}H coupling patterns and the chemical shifts of these candidate compounds were compared to the experimentally determined coupling patterns and chemical shifts of the biosynthesized

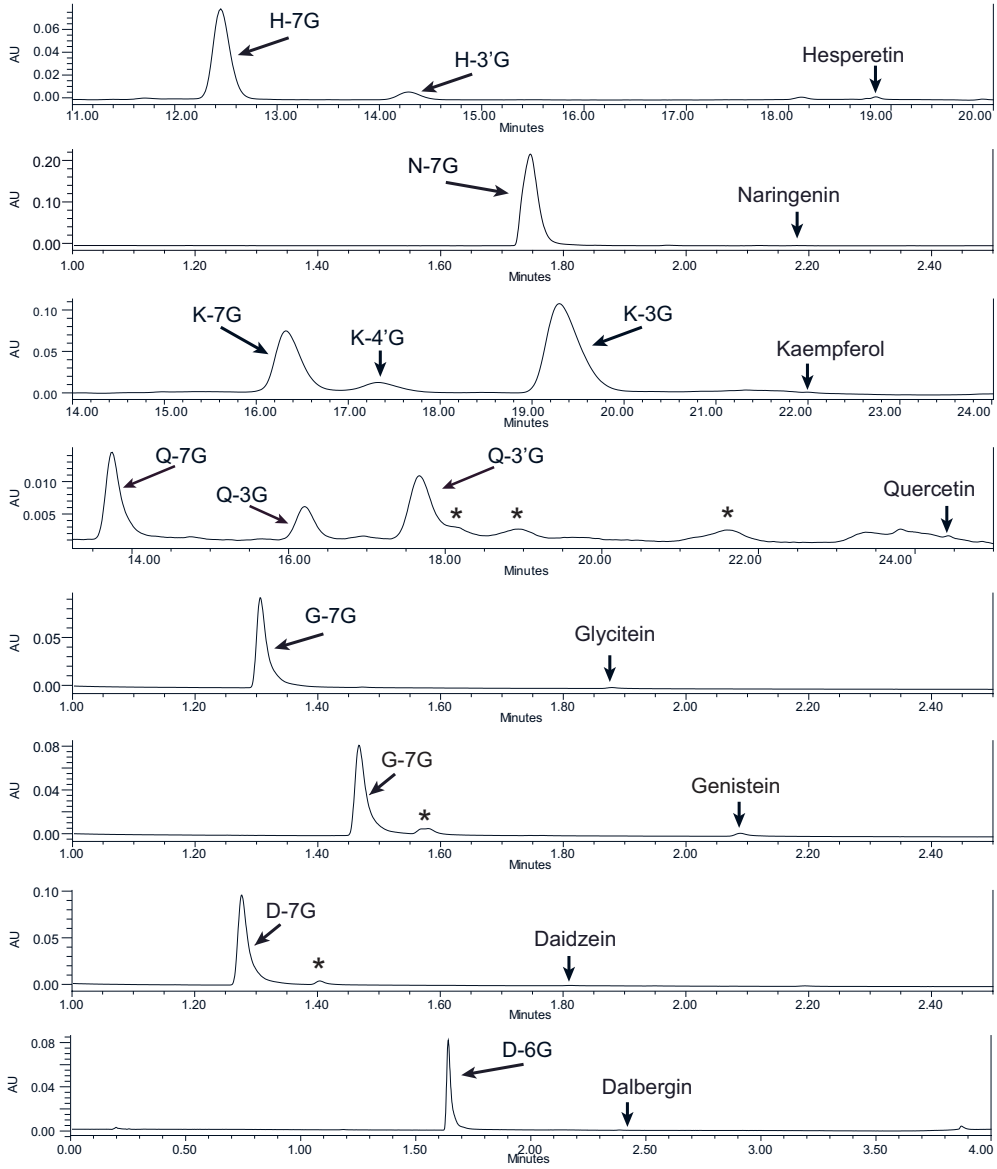

Figure 3.1 Chromatograms of incubations of selected flavonoids with Hepa-1c1c7 cells (i.e. hesperetin, naringenin, kaempferol, quercetin and glycitein) or UGTs (i.e. genistein, daidzein, and dalbergin) showing conjugate formation. Conjugates are labelled according to their identification as described in the text, and the elution positions of the aglycones used as substrate are indicated in the chromatograms. Peaks marked with an asterisk '*' in the chromatograms were not identified.

hesperetin conjugates. Based on this comparison, 75 of the candidate compounds could be excluded leaving 18 possible candidate compounds for both conjugates. For these 18 candidate compounds the differences between the predicted and experimental shifts

were scored and the candidate compounds were ranked according to their score. Table 3.1 shows the ^{1}H chemical shift values and scoring results of the candidate compounds for the hesperetin conjugate with a retention time of 12.5 minutes (Figure 3.1).

Table 3.1 ^{1}H resonances chemical shift values (in ppm) of the experimentally obtained ^{1}H-NMR spectrum of the conjugate of hesperetin with a retention time of 12.5 minutes (Figure 3.1) and of predicted ^{1}H-NMR spectra of candidate compounds. Predicted spectra of candidate compounds are ranked according to similarity of the ^{1}H-NMR data with the experimental ^{1}H NMR data of the conjugate of hesperetin with a retention time of 12.5 minutes

Experimental							
H2'	**H5'**	**H6'**	**H8**	**H6**	**H2**		
6.96	6.94	6.93	6.24	6.19	5.34		
Predicted						*Score*[a]	*Compound*
6.98	6.91	6.85	6.22	6.23	5.39	*0*	**Hesperetin-7-*O*-glucuronide**
7.06	6.83	6.98	6.22	6.26	5.36	*2*	Homoeriodictyol-7-*O*-glucuronide
6.84	6.93	6.76	6.40	6.15	5.37	*3*	Sternbin-5-*O*-glucuronide
6.99	6.91	6.82	5.93	6.24	5.39	*4*	Hesperetin-5-*O*-glucuronide
7.04	7.06	6.81	6.11	5.99	5.33	*4*	Sternbin-3'-*O*-glucuronide
7.08	6.90	6.82	5.93	6.29	5.36	*5*	Homoeriodictyol-5-*O*-glucuronide
6.99	7.18	6.82	6.09	6.03	5.42	*5*	Sternbin-4'-*O*-glucuronide
7.36	6.84	6.95	5.90	5.87	5.40	*8*	Hesperetin-3'-*O*-glucuronide
7.03	7.20	7.04	5.89	5.86	5.40	*9*	Homoeriodictyol-4'-*O*-glucuronide
6.43	7.10	6.51	6.29	6.26	5.12	*11*	Ferreirin-7-*O*-glucuronide
6.72	7.28	6.42	6.14	6.04	5.64	*12*	Artocarpanone-2'-*O*-glucuronide
6.61	6.67	6.36	6.22	6.27	5.06	*12*	Isoferreirin-7-*O*-glucuronide
6.80	6.73	6.68	5.92	5.90	5.05	*12*	Isoferreirin-4'-*O*-glucuronide
6.48	7.08	6.40	6.13	6.45	5.55	*13*	Artocarpanone-5-*O*-glucuronide
6.77	6.72	6.53	5.91	5.90	5.10	*13*	Ferreirin-2'-*O*-glucuronide
6.73	7.39	6.60	6.10	6.07	5.70	*14*	Artocarpanone-4'-*O*-glucuronide
6.52	6.74	6.47	5.93	6.30	5.09	*15*	Ferreirin-5-*O*-glucuronide
6.61	6.65	6.36	5.93	6.29	5.04	*15*	Isoferreirin-5-*O*-glucuronide

a The scoring is a quantitative measure for the differences between the experimental and predicted spectra; a low score is indicative of a good match between the spectra. For further details on the scoring see Materials and Methods.

The candidate compound with the lowest score is hesperetin-7-*O*-glucuronide, which has a score of 0. The next compounds in the ranking are glucuronides of homoeriodictyol and sternbin. Homoeriodictyol and sternbin are structurally very close to hesperetin and only differ in the position of the methoxy group, which gives rise to small but significant differences between measured and predicted ^{1}H NMR chemical shifts.

The conjugate of hesperetin with a retention time of 14.3 minutes (Figure 3.1) was compared to its possible candidate molecules in the same manner as the conjugate with a retention time of 12.5 minutes. The ^{1}H resonances of the predicted and experimentally determined spectra, as well as the scores for the 18 different candidate compounds with similar ^{1}H-^{1}H coupling and chemical shift are presented in Table 3.2. The highest ranking

candidate compound for the conjugate of hesperetin eluting at 14.3 minutes (Figure 3.1) is hesperetin-3'-*O*-glucuronide, which has a score of 2. Also for this conjugate of hesperetin, the candidate compounds following the best matching candidate in the ranking are glucuronides of homoeriodictyol and sternbin.

Table 3.2 ^{1}H resonances chemical shift values (in ppm) of the experimentally obtained ^{1}H-NMR spectrum of the conjugate of hesperetin with a retention time of 14.3 minutes (Figure 3.1) and of predicted ^{1}H-NMR spectra of candidate compounds. Predicted spectra of candidate compounds are ranked according to similarity of the ^{1}H-NMR data with the experimental ^{1}H NMR data of the conjugate of hesperetin with a retention time of 14.3 minutes

Experimental							
H2'	**H6'**	**H5'**	**H8**	**H6**	**H2**		
7.36	7.13	7.03	5.92	5.89	5.39		
Predicted						*Score*[a]	*Compound*
7.36	6.84	6.95	5.90	5.87	5.40	*2*	**Hesperetin-3'-*O*-glucuronide**
7.03	7.20	7.04	5.89	5.86	5.40	*3*	Homoeriodictyol-4'-*O*-glucuronide
7.04	7.06	6.81	6.11	5.99	5.33	*6*	Sterbin-3'-*O*-glucuronide
7.08	6.90	6.82	5.93	6.29	5.36	*9*	Homoeriodictyol-5-*O*-glucuronide
6.99	7.18	6.82	6.09	6.03	5.42	*9*	Sternbin-4'-*O*-glucuronide
6.99	6.91	6.82	5.93	6.24	5.39	*10*	Hesperetin-5-*O*-glucuronide
7.06	6.83	6.98	6.22	6.26	5.36	*10*	Homoeriodictyol-7-*O*-glucuronide
6.98	6.91	6.85	6.22	6.23	5.39	*11*	Hesperetin-7-*O*-glucuronide
6.80	6.73	6.68	5.92	5.90	5.05	*14*	Isoferreirin-4'-*O*-glucuronide
6.84	6.93	6.76	6.40	6.15	5.37	*14*	Sterbin-5-*O*-glucuronide
6.77	6.72	6.53	5.91	5.90	5.10	*15*	Ferreirin-2'-*O*-glucuronide
6.72	7.28	6.42	6.14	6.04	5.64	*16*	Artocarpanone-2'-*O*-glucuronide
6.73	7.39	6.60	6.10	6.07	5.70	*16*	Artocarpanone-4'-*O*-glucuronide
6.43	7.10	6.51	6.29	6.26	5.12	*20*	Ferreirin-7-*O*-glucuronide
6.48	7.08	6.40	6.13	6.45	5.55	*21*	Artocarpanone-5-*O*-glucuronide
6.52	6.74	6.47	5.93	6.30	5.09	*21*	Ferreirin-5-*O*-glucuronide
6.61	6.65	6.36	5.93	6.29	5.04	*21*	Isoferreirin-5-*O*-glucuronide
6.61	6.67	6.36	6.22	6.27	5.06	*23*	Isoferreirin-7-*O*-glucuronide

a The scoring is a quantitative measure for the differences between the experimental and predicted spectra; a low score is indicative of a good match between the spectra. For further details on the scoring see Materials and Methods.

Identification of the conjugates of other flavonoids

Using the same approach as outlined above for the glucuronides of hesperetin, the conjugates formed in incubations with naringenin, kaempferol, quercetin, daidzein, and dalbergin (Figure 3.1) could also be identified. Table 3.3 gives an overview of the number of candidate compounds and the finally selected identities of the conjugates based on the elemental composition and ^{1}H-NMR spectra for all conjugates biosynthesized. Details on the scoring for these compounds can be found in the Supplemental Tables 3.1 to 3.11.

It can be seen from Table 3.3, that for all conjugates but the glucuronide of dalbergin, the best-scoring compounds were the correct molecules. In the case of the glucuronide of dalbergin, isodalbergin-7-*O*-glucuronide had a slightly lower score than dalbergin-6-*O*-glucuronide. Dalbergin and isodalbergin differ only in the position of the methoxy group.

Table 3.3 Overview of the identification of the flavonoid conjugates biosynthesized in this study. The numbers of candidate compounds based on LC-MS and ^{1}H-NMR data, the identity, and the scoring are given in the table.

Substrate Retention time of conjugate (Figure 3.1)	Number of candidate compounds with same elemental composition as derived from LC-MS data	Number of candidate compounds after refinement with similar ^{1}H-^{1}H coupling and chemical shift.	Identity of conjugate	Rank of candidate in scoring (score of conjugate)
Hesperetin				
12.5 min	93	18	*Hesperetin-7-O-glucuronide*	#1 (0)
14.3 min	93	18	*Hesperetin-3'-O-glucuronide*	#1 (2)
Naringenin				
1.67 min	60	3	*Naringenin-7-O-glucuronide*	#1 (0)
Kaempferol				
16.3 min	89	4	*Kaempferol-7-O-glucuronide*	#1 (0)
17.3 min	89	4	*Kaempferol-4'-O-glucuronide*	#1 (0)
19.3 min	89	4	*Kaempferol-3-O-glucuronide*	#1 (0)
Quercetin				
13.7 min	71	15	*Quercetin-7-O-glucuronide*	#1 (3)
16.2 min	71	15	*Quercetin-3-O-glucuronide*	#1 (0)
17.7 min	71	15	*Quercetin-3'-O-glucuronide*	#1 (7)
Glycitein				
1.32 min	72	7	*Glycitein-7-O-glucuronide*	#1 (1)
Genistein				
1.47 min	54	9	*Genistein-7-O-glucuronide*	#1 (2)
Daidzein				
1.28 min	44	8	*Daidzein-7-O-glucuronide*	#1 (0)
Dalbergin				
1.63 min	20	3	*Dalbergin-6-O-glucuronide*	#2 (2)

The results thus obtained also reveal that especially the hydroxyl-group at C7 on the A-ring was the predominant target for conjugation. The chromatograms presented in Figure 3.1 show the nature of the various conjugate peaks thus identified. Even though all of the used compounds except for dalbergin have a hydroxyl group available for conjugation on the B-ring, glucuronidation of this hydroxyl moiety was only observed for three of the substrates. In those cases, conjugation of the B-ring was only observed in addition to and to a lesser extent than conjugation at C7. It can be seen from Figure 3.1, that if different glucuronides of one flavonoid are formed, the conjugates glucuronidated at the 7-position elute before the others.

Discussion

In the present study, glucuronidated conjugates of a series of flavonoids were synthetized and identified. Most of the conjugates that were prepared and identified are reported to be found in the systemic circulation after ingestion of the respective flavonoids. Hesperetin-7-*O*-glucuronide, which was the main conjugate biosynthesized in this study from hesperetin in incubations with Hepa-1c1c7 cells, is reported to be present in human urine after the ingestion of hesperetin and hesperidin, a glycoside of hesperetin. Hesperetin-3'-*O*-glucuronide, however, which was produced to a lesser extent by the Hepa-1c1c7 cells in this study, is reported to be the major conjugate in human urine [39-41]. In human urine, in addition to the two mono-glucuronides, small amounts of di-glucuronides and sulfo-glucuronides are also detected after the ingestion of hesperetin or hesperidin [39-42]. In plasma of rats, hesperetin-7-*O*-glucuronide is reported to be present at slightly higher concentrations than hesperetin-3'-*O*-glucuronide after the consumption of hesperidin [43].

For naringenin, the two glucuronides naringenin-7-*O*-glucuronide and naringenin-4'-*O*-glucuronide are reported to be the main conjugates of naringenin present in plasma and urine after consumption of oranges or orange juice [39, 40, 44, 45]. Under the conditions described, only naringenin-7-*O*-glucuronide was produced by the Hepa-1c1c7 cells used in this study. As different cell types are known to produce different types of flavonoid conjugates [20, 46], it is well possible that also naringenin-4´-*O*-glucuronide can be produced by other cell types derived from different tissues or species.

For kaempferol, only one study reports on conjugates present in human plasma and urine. After the consumption of endive, kaempferol-3-*O*-glucuronide, which was one of the conjugates biosynthesized in our study to the highest extent, is the only kaempferol conjugate reported to be present in human plasma, as well as certain amounts of the aglycone. In human urine, kaempferol-3-*O*-glucuronide is the major conjugate; additionally, a mono-sulfated and a di-sulfated conjugate were reported to be found [47]. For kaempferol, a range of possible conjugates have been identified in vitro. Incubations with Caco-2/TC7 cells (i.e. a clone of the human epithelial colorectal adenocarcinoma cell line Caco-2), co-cultures of Caco-2 and HT-29 (i.e. a human epithelial colorectal adenocarcinoma cell line), and rat liver S9 all yielded glucuronides conjugated at the 3-, 4'-, and 7-positions, which are the same conjugates biosynthesized in our study using Hepa-1c1c7 cells [48, 49]. These studies also report the formation of two sulfates (conjugated at the 3- and 7-position), and a sulfo-glucuronide. Other studies report the formation of various, though not identified, glucuronides and sulfates after incubations of kaempferol with rat liver microsomes and cytosol, primary rat hepatocytes, or recombinant UGT1A9 [50, 51].

Quercetin is one of the most studied flavonoids, and various plasma metabolites in humans have been identified. After the consumption of onions or tomato juice enriched with rutin (i.e. quercetin-3-*O*-rutinoside), the main metabolites in plasma are reported to be quercetin-3-*O*-glucuronide and quercetin-3'-*O*-sulphate; further conjugates found are quercetin-4'-*O*-glucuronide, quercetin-3'-*O*-glucuronide, isorhamnetin-3-*O*-glucuronide, isorhamnetin-4'-*O*-glucuronide, a quercetin di-glucuronide and a quercetin sulfo-glucuronide [52-56]. In our study, glucuronides of quercetin conjugated at the 3-, 7-, and 3'-positions could be biosynthesized using Hepa-1c1c7 cells and identified; additional conjugates of quercetin were formed in small quantities but were not purified and identified. It can be expected that even more conjugates than the three conjugates synthesized and identified in the present study can be biosynthesized in vitro, as van der Woude et al. [20] report the formation of a total of 14 distinct conjugates of quercetin using various in vitro models.

The conjugates of the isoflavones daidzein and genistein that were biosynthesized using UGTs, as well as the conjugate of glycitein that was biosynthesized using Hepa-1c1c7 cells in this study, were all glucuronidated at the 7-position, which is the main position of conjugation for these isoflavones in humans in vivo [57, 58]. Some studies report glucuronidation to be the main type of conjugation for isoflavones [59, 60], while some also mention sulfation as a frequent type of conjugation [57-59, 61, 62]. The K_m and V_{max} for flavonoid sulfation are generally reported to be lower than for glucuronidation [21, 63-65]. Therefore, it can be expected that the preference for the type of conjugation varies depending on the substrate concentration, with glucuronidation being favored at relatively higher dose levels. In addition, many flavonoids are known to inhibit sulfotransferases [21, 66-68], which can additionally impede sulfation at higher concentrations. During the incubations with daidzein and genistein, small amounts of an additional glucuronide were formed. Although not isolated and identified in this study, these glucuronides are likely to be conjugated at the 4'-position, which is generally reported to be glucuronidated to a lesser extent than the 7-position in vivo [57, 58]. Unlike daidzein, which has hydroxyl groups only at the 7- and 4'-positions, genistein has an additional hydroxyl group available for conjugation at the 5-position; this is a less likely position for conjugation as genistein-5-*O*-glucuronide is reported to be found in vivo only in trace amounts in porcine rat urine [61, 69], and glucuronidation of this hydroxyl group is likely to be hindered by intramolecular hydrogen bonding.

There are no studies reporting on the pharmacokinetics of dalbergin. As dalbergin has only one hydroxyl group available for conjugation, dalbergin-6-*O*-glucuronide was the only conjugate that could have been formed during the incubation with UGT in our study.

The amounts of conjugates that can be prepared using the described methods are 0.4 µmol per 75 cm^2 cell culture flask or 10 ml incubation using UGTs. For the conjugates

biosynthesized in this study, this correlates to around 172 µg to 191 µg of flavonoid conjugates, depending on the molecular mass of the substrate used and assuming no losses during sample clean-up. These amounts, equivalent to 10 ml of a 40 µM solution, are sufficient for many types of in vitro studies, given that flavonoids are generally active at micromolar concentrations. The incubation volumes can easily be scaled up and require only little extra sample handling due to concentration of the samples during SPE.

While studies unequivocally report that flavonoids are present in the systemic circulation nearly exclusively as conjugated metabolites, only some studies report the type of conjugation and even less report the positions of conjugation. This is in part due to the low concentrations of circulating conjugates and the technical difficulties of identifying the positions of conjugation. With the strategy for identification described in this study, flavonoid conjugates can be identified based on LC-MS and ^{1}H NMR spectra obtained for samples containing only a few micrograms of the conjugate. While in this study the knowledge of the substrates used for biosynthesis was used for the preparation of the list of candidate compounds, this information can be generated for unknown compounds using tandem mass spectrometry. The employed strategy for the identification of the glucuronides was able to reduce the number of possible candidate molecules from a large set to a small and manageable number, and in all but one case even indicated the correct compound, being the conjugate with the lowest score. While this procedure can be applied in automation, provided that the ^{1}H NMR spectra are sufficiently clean, in practice, however, solvent impurities and bleeding of the columns can impact sample purity. Therefore, especially at low sample concentrations, the automated fitting of the spectra can be hampered and the procedure requires visual comparison and manual fitting of the predicted and measured spectra.

Altogether, the described strategy offers great flexibility for the biosynthesis of relevant flavonoid conjugates, as different cell lines [20, 46, 48, 49], as well as different UGTs and also sulfotransferases [21, 32] can be used for different flavonoid substrates. The production of flavonoid conjugates using this strategy can easily be scaled up to produce sufficient amounts of conjugates for bio-assays. The described strategy for the identification based on the LC-MS and ^{1}H-NMR data using the MetIDB proved to be a powerful tool for the quick and reliable identification of even small amounts of conjugates.

Supplemental data

Supplemental Tables 3.1 - 3.11 can be downloaded from: https://goo.gl/jd7kBJ (case sensitive)

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Chapter 4

The effect of quercetin and kaempferol aglycones and glucuronides on peroxisome proliferator-activated receptor-gamma (PPAR-γ)

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Abstract

The consumption of dietary flavonoids has been associated with a variety of health benefits, including effects mediated by the activation of peroxisome proliferator-activated receptor-gamma (PPAR-γ). Flavonoids are extensively metabolized during and after uptake and there is little known on the biological effects of these conjugated metabolites of flavonoids that are found in plasma. To investigate the effect of glucuronidation on the ability of flavonoids to activate PPAR-γ we studied and compared the activity of quercetin, kaempferol and their relevant plasma conjugates quercetin-3-O-glucuronide (Q-3G) and kaempferol-3-O-glucuronide (K-3G) on different PPAR-γ related endpoints. The flavonoid aglycones increased PPAR-γ mediated gene expression in a stably transfected reporter gene cell line and glucuronidation diminished their effect. To study the intrinsic activity of the test compounds to activate PPAR-γ we used a novel microarray technique to study ligand induced ligand binding domain (LBD) – nuclear receptor coregulator interactions. In this cell-free system we demonstrate that, unlike the known PPAR-γ agonist rosiglitazone, neither the flavonoid aglycones nor the conjugates are agonistic ligands of the receptor. The increases in reporter gene expression in the reporter cells were accompanied by increased PPAR-γ receptor-mRNA expression and quercetin synergistically increased the effect of rosiglitazone in the reporter gene assay. It is concluded that flavonoids affect PPAR-γ mediated gene transcription by a mode of action different from agonist binding. Increases in PPAR-γ receptor mRNA expression and synergistic effects with endogenous PPAR-γ agonists may play a role in this alternative mode of action. Glucuronidation reduced the activity of the flavonoid aglycones.

Introduction

Flavonoids are plant secondary metabolites and ubiquitously present in many plant-derived foodstuffs. As a result, flavonoids are generally consumed on a regular basis via fruits, vegetables and their juices, as well as via wine, tea and cocoa-derived products [1, 2]. Dietary intake of flavonoids has been correlated with the prevention of various degenerative diseases and improvement of disease states [3, 4]. One possible mode of action behind beneficial health effects of flavonoids has been suggested to be the activation of PPAR-γ [5]. PPARs are ligand-activated transcription factors which form obligate heterodimer partners with the retinoid X receptor. The heterodimers bind to peroxisome proliferator-responsive elements (PPREs) in the regulatory region of target genes and upon activation recruit nuclear co-activators required for gene transcription, while dismissing co-repressors that are bound in the unliganded state [6]. Three PPAR isoforms are currently known, i.e. PPARα (NR1C1), PPARβ/δ (NR1C2) and PPAR-γ (NR1C3). Apart from certain overlaps, these isoforms are activated by different ligands and regulate specific target genes [7]. Various health promoting effects are ascribed to PPAR activation and especially PPAR-γ is highlighted for its effects on for example adipogenesis, insulin resistance and inflammation [8]. There are two PPAR-γ splice variants, i.e. PPAR-γ1 and PPAR-γ2 which have different expression levels in tissues [7]. The functional differences between these two are not fully elucidated but there are indications that PPAR-γ2 is of higher importance in adipogenesis and insulin sensitivity [9-11]. Various preferably unsaturated fatty acids serve as endogenous receptor agonists [12], and the receptor is target of a variety of drugs to treat reduced insulin sensitivity and hyperlipidemia such as the well-known class of thiazoledinediones [13]. Several flavonoids are reported to activate PPAR-γ mediated gene transcription and other related endpoints (see Table 4.1).

With only few exceptions, flavonoids occur in nature in their glycosidic form. Upon ingestion, these flavonoid glycosides have to be deconjugated to their respective aglycones before or during uptake in the gastrointestinal tract. During uptake the aglycones are extensively metabolized to sulfated, methylated and/or glucuronidated conjugates in intestinal tissue or the liver before they enter the systemic circulation [14]. As a result, under physiological conditions flavonoids usually do not occur as aglycones in biological fluids. It is widely accepted that conjugation and deconjugation can significantly influence the biological activity of flavonoids [15, 16].

Table 4.1 Effects of flavonoids on common PPAR-γ related endpoints. Regular print: positive association; italic print: negative association; bold print: inactive.

	Reporter gene assays	Competitive binding / coregulator binding	Target gene expression	PPAR-γ expression	Adipocyte differentiation	PPRE binding/ activation
Alpinetin		[53]		[53]		
Apigenin	[34],[35]	[34],[35]		**[34]**	**[34]**,*[54]*	[55]
Baicalin	[56]		***[57]***		*[57]*	
Biochanin A	[34],[35],[58],[59]	**[35]**,[35]				[55]
Calcycosin	[58]					
Chrysin	[34],[60]	[34]	[60]	**[34]**,**[60]**	**[34]**	[55]
Cyanidin		[61]	[61]	[61]		
Daidzein	[58],[59],[62],[63]					
Diosmetin	**[35]**	[35]				
Equol	[59],[63]			[64],*[64]*	***[64]***	
Eriodictyol	**[34]**,**[35]**	[35]				
(-)-Epigallocatechin -3-gallate	**[34]**					
Fisetin	**[34]**			*[65]*	*[65]*,*[66]*	[67]
Flavone	[34]					
Formononetin	[58]					
Formononetin	[68]					
Galangin	[34]					
Genistein	[34],[58],[59],[62]	[62]				[55]
Glycitein	[62]					
Gossypetin				*[69]*		
Hesperetin	**[34]**,[70],[71]		[71]	*[72]*		
Hesperidin						[55],[67],[73]
Isoquercetrin	**[35]**	[35]				
Isosakuranetin	**[34]**					
Kaempferol	[34],**[35]**,[33],[32]	[34],[35]		**[34]**	**[34]**,**[74]**	
Luteolin	[34],**[35]**	[35]	[75]			
Morin	**[34]**	[76]				[67],[76]
Myricetin	**[34]**					[67]
Naringenin	[34],[35],[71],[33],[32],[77]	[35]	[71],[77]	*[72]*	*[72]*	[77]
Naringenin chalcone	[33]					
Naringin	**[34]**					
Odoratin	[78]					
Oroxylin A				**[79]**		*[79]*
Pinocembrin	**[34]**					
Quercetin	**[34]**,**[35]**,**[33]**,**[32]**	[35],[76]		[45]	[80]	[76]
Resveratrol	[81]		[81]			
Rutin	**[34]**					
Sakuranetin	**[82]**					
Tangeretin	**[34]**					

Table 4.1 *continued*

	Reporter gene assays	Competitive binding / coregulator binding	Target gene expression	PPAR-γ expression	Adipocyte differentiation	PPRE binding/ activation
Taxifolin		[35]				
Theaflavin-3,3'-digallate	**[34]**					
Vitexin	[35]	**[35]**		**[83]**		
Wogonin	**[84]**				**[84]**	
3,6-dihydroxyflavone	[85]					
3-hydroxyflavone	[34]					
5,7-dimethoxyflavone	[34]					
5-methoxyflavone	**[34]**					
7,8-dihydroxyflavone	[34]					

The aim of the present study was to investigate the effect of flavonoid conjugation on the reported activity of flavonoids to induce PPAR-γ mediated gene expression. To this end we selected the dietary flavonoids quercetin and kaempferol as model flavonoids to compare their activity with their respective 3-O-glucuronidated conjugates. Q-3G and K-3G belong to the most abundant conjugates of quercetin and kaempferol found in plasma and urine [17-22]. In this study we describe the effect of these flavonoid aglycones and conjugates on PPAR-γ mediated gene expression, receptor mRNA expression and PPAR-γ LBD-coregulator interaction.

Materials and Methods

Chemicals

Rosiglitazone (CAS no: 122320-73-4) was obtained from Cayman Chemical (Ann Arbor, USA). Kaempferol (CAS no: 520-18-3), K-3G (CAS no: 22688-78-4), quercetin (CAS no: 117-39-5), Q-3G (CAS no: 22688-79-5), DL-dithiothreitol (DTT, CAS no: 3483-12-3) and L-ascorbic acid (VitC, CAS no:50-81-7) were purchased from Sigma Aldrich (Missouri, USA). Stock solutions of the flavonoids were prepared in dimethylsulphoxide (DMSO, 99.9% purity) obtained from Acros (Geel, Belgium) and stored at –20 °C. G418 solution and fetal bovine serum (FBS) were purchased from PAA (Pasching, Austria). Acetic acid was purchased from VWR International (Darmstadt, Germany). Acetonitrile (ULC/MS grade) and methanol (HPLC supra-gradient) were purchased from Biosolve BV (Valkenswaard, the Netherlands). Phosphate buffered saline (PBS), Dulbecco's Modified Eagle Medium with Ham´s Nutrient Mixture F-12 (1:1) (DMEM/F12), DMEM/

F12 without phenol red, nonessential amino acids (NEAA) and trypsin were purchased from Invitrogen (Breda, The Netherlands).

Dextran-coated charcoal-stripped fetal calf serum (DCC-FCS) was purchased from Thermo Scientific (Waltham, Missouri, USA). Nanopure water was prepared with a Barnstead Nanopure Type I ultrapure water system.

Cell cultures

The PPAR-γ2 CALUX cells (provided by BioDetection Systems BV, Amsterdam, the Netherlands) are human osteosarcoma U2OS cells stably transfected with an expression vector for PPAR-γ2 and a firefly luciferase reporter construct under control of the peroxisome proliferator responsive element [23]. The cells were cultured in DMEM/F12 GlutaMAX supplemented with 7.5% FBS and NEAA. To maintain selection pressure 200 µg/mL G418 was added once per week. The cells were maintained at 37°C in a humidified atmosphere with 5% CO_2.

All compounds were tested for cytotoxicity and potential effects on luciferase stability using the Cytotox CALUX cell line (provided by BioDetection Systems BV) as described before [24]. The Cytotox CALUX cells show an invariant luciferase expression and a decrease in luciferase activity therefore indicates a cytotoxic effect. Moreover, an increase in luciferase activity in the Cytotox CALUX cells may indicate stabilization of the luciferase enzyme and possible false positives for reporter gene expression in the PPAR-γ2 CALUX assay [25]. Only non-cytotoxic concentrations of the test compounds were used in the PPAR-γ2 CALUX assay.

The Cytotox CALUX cells were cultured in DMEM/F12 supplemented with 7.5% FBS and NEAA. Once per week 200 µg/ml G418 was added to the culture medium in order to maintain the selection pressure.

PPAR-γ2 CALUX and cytotox CALUX assay

The ability of the tested flavonoids to induce PPAR-γ2 mediated luciferase expression at protein level in an intact cell system was tested by measuring luciferase activity in the PPAR-γ2 CALUX reporter cells. To this end PPAR-γ2 CALUX cells were seeded in a white 96-wells microtiter plate with clear bottom (View Plate-96 TC, PerkinElmer) at a density of 10,000 cells per well in 100 µl exposure medium (DMEM/F12 without phenol red +5 % (v/v) DCC- FCS +1% (v/v) NEAA). The seeded cells were incubated for 24 h to allow them to attach and form a confluent monolayer. Subsequently, the 60 inside wells of the plate were exposed for 24 h to the test compounds in exposure medium at the concentrations indicated. The final DMSO concentration in the exposure medium was 0.5%. On each plate, 100 nM rosiglitazone, a known PPAR-γ agonist [26] was included as positive control. Quercetin was co-incubated with 500 µM VitC to

prevent auto oxidation; this concentration of VitC was determined not to interfere with cell viability, luciferase expression or luciferase stabilization.

After 24 h of exposure, medium was removed and the cells were washed twice with 100 µl 0.5x PBS. Subsequently, cells were lysed by addition of 30 µl low salt lysis buffer [27] and stored overnight at −80 °C. Luciferase activity in the lysate was measured using a luminometer (Luminoscan Ascent, Thermo Scientific, Waltham, MA) and flash mix as described previously [27]. Background light emission and luciferase activity was measured per well and expressed in relative light units (RLU). Background values were subtracted prior to data analysis. Data and statistical analyses were conducted using Microsoft Excel (Version 14.0.7106.5003; Microsoft Corporation) and GraphPad Prism software (version 5.00 for windows, GraphPad software, San Diego, USA). The depicted graphs are representative curves giving mean and standard deviations of sextuplicate measurements. The Cytotox CALUX cells were cultured, exposed, lysed and measured in the same manner as the PPAR-γ2 CALUX cells.

Quantitative polymerase chain reaction (qPCR)

For qPCR the PPAR-γ2 CALUX cells were propagated as described above with some minor modifications. Cells were seeded in 12 well plates, at 100,000 cells in 1 ml of exposure medium per well. After 24h of incubation, cell culture medium was removed and 750 µl of exposure medium were added containing the test compounds (added from a 200 times concentrated stock solution in DMSO). Each test compound was tested in two independent experiments in triplicates giving a total of six replicates.

RNA isolation

For the isolation and purification of mRNA QIAshredder spin columns and the RNeasy mini kit from QIAGEN (Venlo, the Netherlands) were used. After 24h of exposure of the PPAR-γ2 CALUX cells medium was aspirated and the cells were washed with 600 µl PBS. Subsequently, 300 µl of RLT lysis buffer (RNeasy Mini Kit, Qiagen, Venlo, the Netherlands) were added and the plates were placed on an orbital shaker. The lysate was added to QIAshredder spin columns and centrifuged at 8,000x g for 15 seconds. Then 350 µl of 70% ethanol were added to the flow through of the spin columns and the samples were mixed thoroughly. These mixtures were transferred to RNeasy spin columns and centrifuged at 8,000 rcf for 20 seconds. The flow through was discarded. Then 700 µl RW1 buffer (RNeasy Mini Kit) were added to the columns and the columns were centrifuged at 8,000 rcf for 20 seconds. The flow through was discarded. Next, 500 µl of RPE buffer (RNeasy Mini Kit) were added to the columns and the columns were centrifuged at 8,000 rcf for 20 s. The flow through was discarded. The previous step was repeated and followed by 2 min of centrifugation. Subsequently, the columns were placed in new tubes and centrifuged at 14,000 rcf for 1 minute to dry the columns. Next,

the columns were transferred to new tubes and 30 µl RNase-free water were added. The columns were kept at room temperature for 5 minutes and subsequently centrifuged at 8,000 rcf for 1 minute to elute total RNA. The concentration of total RNA in the flow through was determined spectrophotometically at 260 nm using a Nanodrop (ND-1000, Thermo scientific, Wilmington, Delaware).

Reverse transcriptase reaction and real-time PCR with SYBR green

To obtain cDNA, a QuantiTect Reverse Transcription Kit (Qiagen) was used according to the manufacturer's protocol. Total RNA samples were diluted to 50 ng/µl in RNase-free water. To eliminate genomic DNA, 2 µl of gDNA Wipeout Buffer (7x) were added to 8 µl of sample and 4 µl of RNase-free water per reaction. Mixtures were incubated for 2 minutes at 42 °C and subsequently put on ice. Per reaction, 1 µl of Quantiscript Reverse Transcriptase, 4 µl of Quantiscript ER Buffer (5x) and 1 µl RT Primer mix were added. These mixtures were incubated for 15 minutes at 42 °C and subsequently for 3 minutes at 95 °C to inactivate the reverse transcriptase. After incubation the samples were immediately cooled to 4°C and used for gene expression analysis.

The expression of the reporter gene pGL4 mRNA (FW: ATCAGCCAGCCCACCGTCGTATTC, RV: ACAAGCGGTGCGGTGCGGTAGG) and PPAR-γ2 mRNA (FV: GCGATTCCTTCACTGATAC, RV: CTTCCATTACGGAGAGATCC; from [28]) was measured by real-time quantitative chain polymerase reaction (RT-qPCR) using Rotor-Gene Q (Qiagen) and normalized against the expression of beta-actin (FW: GCAAAGACCTGTACGCCAACAC, RV: TCATACTCCTGCTTGCTGATCCCAC) and GAPDH (FW: TGATGACATCAAGAAGGTGGTGAAG, RV: TCCTTGGAGGCCATGTGGGCCAT). For every reaction 5 µl of 20 times diluted sample cDNA, 1 µl forward primer (10 µM), 1 µl reverse primer (10 µM), 12.5 µl of Rotor-Gene SYBR Green PCR Master Mix (Qiagen) and 5.5 µl of RNase free water were used. The plate was incubated at 95 °C for 10 min, and then for 40 cycles each consisting of incubation at 95 °C for 10 s, at 60 °C for 15 s, at 72 °C for 20 s. This was followed by pre-melt conditioning at 72 °C for 90 s, increasing by 1°C every 5 seconds to 95°C for the melting curve. Every reaction was carried out in technical duplicates.

qPCR data analysis

Threshold cycle (Ct)-values were derived using Rotor-Gene 6000 Series Software (Qiagen). For the data and statistical analyses Microsoft Excel and GraphPad Prism software were used. The formulas used are adapted from literature [29].

The efficiencies (E) of the primer pairs were calculated using the formula

$$E = 10\,\hat{}\left(-\frac{1}{slope}\right)$$

where slope is the slope of the standard curve (crossing threshold (Ct) versus cDNA input). An E value of 2 is reached when there is exact doubling of the cDNA every cycle.

The relative quantity of a given sample (RQ_{sample}) and gene of interest or reference gene was calculated using the formula

$$RQ_{sample} = E \wedge (Ct_{(control)} - Ct_{(sample)})$$

where $Ct_{(control)}$ is the average of the Ct values of the solvent control reactions of a certain gene of interest and $Ct_{(sample)}$ the Ct value of the sample to be quantified.

The relative normalized expression (RNE) or fold change for a specific sample and gene of interest (GOI) against the two reference genes (REF) used is calculated using the following formula

$$RNE = RQ_{sample(GOI)} / (RQ_{sample(REF1)} \times RQ_{sample(REF2)}) \wedge 1/n$$

where n is the number of reference genes.

PamGene Nuclear Receptor-Coregulator Interaction Profiling

Ligand-modulated interaction of the PPAR-γ ligand binding domain (LBD) with coregulators (154 different binding motifs of 66 different coregulators) was assessed using PamChip 4 microarray chips for nuclear hormone receptors (PamGene International B.V., 's-Hertogenbosch, The Netherlands) as described previously [30]. Briefly, the PPAR-γ LBD (His-tagged #P1065; Protein One, Rockville, MD, USA) was mixed with an anti-His antibody conjugated to Alexa Fluor 647 (Penta·His Alexa Fluor 647 Conjugate #35370; Qiagen, Venlo, the Netherlands) in the absence and presence of the potential ligands added from a stock solution in DMSO (2% final concentration) to the reaction buffer (Nuclear Receptor Buffer F #PV4547; Invitrogen, Breda, the Netherlands) containing 5 mM DTT. Ligand concentrations used were EC_{90} concentrations obtained in the PPAR-γ reporter gene assays. All assays were performed in a fully automated microarray processing platform (PamStation12, PamGene International B.V.) at 20 °C. After incubation, excess incubation mix was removed and the arrays washed prior to acquisition of images.

Image analysis was performed using BioNavigator software (PamGene International B.V.) which performs automated array grid finding and subsequent quantification of signal and local background for each individual peptide. The median signal-minus-background values were used as the quantitative parameter of binding. For data and statistical analyses Microsoft Excel was used. Experiments were performed in triplicate and the graphs are corrected for binding levels obtained in the solvent control; coregulators for which none of the tested compounds induced an effect that was statistically significantly different from the solvent control ($p \leq 0.05$) are excluded from the figure.

Results

PPAR-γ2 CALUX reporter gene expression

The effect of quercetin and kaempferol on PPAR-γ2 mediated gene expression was measured in U2OS cells stably transfected with the PPAR-γ2 receptor and the firefly luciferase gene regulated by the PPRE. Quercetin and kaempferol, as well as the known PPAR-γ agonist rosiglitazone increase luciferase activity in a concentration-dependent way (Figure 4.1).

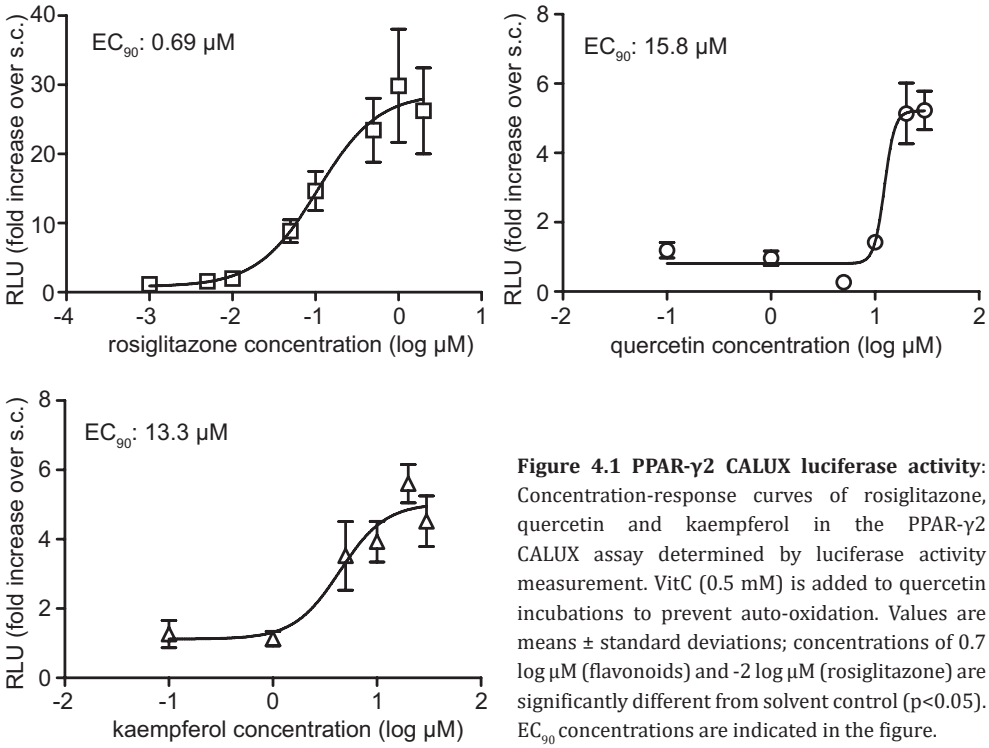

Figure 4.1 PPAR-γ2 CALUX luciferase activity: Concentration-response curves of rosiglitazone, quercetin and kaempferol in the PPAR-γ2 CALUX assay determined by luciferase activity measurement. VitC (0.5 mM) is added to quercetin incubations to prevent auto-oxidation. Values are means ± standard deviations; concentrations of 0.7 log µM (flavonoids) and -2 log µM (rosiglitazone) are significantly different from solvent control ($p<0.05$). EC_{90} concentrations are indicated in the figure.

In addition, the compounds were tested in a control cell line that invariably expresses firefly luciferase to measure effects on cell viability and post-transcriptional stabilization of luciferase. Quercetin and rosiglitazone did not affect the luciferase signal in the control cell line at the concentrations tested. Kaempferol increased the luminescence signal indicating stabilization of the luciferase enzyme – an effect that is likely to cause artificially increased luciferase activity in the PPAR-γ2 reporter gene assay. To avoid false positive results through post-translational stabilization of the luciferase reporter-protein [31] the effect of glucuronidation on the induction of PPAR-γ mediated gene expression was studied on mRNA-expression level by qPCR. The results of these experiments are expressed in Figure 4.2. Rosiglitazone, quercetin and kaempferol

significantly increased pGL4 reporter gene expression also at the mRNA level in the PPAR-γ reporter gene assay. The glucuronidated conjugates of quercetin and kaempferol, i.e. Q-3G and K-3G did not significantly affect pGL4 reporter gene expression (Figure 4.2). The stability of all tested compounds during the 24h of incubation was determined by UPLC and the UPLC chromatograms obtained revealed that all tested compounds remained stable in the exposure medium during incubation (data not shown).

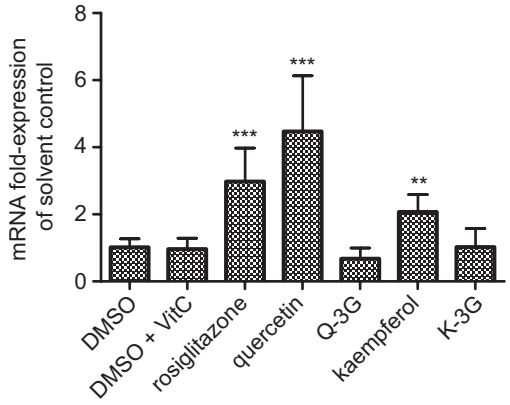

Figure 4.2 PPAR-γ2 reporter gene expression: Induction of the reporter gene expression (i.e. pGL4) by rosiglitazone (0.5 μM) and flavonoids (30 μM). VitC (0.5 mM) is added to quercetin and Q-3G incubations to prevent auto-oxidation. Rosiglitazine, quercetin and kaempferol increase pGL4 expression. Values are means ± standard deviations. Statistically significant differences from solvent control: ** $p<0.01$, *** $p<0.001$.

4

PPAR-γ coregulator binding

Given that the lower activity of the flavonoid glucuronides to activate PPAR-γ in the cell based reporter gene assay and the cell based qPCR assay might be due to their lower cellular bioavailability, additional studies were performed to investigate the intrinsic ability of the tested flavonoid aglycones and their glucuronidated conjugates to activate PPAR-γ. To that end subsequent experiments were performed in a cell-free assay system characterizing PPAR-γ LBD activation using a microarray technique to analyze nuclear receptor - coregulator interactions. The assay employs microarrays containing a total of 154 distinct binding motifs of 66 different nuclear receptor-coregulators that are immobilized on a porous membrane. Figure 4.3 shows the binding patterns of the ligand binding domain of PPAR-γ to these coregulator binding motifs in the presence of quercetin, kaempferol, Q-3G, K-3G and the positive control rosiglitazone for comparison. Quercetin, kaempferol and rosiglitazone were tested at their EC_{90} concentrations derived from the reporter gene assay and the glucuronides were tested at equimolar concentration as the respective aglycones. The results presented reveal that incubation with rosiglitazone increases LBD binding to specific coactivator binding motifs (e.g. CREP-binding protein (CBP), E1A binding protein p300 (EP300), nuclear receptor coactivators 1 and 2 (NCOA1, NCOA2) etc.) and decreases binding to corepressor motifs (nuclear receptor corepressors 1 and 2 (NCOR1, NCOR2)). Incubation with quercetin, kaempferol, Q-3G and K-3G does not affect LBD binding to coregulators in a comparable

manner and resulted in binding patterns similar to the solvent control. These results indicate that the observed effects of the flavonoids on PPAR-γ mediated gene expression cannot be ascribed to an agonistic effect of the flavonoids on the PPAR-γ LBD.

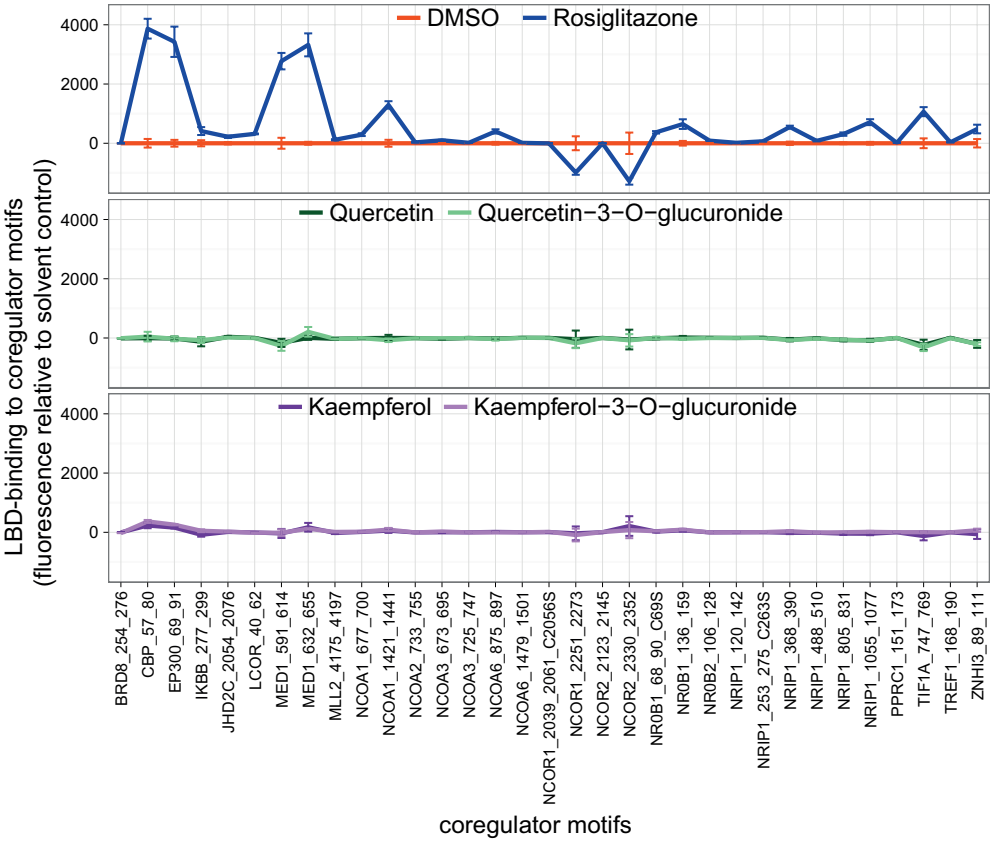

Figure 4.3 PPAR-γ LBD – coregulator interactions: Binding patterns of PPAR-γ LBD to coregulator-derived binding peptides exposed to rosiglitazone (red), quercetin (dark green), kaempferol (dark purple), Q-3G (light green) and K-3G (light purple) at EC_{90} concentrations derived from the reporter gene assay. Coregulator-derived binding peptides are plotted on the x-axis, the fluorescence signal indicating coregulator peptide binding is given on the y-axis. Rosiglitazone induces changes in binding to coregulator-derived peptides; quercetin, kaempferol, Q-3G and K-3G do not induce comparable changes. Values are means ± standard deviations.

PPAR-γ receptor-mRNA expression

As the tested flavonoids were active in the PPAR-γ reporter gene assay but did not activate the LBD of PPAR-γ we investigated other endpoints that could affect the observed activity. To this end the effect of the compounds on PPAR-γ2 receptor-mRNA expression in the reporter gene cell line by qPCR was quantified. Figure 4.4 shows that quercetin and kaempferol significantly increase the expression of PPAR-γ2 receptor mRNA, Q-3G

increases gene expression to a lesser extent than the aglycone, and rosiglitazone and K-3G do not significantly affect receptor mRNA expression. These results show that the effects of quercetin and kaempferol on reporter gene expression in the PPAR-γ2 CALUX cell line are accompanied by an increase in PPAR-γ2 receptor mRNA transcription.

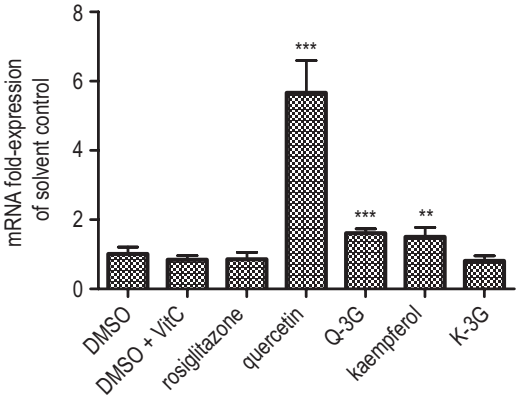

Figure 4.4 PPAR-γ2 receptor-mRNA expression: Induction of PPAR-γ2-mRNA expression by rosiglitazone (0.5 μM) and flavonoids (30 μM). VitC (0.5 mM) is added to quercetin and Q-3G incubations to prevent auto-oxidation. Quercetin, kaempferol and Q-3G increase PPAR-γ2 receptor-mRNA expression. Values are means ± standard deviations. Statistically significant differences from solvent control: ** $p<0.01$, *** $p<0.001$.

In additional experiments the PPAR-γ2 reporter gene cells were exposed to rosiglitazone in the presence of quercetin (Figure 4.5). Figure 4.5 presents a full concentration response curve of rosiglitazone in the presence of a low concentration of quercetin that by itself causes only a low increase in reporter gene expression (i.e. 10 μM). The results obtained reveal that quercetin synergistically increased the effect of rosiglitazone by about 3-fold over the complete range of concentrations tested. This further supports that quercetin has a different mode of action from that of rosiglitazone, and reveals that quercetin can synergistically increase the response of a regular PPAR-γ2 agonist.

Discussion

The objective of this study was to investigate and compare the effect of the dietary flavonoids quercetin and kaempferol and their relevant glucuronidated conjugates Q-3G and K-3G on PPAR-γ mediated gene expression. We observed increased luciferase activity and pGL4 reporter gene expression in the PPAR-γ2 reporter gene assay upon exposure to quercetin and kaempferol. Other studies reported that quercetin does not activate PPAR-γ mediated gene expression in reporter gene assays at concentrations reaching up to 300 μM [32-35]. This difference compared to our results can be explained by the instability of quercetin in vitro where it is known to oxidize rapidly [36]. As already described earlier [27, 37], the addition of ascorbic acid can prevent the auto-oxidation of quercetin.

Of the tested glucuronides, Q-3G increased gene expression to a lesser extent than the aglycone, while K-3G did not significantly affect reporter gene expression. Based on these results it can be concluded that glucuronidation reduces the ability of the flavonoids

to activate PPAR-γ mediated gene expression. Given that this effect was observed in a reporter gene assay with intact cells this can be due either to a lower intrinsic activity to induce PPAR-γ mediated gene expression or a reduced uptake of the conjugates into the cells. It has been well recognised that flavonoid conjugates may have to be deconjugated to enter cells and exert their biological activities [37, 38], although there are cell types that appear to be able to take up flavonoid glucuronides [39].

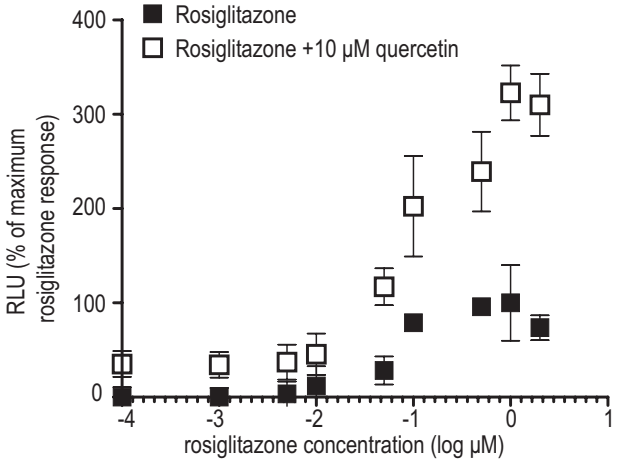

Figure 4.5 PPAR-γ2 CALUX co-incubation of quercetin and rosiglitazone: Concentration-response curves of rosiglitazone in the absence and presence of 10 µM quercetin in the PPAR-γ2 CALUX determined by luciferase activity measurement; luciferase activity is expressed as percentage of maximum response by rosiglitazone alone. Data points on the y-axis are solvent control values in the absence of rosiglitazone; all concentrations of -1.3 log µM and higher are significantly different from solvent control ($p<0.01$). Quercetin synergistically increases reporter activity about 3-fold ($p<0.05$ at all concentrations). VitC (0.5 mM) is added to incubations to prevent auto-oxidation of quercetin. Values are means ± standard deviations.

To investigate the potential inherent activity of the tested flavonoids to activate PPAR-γ the possible effect of the flavonoids on the interaction of the LBD of PPAR-γ with nuclear receptor coregulators was studied in a cell free model system. Our results show that, surprisingly, none of tested flavonoids interacts with the LBD inducing conformational changes of the LBD comparable to the well-known PPAR-γ agonist rosiglitazone. The observed effects of the flavonoids on PPAR-γ mediated reporter gene expression are therefore likely due to another mode of action. While LBD agonism is the key step to receptor activation, there are other ways to interfere with PPAR-γ activity, for example PPAR-γ modification through receptor phosphorylation, deacetylation, and sumoylation can modulate its activity [40-42]. In addition, the expression of PPAR-γ itself can be regulated by kinase activities [41, 43] and flavonoids are reported to directly and indirectly affect protein kinase activities [44]. Thus the results of the present study lead to the conclusion that flavonoids activate PPAR-γ mediated gene expression by a mode of action different from that of regular PPAR-γ agonists.

We observed increased PPAR-γ2 mRNA expression upon flavonoid exposure, an effect that is not exerted by the known agonist rosiglitazone. Various flavonoids are reported to affect PPAR-γ expression in a variety of in vitro and vivo systems. Quercetin has been reported to increase PPAR-γ mRNA and protein level in spontaneously hypertensive rats [45], as well as in primary human adipocytes [46], H9C2 cells [45] and THP-1 macrophages [47]. Interestingly, quercetin downregulates PPAR-γ in 3T3-L1 cells [48, 49]; this is also in line with the general observation that flavonoids can inhibit PPAR-γ dependent adipocyte differentiation in vitro in 3T3-L1 cells (see Table 4.1). Treatment with quercetin can also prevent up-regulated PPAR-γ levels in liver [50] and adipose tissue [51] in laboratory animals fed a high fat diet. One study reports the effect of quercetin conjugates on PPAR-γ expression [52]. In A549 cells, quercetin-3-glucuronide and quercetin-3'-sulfate slightly but significantly increased PPAR-γ expression; the aglycone however did not affect PPAR-γ expression [52]. The inactivity of the aglycone in this study is likely to be due to the instability of quercetin, as discussed above.

Flavonoid-induced increases in PPAR-γ receptor levels combined with receptor activation by endogenous agonists is a likely mechanism behind the observed activity of the flavonoids in the reporter gene assay. It is of interest to note that while kaempferol significantly affects both PPAR-γ mediated PGL4 mRNA expression (Figure 4.2) and PPAR-γ receptor mRNA expression (Figure 4), for Q-3G only the latter endpoint is significantly modulated. Such differences may be due to as yet undefined additional modulatory effects of the flavonoids on for example endogenous PPAR-γ ligands (i.e. fatty acids) and/or the aforementioned modulation of receptor activities by phosphorylation, deacetylation, and/or sumoylation which could altogether further contribute to the flavonoids' effects on PPAR-γ. Further, we also show that quercetin synergistically enhances the effect of rosiglitazone in the PPAR-γ reporter gene assay which may also be due to increased cellular receptor levels. The observed synergistic effects underline that the tested flavonoids have a different mode of action compared to the agonist rosiglitazone and that flavonoids can potentially increase the effect of PPAR-γ ligands.

Conclusion

Our results show that glucuronidation reduces the activity of quercetin and kaempferol on cellular PPAR-γ mediated gene expression. These differences in activity between the aglycone and the conjugated forms that are present in biological fluids highlight the importance of using relevant flavonoid conjugates in in vitro studies. We further observed that none of the tested flavonoid compounds act as agonists on PPAR-γ LBD. It is concluded that flavonoids affect PPAR-γ mediated gene transcription by a mode of action different from agonist binding. Increased PPAR-γ receptor mRNA expression and synergistic effects with endogenous PPAR-γ agonists are likely to play a role in this alternative mode of action.

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Chapter 5

The effect of glucuronidation on isoflavone induced estrogen receptor (ER)α and ERβ mediated coregulator interactions

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Abstract

Non-prenylated isoflavone aglycones are known to have phyto-estrogenic properties and act as agonistic ligands on ERα and ERβ due to their structural resemblance to 17β-estradiol (E2). Genistein and daidzein are the two main dietary isoflavones; upon uptake they are extensively metabolized and exist nearly exclusively as their conjugated forms in biological fluids. Little is known about the effect of conjugation on the intrinsic estrogenic activities of these isoflavones. To characterize and compare the intrinsic estrogenic activities of genistein and daidzein, and their respective 7-O-glucuronide metabolites a cell-free assay system was employed that determines the ligand-induced changes in ERα- and ERβ-ligand binding domain (LBD) interactions with 154 different binding motifs derived from 66 different nuclear receptor coregulators. The glucuronides were 8 to 4,400 times less potent than their respective aglycones to modulate ERα-LBD and ERβ-LBD - coregulator interactions. Glucuronidation changed the preferential activation of genistein from ERβ-LBD to ERα-LBD and further increased the slightly preferential activation of daidzein for ERα-LBD. The tested isoflavone compounds were less potent than E2 (around 5 to 1,580 times for the aglycones) but modulated the LBD-coregulator interactions in a manner similar to E2. Our results show that genistein and daidzein remain agonistic ligands of ERα-LBD and ERβ-LBD in their conjugated form with a higher relative preference for ERα-LBD than the corresponding aglycones. This shift in receptor preference is of special interest as the preferential activation of ERβ is considered one of the possible modes of action underlying the supposed beneficial instead of adverse health effects of isoflavones.

Introduction

Isoflavones are a class of polyphenols that are found nearly exclusively in leguminous plants. The main dietary intake of isoflavones is through soy products or soy based food supplements with the most common dietary isoflavones being genistein and daidzein [1, 2]. Due to their structural resemblance to mammalian estrogens, isoflavones and their various conjugates are long known to possess (ant)agonistic or selective estrogen receptor modulating properties [3-7]. Isoflavone consumption is suggested to be correlated with a variety of beneficial health outcomes affecting amongst others atherosclerosis, menopausal symptoms, bone health and cancer at different sites [8-11]. Because of their phyto-estrogenic activity, isoflavones also carry the potential to cause possible adverse health effects for example those related to induction of proliferation of especially estrogen receptor-α (ERα) positive cells [12]. One of the possible reasons underlying this dualistic mode of action of isoflavones might be related to their effects on the two functionally different estrogen receptors i.e. ERα and ERβ. ERs are the main targets of estrogenic compounds, and upon their activation different transcriptional responses with opposite effects on cell proliferation are elicited; ERα activation stimulates cell proliferation, while ERβ activation causes apoptosis and reduces ERα mediated induction of cell proliferation [13-16]. Dietary isoflavones are reported to bind to and activate both ERα and ERβ, while having a relatively higher affinity for ERβ than for ERα [17-19]. Given the different activation of ERα and ERβ and their different biological responses the ratio of the two receptors in a cell may influence the ultimate outcome of exposure to an estrogen active compound [20]. In addition, the response following binding of an estrogen to the ERs is dependent on the type of coregulators recruited and/or dismissed by the ER ligand complex once bound to the estrogen responsive element (ERE) in the promoter region of responsive genes [21, 22]. Coregulator interactions play a crucial role in the transcriptional activity of nuclear receptors [23, 24]. Ligand-induced activation of nuclear receptors is characterized by a conformational change of the LBD to its active state upon ligand binding leading to the recruitment of coregulator complexes and the dismissal of corepressor complexes. This activation of the LBD is the initial event in ligand-induced transcriptional activation of nuclear receptors [25, 26] as opposed to ligand-independent mechanisms of receptor activation [27, 28]. Ligand-induced coregulator interactions of ER are correlated well with other established estrogenicity tests and the isoflavone genistein has been shown to induce ER-LBD - coregulator interactions [29, 30].

Upon ingestion the isoflavones, which occur mostly as conjugated glycosides in plants, are deconjugated to their respective aglycone in the gastrointestinal tract. During uptake, these aglycones are then extensively metabolized before entering

the systemic circulation where they can be found as glucuronidated and/or sulfated conjugates while only marginal amounts of the aglycones are present [31-33]. The biological activity of circulating flavonoid metabolites can significantly differ from their respective aglycones [34]. The studies reporting on the effect of isoflavone conjugates on ER-related endpoints in assays such as competitive receptor binding and different cellular based assays give somewhat contradicting results [2, 35, 36]. In competitive binding studies it was shown that the 7-O-glucuronides of daidzein and genistein have nearly the same affinity for human ERα as their respective aglycones, while the affinity of these glucuronides for human ERβ were lower than that of their respective aglycones [35]. In yeast-based ER subtype-specific reporter gene assays genistein and daidzein activated ERβ more potently than ERα and glucuronidation at the 7-position reduced the effect of the two isoflavones on ERα (effect not quantifiable due to incomplete concentration response curves of the glucuronides in this assay) [35]. The effect of genistein, the more potent of the two isoflavones, on ERβ was reduced around 10-fold by glucuronidation at the 7-position, while glucuronidation at the 7-position did not affect the potency of daidzein to activate ERβ [35]. In ERα and ERβ-specific reporter gene assays in transfected human osteosarcoma cells the activities of genistein and daidzein were strongly reduced by glucuronidation (around 240 to 1,660 times), as was their effect on proliferation of T47D cells (around 112 to 575 times) [2]. Islam and coworkers concluded that in the cellular models applied the glucuronides of genistein and daidzein are not estrogenic, and that the observed effects in their studies using human cell lines were caused by small amounts of the aglycones that were released during incubation [2]. Taken together, the isoflavone conjugates appear to be able to bind to and displace 17β-estradiol (E2) from the LBDs of ERα and ERβ, while the ultimate effects vary with the model system applied. There are several factors that can influence the ultimate effects of the test compounds in the model systems of the reported studies and these may explain the differential results to some extent. These factors include possible (de)conjugation of the test compounds, an effect of glucuronidation on the cellular uptake of the compounds, as well as receptor subtype-specific characteristics of the test system. Especially in intact cell models effects of (de)conjugation on cellular uptake may influence the responses detected which hampers concluding on the intrinsic estrogenic activities of the glucuronides. None of the model systems applied so far could actually characterize the intrinsic effects of the isoflavone conjugates on ER-coregulator interactions and possible subsequent effects. The objective of the present study was to characterize and compare the intrinsic estrogenic activities of the two main dietary isoflavones genistein and daidzein, and their respective 7-O-glucuronide metabolites. Given that ER binding alone does not necessarily result in an agonistic or antagonistic effect, the endpoints characterized should go beyond the binding of the isoflavones and their conjugates to the ERs. To this end the cell-free MARCoNI (Microarray Assay for

Real-time Co-regulator – Nuclear receptor Interaction) assay system determining the ligand-induced changes of ERα- and ERβ-LBD interactions with 154 different binding motifs derived from 66 different nuclear receptor coregulators was used. Because the MARCoNI assay is a cell free model system the results obtained will not be confounded by cellular (de)conjugation and uptake processes; the assay allows the detection of estrogen mediated activity as a response to ER-LBD agonism.

Materials and Methods

Chemicals

Genistein, daidzein-7-O-glucuronide (D-7G) and genistein-7-O-glucuronide (G-7G) were obtained from Extrasynthese (Genay Cedex, France). 17β-Estradiol (E2), daidzein and DL-dithiothreitol (DTT) were purchased from Sigma Aldrich (Missouri, USA). Stock solutions of the test compounds were prepared in dimethylsulfoxide (DMSO, 99.9% purity) from Acros (Geel, Belgium) and stored at –20 °C.

MARCoNI Nuclear Receptor-Coregulator Interaction Profiling

Ligand-modulated interaction of the ERα and ERβ LBDs with coregulators (154 different binding motifs of 66 different coregulators) was assessed using PamChip 4 microarray chips for nuclear hormone receptors (PamGene International B.V., 's-Hertogenbosch, The Netherlands) as described previously [37]. Briefly, for ERα the polyhistidine (His) tagged ERα LBD (amino acids 302–552, partly purified from *Escherichia coli*, final concentration between 1 and 10 nM) was mixed with anti-His antibody penta-His Alexa Fluor 488 conjugate (Qiagen, Germantown, MD, USA, #35310, final concentration 25 nM) and for ERβ glutathione S-transferase (GST) tagged ERβ subtype specific LBD (amino acids 243–530 final concentration 10 nM, AB Vector, San Diego, CA, USA #N3A2) was mixed with anti GST Alexa Fluor 488 conjugate (Molecular probes, Life Technologies Ltd, UK, #A11131, final concentration 50 nM) in the absence and presence of the potential ligands added from a stock solution in DMSO (2% final concentration) in reaction buffer (TR-FRET Coregulator Buffer E #PV4540; Invitrogen, Breda, the Netherlands) containing 5 mM DTT. To calculate the modulation indices (MIs), which express the modulation of LBD binding to coregulator motifs relative to LBD binding in the solvent control (2% DMSO), LBD-binding values (fluorescence) were log10 transformed and the fold difference between exposed reactions and solvent control reactions calculated. All assays were performed in a fully automated microarray processing platform (PamStation12, PamGene International B.V.) at 20 °C. After incubation, excess incubation mix was removed and the arrays were washed prior to acquisition of images. Concentration-response curves were tested in singular; experiments for the calculation of MIs were

performed at least in triplicate.

Image analysis was performed using BioNavigator software (PamGene International B.V.) which performs automated array grid finding and subsequent quantification of signal and local background for each individual peptide. The median signal-minus-background values were used as the quantitative parameter of binding. For data and statistical analyses BioNavigator, Microsoft Excel (Version 14.0.7106.5003; Microsoft Corporation) and IBM SPSS Statistics (Version 22, International Business Machines Corporation) were used.

Results

Isoflavone mediated modulation of ERα and ERβ binding to coregulator motifs

The isoflavone aglycones genistein, daidzein, and their relevant glucuronide conjugates G-7G and D-7G, as well as the reference compound E2 were tested in the MARCoNI coregulator binding assay to evaluate their intrinsic capacity to modulate ERα-LBD and ERβ-LBD binding to nuclear receptor-coregulator derived motifs. Figure 5.1 shows a schematic representation of the MARCoNI assay. Coregulator-derived binding peptides containing the LXXLL coactivator motif or the LXXXIXXXL corepressor motif are immobilized on a porous membrane. The reaction mix containing the LBD, potential ligand, and fluorescently labeled antibody is pumped through the porous carrier allowing the LBD to interact with the coregulator-derived peptides. LBD binding to the peptides is measured by fluorescence. Figure 5.2 shows the MIs of the tested compounds for ERα-LBD and ERβ-LBD; these are the changes in LBD binding to the coregulator motifs expressed relative to the solvent control. Positive values on the y-axis denote higher binding than the solvent control, negative values denote lower binding. The figure shows that the isoflavone aglycones and glucuronides modulate LBD binding in a way similar to the modulation induced by the reference compound E2 as apparent from the comparable patterns of modulation. Differences can be observed in the height of the patterns on the y-axis which can be due to differences in activation potency (i.e. the respective effective concentration needed to induce the effect) and the maximum ligand-induced response of the compounds. To study the differences in activation potency and maximum response between the isoflavone aglycones and their respective glucuronides, all compounds were tested at increasing concentrations and sigmoidal concentration-response curves thus obtained were fitted using least squares fit for the binding to each coregulator peptide. For concentration response-curves with a coefficient of determination (R^2) of >0.90 half-maximal effect levels (EC_{50}) as measure for the activation potency and the

maximum responses were determined. Figure 5.3 shows exemplary concentration-response curves of ERα-LBD and ERβ-LBD binding to coactivator derived peptides from NCOA3 and NRIP1 induced by E2, genistein, daidzein, G-7G and D-7G. The figure shows that the tested compounds induce the binding of ERα- and ERβ-LBD to the binding motifs derived from NCOA3 and NRIP1 in a concentration-dependent manner; the increasing relative fluorescence units (RFU) are a measure for increased binding. Supplemental Figures 5.1 to 5.5 show concentration-response curves of all compounds and coregulator motifs; Supplemental Tables 5.1 to 5.5 give EC_{50} values and maximum response of all concentration-response curves with an R^2 value of > 0.90.

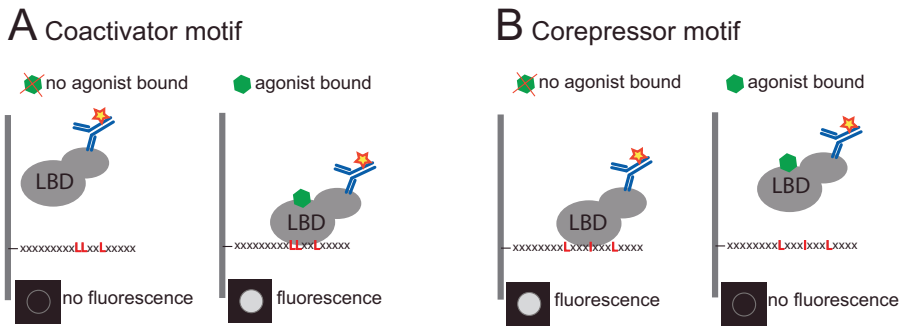
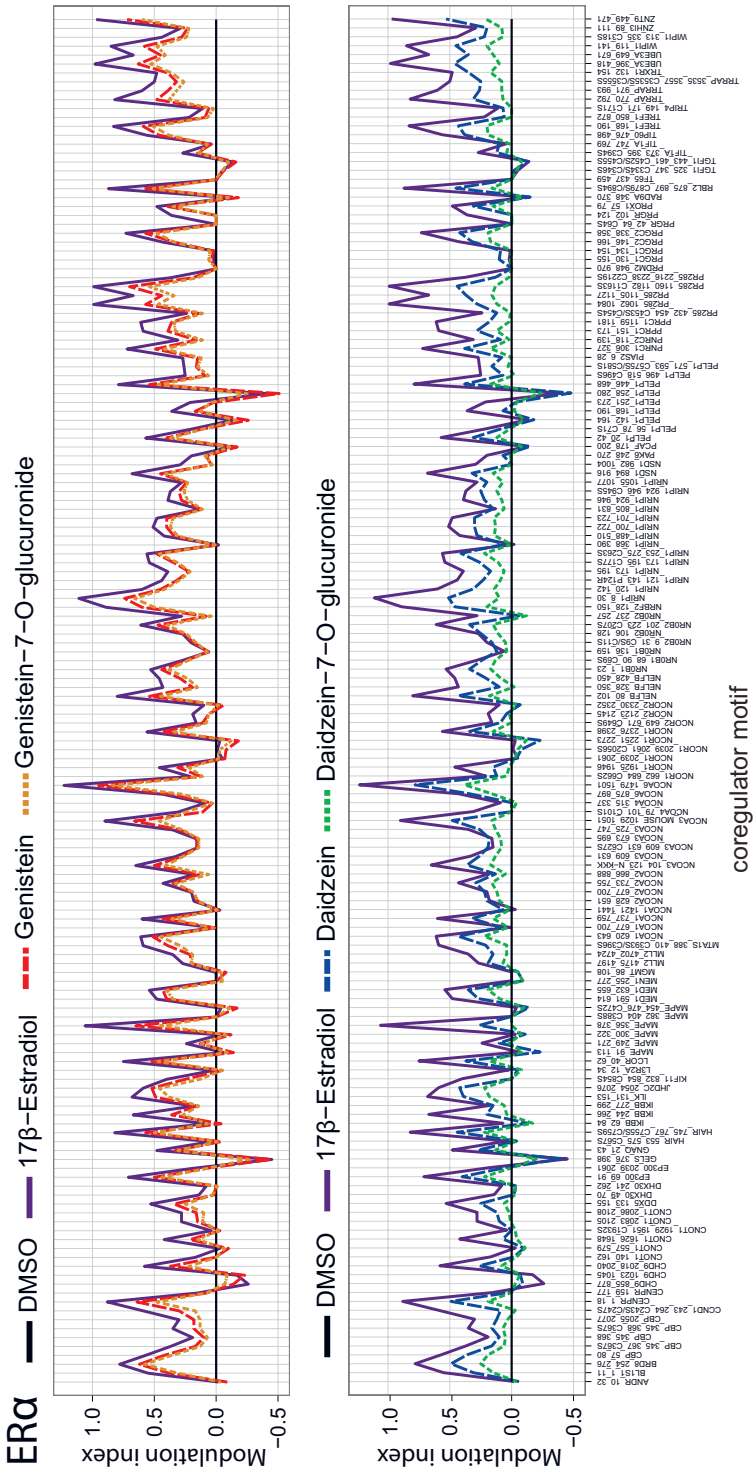


Figure 5.1 The MARCoNi assay detects binding of a nuclear receptor-LBD to coregulator motifs. Schematic description of MARCoNI coregulator binding assay. (*A*) *Coactivator motif* Binding of an agonistic ligand to the LBD increases LBD - coactivator motif interactions which increases the fluorescence signal of the corresponding spot. (*B*) *Corepressor motif* Binding of an agonistic ligand to the LBD decreases LBD - corepressor motif interactions which decreases the fluorescence signal of the corresponding spot.

To facilitate comparison of the different compounds Figure 5.4 presents histograms of the EC_{50} values of all concentration-response curves that fit the criterion of R^2 > 0.90 per compound and receptor-subtype. From this figure it can be seen that the compounds differ in potency to activate the ER-LBDs. E2 induced coregulator motif binding at lower concentrations than the isoflavone compounds indicating a higher potency (lower EC_{50} values) of E2 to activate the LBDs. The figure further shows that glucuronidation reduced the potencies of the isoflavones to activate ERα-LBD and ERβ-LBD, reflected by the fact that the conjugates had the highest EC_{50} values of the tested compounds. Table1 gives the median EC_{50} values and the corresponding 5^{th} and 95^{th} percentiles of the tested compounds per receptor subtype; the number of concentration-response curves meeting the standard for the R^2 value differed per compound and receptor subtype.


ERα
DMSO
17β−Estradiol
Genistein
Genistein−7−O−glucuronide
Modulation index
1.0
0.5
0.0
−0.5
DMSO
17β−Estradiol
Daidzein
Daidzein−7−O−glucuronide
Modulation index
coregulator motif

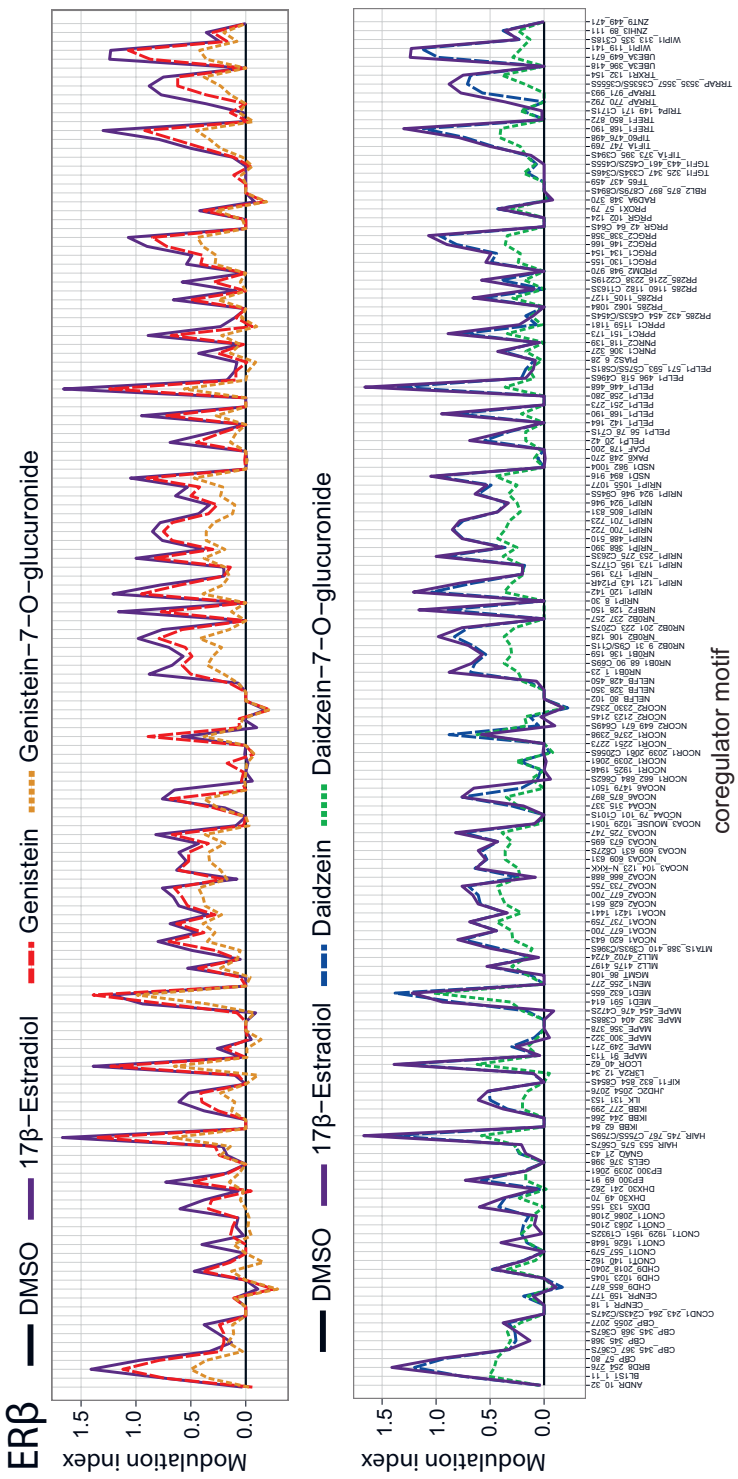


Figure 5.2 Isoflavone aglycones and glucuronides cause similar ER-LBD - coregulator binding patterns compared to E2. These MI plots depict the ligand-induced modulation of ERα-LBD and ERβ-LBD binding to coregulator motifs by estradiol (10^{-6} M) and isoflavones (10^{-4} M). Incubation of the ER-LBDs with isoflavone aglycones and glucuronides cause changes in coregulator binding patterns similar to E2 indicating a comparable mode of action.

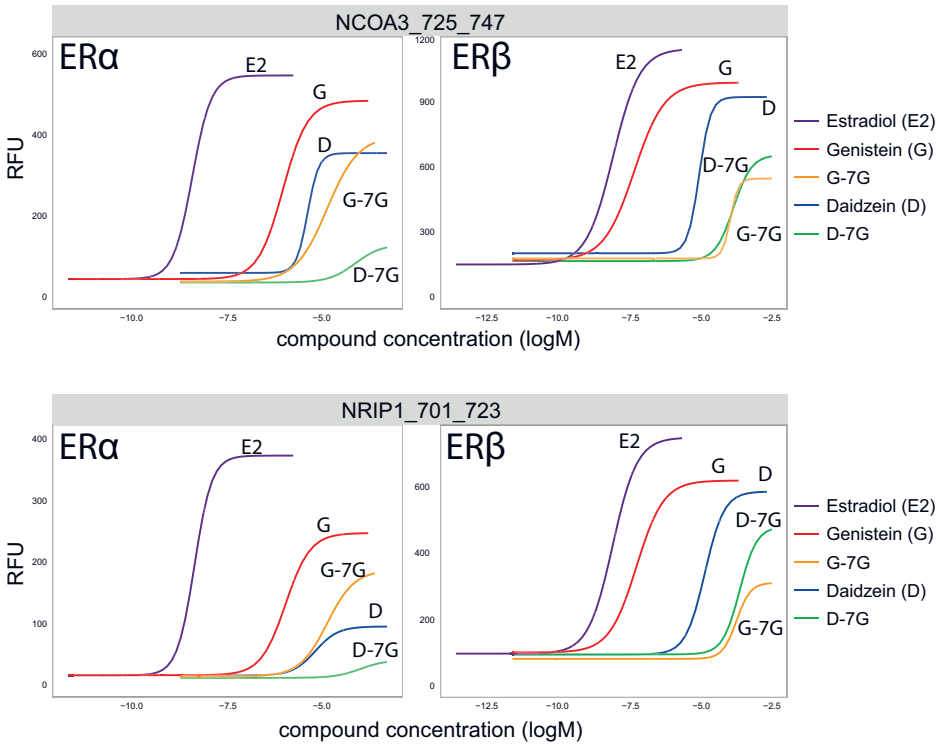


Figure 5.3 The test compounds increase ER-LBD binding to coregulator motifs in a concentration-dependent manner. The figure shows concentration-response curves of ERα-LBD and ERβ-LBD binding to the coactivator derived motif NCOA3 (amino acids 725 to 747) and NRIP1 (amino acids 701 to 723) induced by E2 and isoflavones. E2 increases ER-LBD - coregulator motif binding at lower concentrations than genistein, daidzein and their glucuronides. Glucuronidation of the isoflavones increases the concentrations needed for activity.

5

Comparison of the effects of genistein and daidzein to E2

To further compare the effects of the flavonoid aglycones to E2 relative potency factors (RPF_{E2}) and relative maximum responses (RMR_{E2}) were calculated. To derive the RPF_{E2}, the EC_{50} values of genistein and daidzein were divided by the EC_{50} values of E2 per coregulator binding motif, respectively. The RMR_{E2} were derived by dividing the maximum responses induced by genistein and daidzein by the maximum responses induced by E2 per coregulator motif. For the calculations of the RPF_{E2} and RMR_{E2} only coregulator motifs were used where the concentration response curves for both the respective isoflavone aglycone and E2 met the criterion of R^2>0.90. Figure 5.5 presents a summarized overview of the RPF_{E2} and RMR_{E2} of genistein and daidzein compared to E2 for both receptor subtypes; the figure depicts histograms of the derived values and the median of the values. Genistein is around 200 times less potent than E2 to activate ERα-LBD coregulator interactions (median RPF_{E2} = 203); for ERβ, however, genistein

is only around 5 times less potent than E2 (median RPF_{E2} = 5.4). This relatively higher affinity of genistein for ERβ-LBD over ERα-LBD is also reflected in the lower EC_{50} values for ERβ-LBD activation than for ERα-LBD activation (see Table 5.1). Daidzein appears to be a less potent agonist of ERα-LBD and ERβ-LBD than the structurally related isoflavone genistein; Figure 5.5 shows that daidzein is around 1,300 times less potent than E2 for ERα-LBD (median RPF_{E2} = 1,336) and around 1,600 times less potent than E2 for ERβ-LBD (median RPF_{E2} = 1,580). The maximum responses induced by the isoflavones were lower than those induced by E2 (Figure 5.5). The maximum responses of genistein were around 70% of those for E2 for both ERα-LBD and ERβ-LBD (median RMR_{E2} = 0.66 and 0.71, respectively). The maximum responses of daidzein for ERβ-LBD were also around 70% of those of E2 (median RMR_{E2} = 0.75); for ERα-LBD, however, the maximum responses of daidzein were only around 30% of those of E2 (median RMR_{E2} = 0.29). While genistein is a more potent agonist for ERβ-LBD than ERα-LBD, the EC_{50} values of daidzein indicate that daidzein is a slightly more potent agonist for ERα-LBD than for ERβ-LBD (see also Table 5.1).

Table 5.1 The test compounds activate ERα-LBD and ERβ-LBD with different potencies. The median EC_{50} values and 5^{th} – 95^{th} percentiles for ERα-LBD and ERβ-LBD activation of the test compounds. The number of concentration-response curves that fitted the criterion of R2 > 0.90 is given in brackets. E2 is the most potent agonist of ERα-LBD and ERβ-LBD, followed by genistein, daidzein, and their glucuronides. Genistein shows a clear preference for ERβ-LBD activation. Glucuronidation increases the EC_{50} values of genistein and daidzein and affects the receptor subtype preferences of the isoflavones

Receptor	Compound	Median EC_{50}		5^{th} - 95^{th} percentile
ERα	E2	3.8 nM	(n=95)	0.9 – 18.7 nM
	Genistein	586.5 nM	(n=76)	237.3 – 1,055.3 nM
	G-7G	9.2 µM	(n=65)	3.0 – 20.9 µM
	Daidzein	3.2 µM	(n=68)	1.7 – 4.8 µM
	D-7G	10.6 µM	(n=76)	1.9 – 167.4 µM
ERβ	E2	3.8 nM	(n=87)	1.9 – 18.8 nM
	Genistein	20.1 nM	(n=72)	8.1 – 38.8 nM
	G-7G	78.91 µM	(n=49)	31.0 – 285.7 µM
	Daidzein	5.8 µM	(n=79)	3.2 – 15.7 µM
	D-7G	92.7 µM	(n=63)	43.8 – 368.0 µM

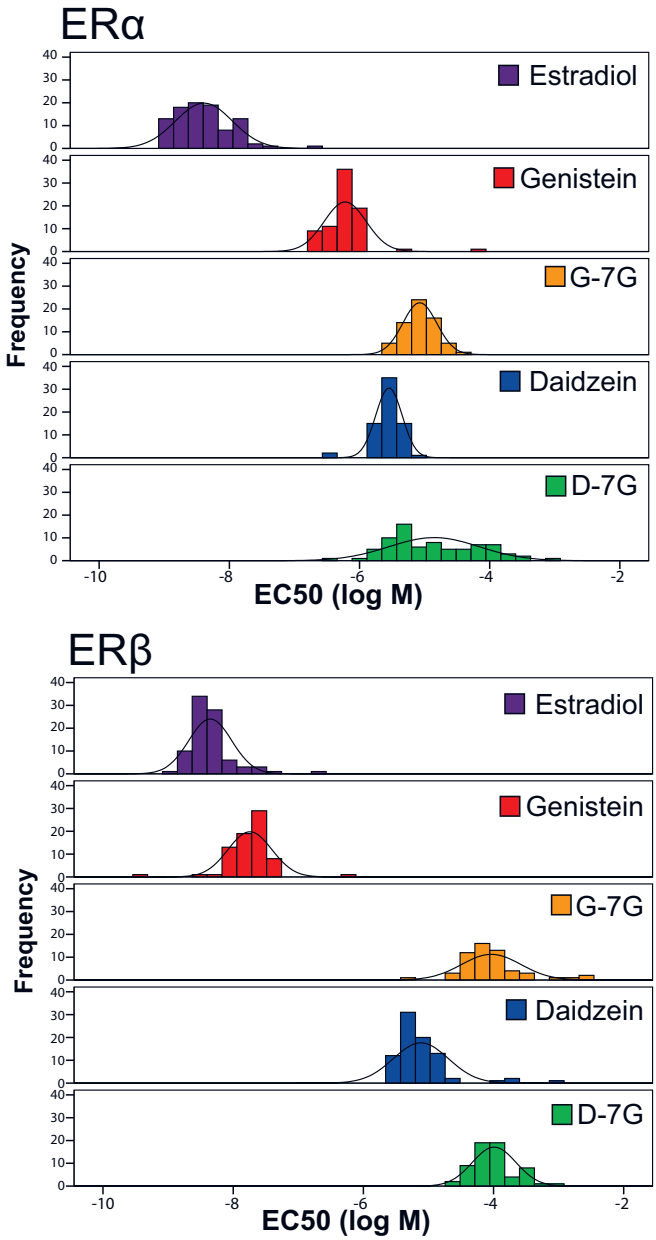


Figure 5.4 Glucuronidation increases the EC_{50} values of genistein and daidzein for ER-LBD binding to coregulator motifs. The figure depicts histograms of EC_{50} values of coregulator concentration-response curves with $R^2 > 0.90$. Estradiol has the lowest EC_{50} values of the tested compounds, followed by genistein, daidzein, and their respective glucuronides.

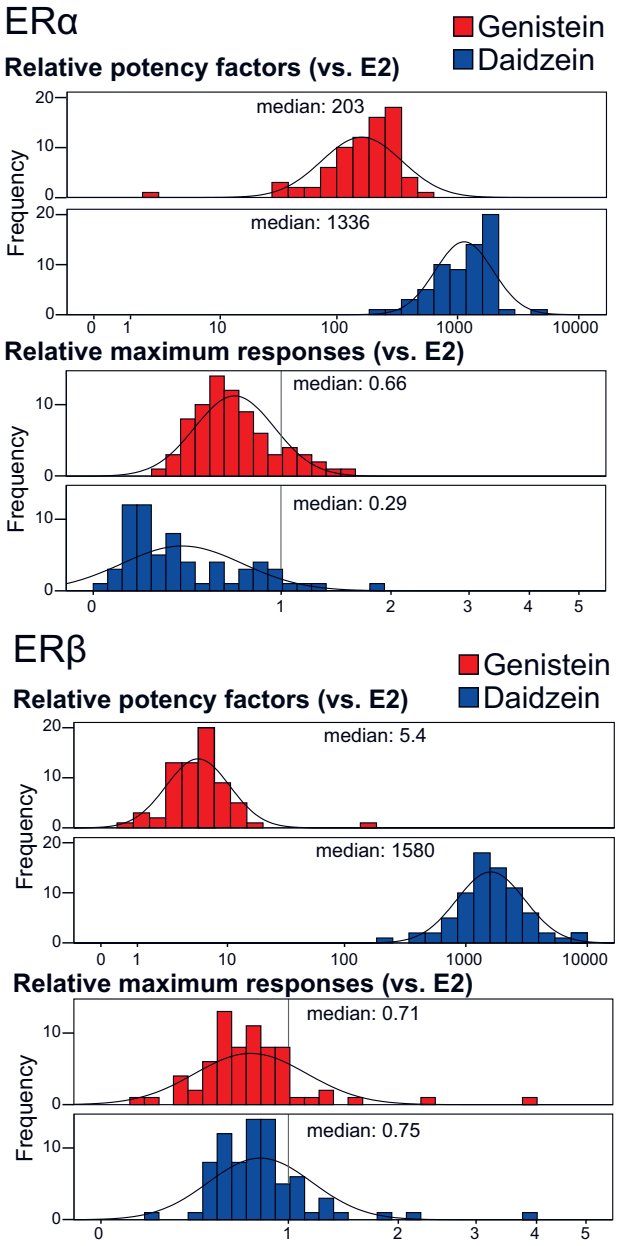


Figure 5.5 The isoflavone aglycones show different relative potencies and maximum responses for ERα-LBD and ERβ-LBD activation compared to E2. The figure shows histograms of relative potency factors of genistein and daidzein aglycone compared to E2 (RPF_{E2}) and relative maximum responses of genistein and daidzein aglycone compared to E2 (RMR_{E2}) for ERα-LBD and ERβ-LBD; median values are given in the figure. Genistein is a more potent agonist of both ERα-LBD and ERβ-LBD than daidzein and shows a clear preference for ERβ-LBD activation over ERα-LBD activation. Daidzein has a slightly higher relative potency to activate ERα-LBD than ERβ-LBD. Both isoflavone aglycones induce lower relative maximum responses than E2; daidzein shows the lowest relative maximum responses for ERα-LBD.

The effect of glucuronidation on the activity of genistein and daidzein

The effect of the glucuronidated isoflavones on ERα-LBD and ERβ-LBD activation were compared to the effect of their respective aglycones. The glucuronide samples were analyzed chromatographically for the presence of aglycone impurities; in the chromatograms of the glucuronide samples no aglycones were present (detection limit < 0.05%) and no detectable amounts of the respective aglycones were formed during the incubation in the MARCoNI assay buffer (for chromatograms see Supplemental Figure 5.6). Figure 5.6 shows histograms of the relative potency factors of the glucuronides compared to their respective aglycones (RPF_{agl}) and relative maximum responses compared to their respective aglycones RMR_{agl}. The RPF_{agl} were derived by dividing the EC_{50} values of G-7G and D-7G by the EC_{50} values of their respective aglycone forms per coregulator binding motif. The RMR_{agl} for the glucuronides were calculated by dividing the maximum responses induced by G-7G and D-7G by the maximum responses induced by their respective aglycones per coregulator motif. For the calculations of the RPF_{agl} and RMR_{agl} only coregulator motifs were used where the concentration response curves for both the respective isoflavone aglycones and glucuronides met the criterion of R^2>0.90. Glucuronidation lowered the potency of genistein to activate ERα-LBD and ERβ-LBD. The glucuronidation of genistein reduced its potency to activate ERβ-LBD around 4,400-fold (median RPF_{agl} = 4,391) while it reduced genistein's potency to activate ERα-LBD only around 15 times (median RPF_{agl} = 14.7). Not only the potencies were affected by glucuronidation, but also the relative preference for the ER subtype.

Based on the median EC_{50} values, genistein activated ERβ-LBD around 29-times more potently than ERα-LBD, whereas upon glucuronidation the ER subtype preference changed since G-7G activated ERα-LBD about 8.5 times more potently than ERβ-LBD. The EC_{50} values for ER activation of daidzein, though in general being less potent than genistein, were less affected by glucuronidation than those of genistein. D-7G activated ERα-LBD around 8 times less potently than daidzein (median RPF_{agl} = 8.1) and ERβ-LBD around 16 times less potently than daidzein (median RPF_{agl} = 16.1). Daidzein and D-7G both activated ERα-LBD more potently than ERβ-LBD, and for this isoflavone glucuronidation further increased the relative preference for ERα-LBD over ERβ-LBD; the median EC_{50} concentrations of daidzein and D-7G for ERα-LBD activation were around 2 and 9 times lower than for ERβ-LBD activation, respectively. Glucuronidation also reduced the maximum responses of the isoflavones. As can be seen from Figure 5.6, glucuronidation lowered the maximum responses of genistein by 30-40% for ERα-LBD and ERβ-LBD (median RMR_{agl} = 0.71 and 0.62, respectively). Glucuronidation reduced the maximum responses of daidzein by around 25% for ERβ-LBD (median RMR_{agl} = 0.76) and by around 55% for ERα-LBD (median RMR_{agl} = 0.43) which already showed relatively low maximum responses in comparison to E2 and genistein.

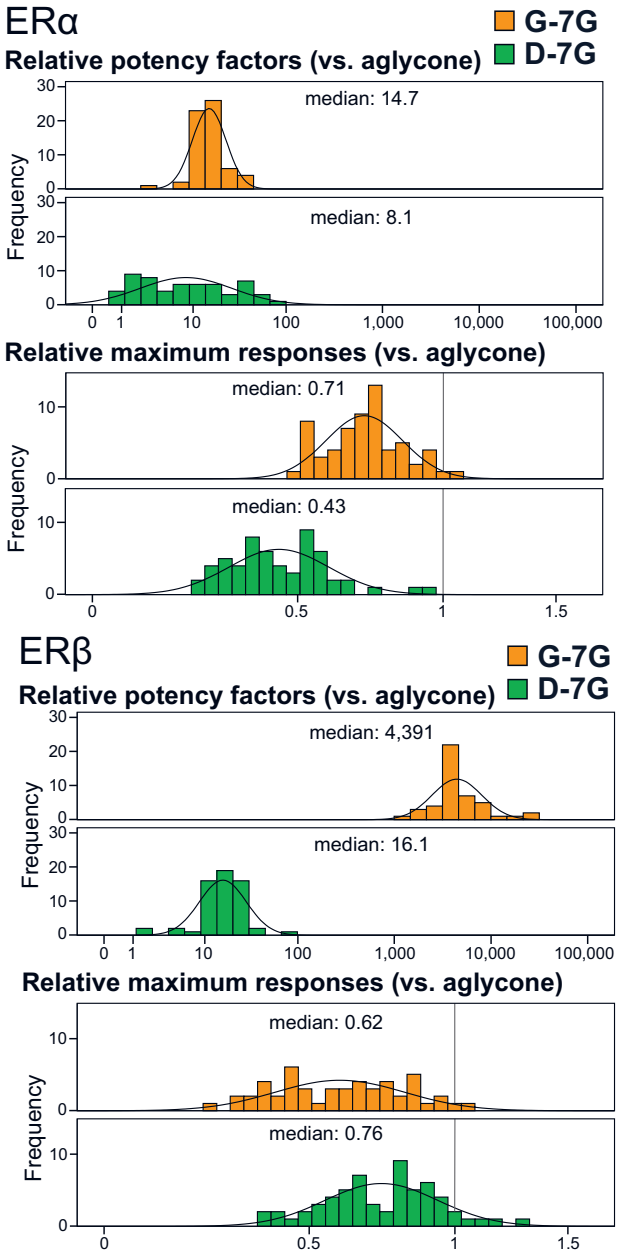


Figure 5.6 Glucuronidation of genistein and daidzein reduces their potencies and maximum responses for ERα-LBD and ERβ-LBD to a different extent. The figure shows histograms of relative potency factors of G-7G and D-7G compared to the respective aglycones (RPF_{agl}) and relative maximum responses of G-7G and D-7G compared to the respective aglycones (RMR_{agl}) for ERα-LBD and ERβ-LBD; median values are given in the figure. Glucuronidation strongly reduced the potency of genistein to activate ERβ-LBD while its potency to activate ERα-LBD, as well as the potency of daidzein to activate both ERα-LBD and ERβ-LBD were affected to a much lesser extent. The maximum responses induced by the isoflavone aglycones were reduced by glucuronidation, most notably those of daidzein on ERα-LBD.

Altogether, even though glucuronidation decreases the relative potencies of genistein and daidzein, the glucuronides remain active. It appears that glucuronidation of genistein changes its relative preference for ERβ-LBD activation towards ERα-LBD activation and glucuronidation of daidzein increases its slight relative preference for ERα-LBD activation over ERβ-LBD activation.

Discussion

The objective of this study was to characterize and compare the intrinsic activities of the two main dietary isoflavones genistein and daidzein, and their respective 7-O-glucuronide metabolites to activate ERα-LBD and ERβ-LBD mediated responses. To this end the ligand-induced changes of ERα-LBD and ERβ-LBD interactions with nuclear-receptor coregulator binding motifs were studied in the MARCoNI assay. All of the tested isoflavone compounds, including the two glucuronide metabolites, induced concentration-dependent changes in ERα-LBD and ERβ-LBD binding to coregulator motifs. The observed MI-patterns induced by the isoflavones and their glucuronide conjugates were comparable to the MI-patterns of the reference compound E2 (Figure 5.2), while the potencies (reflected by the EC_{50}) and the maximum responses of the isoflavones were generally lower than those of E2 (Figures 2-5). Glucuronidation reduced the potential of both genistein and daidzein to activate ERα-LBD and ERβ-LBD, but the conjugates retained significant activity. Kinjo and co-workers [35] report that glucuronides of daidzein and genistein can bind to ERα and ERβ. Based on the modulation indices (Figure 5.2) our results show that the isoflavone compounds induce coregulator binding patterns of ERα-LBD and ERβ-LBDs that are comparable to those induced by E2. There are differences between the conjugates and their respective aglycones in the potencies and the maximum responses induced. Islam and co-workers [2] concluded that in their experiments using human cell lines to study the estrogenic effects of isoflavone glucuronides the glucuronides of genistein and daidzein needed to be deconjugated to their respective aglycones to become bioactive. It is likely that in experiments with intact cell models the isoflavone glucuronides are not as efficiently taken up by the cells as the respective aglycones and therefore no or only a very low activity of the glucuronides can be observed. While the reported potencies of the glucuronides in the U2OS based reporter gene assays for ERα and ERβ mediated gene expression was at least 240x lower than that of the aglycones [2], the potencies to induce T47D cell-proliferation were, in the case of G-7G, only around 40-100x lower than genistein; this is surprising as the T47D-cells were reported to deconjugate the isoflavone glucuronides less efficiently than the U2OS cells [2]. Taken together, these data suggest that, depending on the cell type, the deconjugation of the glucuronides might play a role in the activity of the conjugates especially when cellular uptake of the conjugates is otherwise low; we show that the glucuronides retain a significant part of the intrinsic activities of the respective aglycones on ERα-LBD and ERβ-LBD.

Our studies further revealed that in the coregulator binding assay E2 has comparable EC_{50}s for ERα-LBD and ERβ-LBD activation. In line with these results, binding studies show that E2 has the same binding affinities for ERα and ERβ [38, 39]. In contrast to this, in reporter gene assays E2 has lower EC_{50} values for ERα activation than ERβ activation [2, 29]. The relative differences in receptor subtype activation and the reported transcriptional activities might be due to different activities of the ligand-independent activation function (AF)-1 of ERα and ERβ. Especially for ERα it is reported that there is a strong synergy between AF-1 and AF-2 (i.e. the ligand-activated part of the receptor), which can increase the transcriptional activity of ERα upon ligand activation while ERβ is reported to have a non-functional AF-1 [40-44].

Our results show that the isoflavone aglycones were less potent than E2 in activating the LBDs of ERα and ERβ, and that genistein more potently activated the ER-LBDs than daidzein. This is in line with previous reports stating that genistein and daidzein bind to ERα and ERβ with a lower affinity than E2 [19, 45] and that genistein and daidzein induce ERα and ERβ mediated gene transcription, as well as cell proliferation at higher concentrations than E2 with genistein often, though not consistently, being more potent than daidzein [2, 17, 18, 45].

In our experiments lower concentrations of genistein were needed to activate ERβ-LBD than ERα-LBD. This is in line with reports in literature that genistein preferentially binds to and transcriptionally activates ERβ over ERα [2, 19, 45, 46]. For daidzein we observed a slightly lower median EC_{50} value for ERα-LBD than for ERβ-LBD activation. According to literature, daidzein has a slightly higher binding affinity for ERβ over ERα [19, 45] and it induces ERβ-mediated gene transcription more potently than ERα-mediated gene transcription [2, 45, 46]. While there were only slight differences in EC_{50} values for ERα-LBD and ERβ-LBD activation by daidzein, the maximum responses induced were considerably lower for ERα-LBD than for ERβ-LBD. Differences in maximum responses for ER-coregulator binding between different agonists have been reported [30]. We speculate that the reduced maximum responses might be a consequence of different positioning of the LBDs' helix-12 (H12) upon agonist binding. The positioning of H12 is a key determinant for the recruitment of coregulators. When bound to an agonist ligand like E2, H12 folds over the ligand binding pocket and in that position is an integral part of the interaction surface for co-activator binding [25]. It is reported for ERβ that when bound to genistein, H12 is not fully positioned over the ligand binding pocket and that coregulators must displace H12 into the correct agonist position before binding [47]. Compared to E2 genistein induces lower maximum responses and it is possible that the positioning of H12 might be the reason for this lower binding induced by the isoflavone compounds. If lower maximum responses observed in the MARCoNI assay correspond to lower transcriptional activation, then this could serve as an explanation why daidzein is reported to have a relatively higher transcriptional activation of ERβ over ERα while

it is activating both receptor subtypes at similar concentrations.

It is of importance to note that in addition to intracellular receptors also membrane bound receptors may be involved in the effects induced by estrogens. Membrane bound estrogen receptors are reported to be structurally identical to the nuclear ERs [48-50]; they derive from the same genes as the nuclear receptor ERs [51] while a posttranslational modulation on Cys447 allows the receptors to associate with the membrane [52]. Membrane bound ERs are G protein coupled and induce kinase signaling cascades upon activation [53, 54]. Interestingly, only the E-domain (i.e. the LBD) of the receptor is required for activity of membrane bound ERs [55]. These membrane-bound ERs are not to be confused with G-protein coupled estrogen receptors (also known as GPR30) that are mainly located intracellularly [56, 57]. Based on their structural similarity to nuclear ERs it is likely that the membrane bound ERs can also be activated by isoflavones and their circulating metabolites.

In conclusion, our results show that the isoflavone compounds cause changes in ER-LBD coregulator binding patterns comparable to E2 while having lower potencies and maximum responses. The extent and nature of the effects of glucuronidation on the activities of daidzein and genistein differs, but in both cases the conjugates retain the ability to activate ERα-LBD and ERβ-LBD, while conjugation appeared to shift the preference for activation more in favor of ERα-LBD over ERβ-LBD. This shift in receptor preference is of special interest as the preferential activation of ERβ is considered one of the possible modes of action underlying the supposed beneficial instead of adverse health effects of isoflavones [12].

Supplemental data

Supplemental Figures 5.1 - 5.6 and Supplemental Tables 5.1 - 5.5 can be downloaded from: https://goo.gl/jd7kBJ (case sensitive)

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Chapter 6

The effect of glucuronidation on the potential of kaempferol to inhibit serine/threonine protein kinases

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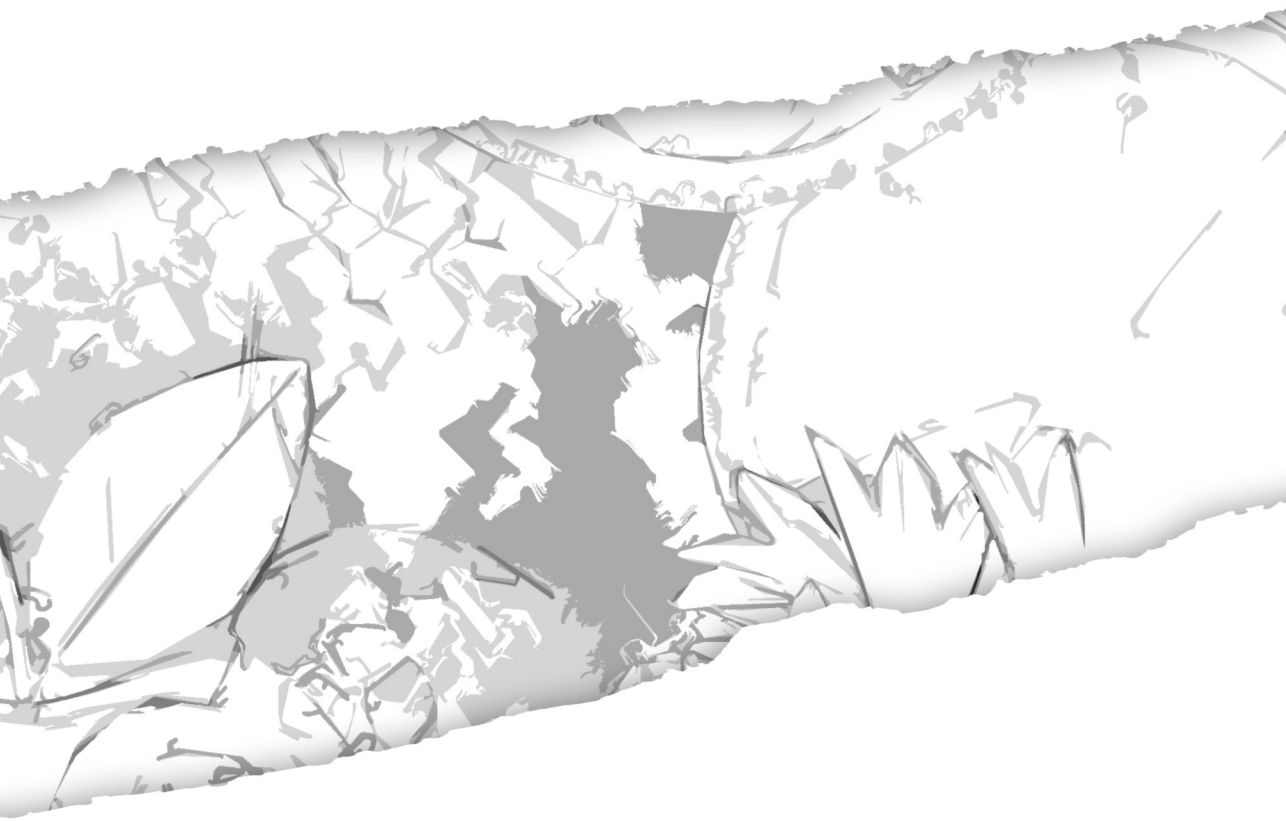

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Abstract

To study the effect of metabolic conjugation of flavonoids on the potential to inhibit protein kinase activity, the inhibitory effects of the dietary flavonol kaempferol and its major plasma conjugate kaempferol-3-*O*-glucuronide on protein kinases were studied. To this end, the inhibition of the phosphorylation activity of recombinant protein kinase A (PKA) and of cell lysate from the hepatocellular carcinoma cell line HepG2 on 141 putative serine/threonine phosphorylation sites derived from human proteins was assessed. Glucuronidation reduced the inhibitory potency of kaempferol on the phosphorylation activity of PKA and HepG2 lysate on average about 16 and 3.5 times, respectively, but did not appear to affect the target selectivity for kinases present in the lysate. The data demonstrate that upon glucuronidation, kaempferol retains part of its intrinsic kinase inhibition potential which implies that K-3G does not necessarily need to be deconjugated to the aglycone for a potential inhibitory effect on protein kinases.

Introduction

Flavonoids are ubiquitously present in plants as secondary metabolites. Important dietary sources of flavonoids are fruits, vegetables and their juices, as well as tea, wine, and cocoa-derived products [1, 2]. Diets rich in flavonoids are associated with the prevention of various degenerative diseases and improvement of disease states [1, 3-5]. Most flavonoids of relevance for the human diet occur in nature in their glycosidic form with some exceptions such as catechins in tea, and leaf surface flavonoids of certain herbs. Upon ingestion, these flavonoid glycosides have to be hydrolyzed to their respective aglycones before or during uptake in the gastrointestinal tract. During uptake the aglycones are extensively metabolized to sulfated, methylated and/or glucuronidated conjugates in intestinal tissue or the liver before they enter the systemic circulation [6]. As a result, under physiological conditions, most flavonoids usually do not occur as aglycones in biological fluids. It is widely accepted that conjugation and deconjugation can significantly influence the biological activity of flavonoids [7, 8].

Flavonoids are suggested to induce their biological effects amongst others through the inhibition of protein kinases [9-13]. The effect on protein kinases is of special interest as protein kinases are involved in a wide range of physiological processes by controlling signaling cascades and regulating protein functions [14-19]. Protein kinases transfer the terminal phosphate of ATP (i.e. the gamma phosphate) to a hydroxyl-group of a substrate. In eukaryotes protein kinases phosphorylate either tyrosine residues (tyrosine-specific protein kinases; PTKs), serine/threonine residues (serine/threonine-specific protein kinases; STKs) or both tyrosine and serine/threonine residues (dual-specificity protein kinases) [20, 21]. STKs are the most common eukaryotic protein kinases and phosphoserine and phosphothreonine are more abundant than phosphotyrosine in eukaryotic proteins [22]. Protein kinase inhibition is studied especially because of its pharmacological relevance; notably, most protein kinase inhibitors with clinical approval are used for cancer treatment [23, 24]. Protein kinase inhibitors can bind either covalently or non-covalently to protein kinases; inhibitors that interfere with the ability of the kinase to bind ATP are in general less selective than allosteric inhibitors which usually show very high selectivity for specific protein kinases [22].

Flavonoids are reported to act as inhibitors of protein kinases [9, 25, 26]; however, these in vitro experiments on protein kinase inhibition by flavonoids are generally conducted using their aglycone forms and not the conjugated metabolites that can be found in plasma after uptake. Very little information can be found on the effect of flavonoid conjugates on protein kinases. While certain methylated conjugates of several flavonoids are equally, less or more potent than the respective aglycone to inhibit p38α and JNK3, depending on the amount and position of the methyl groups, glucuronidation of quercetin at the 3-position was reported to reduce its potency to inhibit p38α and

JNK3 [27]. Given that glucuronidation is generally the major metabolic conjugation reaction for flavonoid aglycones [2] the aim of the present study was to characterize the effect of flavonoid glucuronidation on protein kinase inhibition. To this end we selected the dietary flavonol kaempferol and its main plasma conjugate K-3G [28] as model compounds; kaempferol is reported to inhibit a range of protein kinases with different potencies (see Table 6.1). The potential of these compounds to inhibit protein kinase activity to that of the standard kinase inhibitor staurosporine was compared. The effects on human PKA as a model kinase, and cell lysate of HepG2 cells containing many different cellular kinases were studied in a microarray system that simultaneously determines the phosphorylation of 141 putative serine/threonine phosphorylation sites derived from human proteins.

Materials and Methods

Materials

Kaempferol was obtained from Sigma-Aldrich (St. Louis, MO, USA). K-3G was purchased from Extrasynthese (Genay Cedex, France). Staurosporine was acquired from Enzo Life Sciences (Farmingdale, NY, USA). Stock solutions of these chemicals were prepared in dimethylsulfoxide (DMSO, 99.9% purity) which was obtained from Acros (Geel, Belgium). Acetic acid was purchased from VWR International (Darmstadt, Germany). Acetonitrile was acquired from Biosolve BV (Valkenswaard, the Netherlands). Sodium hydroxide (NaOH) was purchased from Merck Millipore (Darmstadt, Germany). M-PER Mammalian Protein Extraction Reagent, Halt Phosphatase Inhibitor Cocktail, and Halt Protease Inhibitor Cocktail (EDTA-Free) were purchased from Fisher Scientific (Pittsburgh, PA, USA). The human hepatoma cell line HepG2 was purchased from the American Type Culture Collection (Manassas, VA, USA). Fetal calf serum (FCS), DMEM/F12 with glutamax and phosphate buffered saline (PBS) were obtained from Gibco (Paisley, United Kingdom).

Cell cultures

HepG2 cell cultures were maintained in DMEM/F12 +10% FCS at 37° C 5% CO_2. Cells were plated at $2x10^5$ cells / 2 mL per well in 6-wells plates. After 24h the medium was aspirated and the cells washed twice with 2 mL PBS. After aspiration of PBS, 300 µL M-PER (Mammalian Protein Extraction Reagent) containing 2x Halt Phosphatase Inhibitor Cocktail and 2x Halt Protease Inhibitor Cocktail (EDTA-Free) were added per well. The plates were left for 10 minutes on ice and the cells were subsequently scraped and harvested into microcentrifuge tubes. The lysate was centrifuged at 16,000 RCF, at 4 °C for 15 minutes. The supernatant was pooled, aliquoted and stored at -80 °C until

Table 6.1 Literature based overview of protein kinase inhibition by kaempferol. Where available, IC_{50} values are given, otherwise the lowest effective concentration tested is given

Protein kinase	IC_{50}	Lowest effective concentration tested	Reference
CDK1		40 µM	[56]
CDK1/cyclinB	41 µM		[57]
CDK5	66 µM		[58]
CDK5/p25	59 µM		[59]
CDK5/p25	52 µM		[57]
CDK6/Vcyclin	22 µM		[57]
CK (G-type)		2.5 µM	[60]
CK1α	> 40 µM		[61]
CK1γ1	> 40 µM		[61]
CK1δ	27 µM		[61]
CK2	1.9 µM		[62]
CK2	1.9 µM		[63]
CK2	0.40 µM		[61]
CK (Golgi apparatus)	> 40 µM		[61]
GSK-3	3.5 µM		[57]
GSK-3β	3.5 µM		[64]
GSK-3β	4.5 µM		[59]
IRAK1		100 µM	[65]
IRAK4		100 µM	[65]
JAK3		20 µM	[66]
JNK		1 µM	[67]
JNK3	19 µM		[27]
MSK1		12.5 µM	[68]
p38α	18 µM		[27]
PI3K		60 µM	[69]
PI3K		10 µM	[70]
PIM1	1.3 µM		[71]
PKA	150 µM		[72]
PKC		60 µM	[69]
PKC	15 µM		[72]
PKC	0.025-0.1 µM		[73]
PKC	34 µM		[74]
PKG	17 µM		[75]
RSK	15 µM		[76]
RSK2		1.5 µM	[77]
RSK2		12.5 µM	[68]
RSK2	7 µM		[78]
RSK2	1.7 µM		[79]
Src		10 µM	[80]
Src		100 µM	[65]
Syk		100 µM	[65]

further analysis. The protein content of the cell lysate was quantified using a BCA Protein Assay Kit (Pierce Biotechnology, Rockford, IL, USA) according to the manufacturer's instructions.

UPLC analysis

The purity and stability of the flavonoid compounds were analyzed chromatographically using a Waters ACQUITY UPLC H-Class System with an ACQUITY UPLC BEH C18 1.7 µm (2.1 x 50 mm) column, connected to a Waters ACQUITY UPLC photodiode array detector. The mobile phases used were nanopure water (+0.1% acetic acid) and acetonitrile (+0.1% acetic acid).

As kaempferol was not entirely soluble at the highest concentrations used the actual concentrations of kaempferol in solution were determined as follows. Samples were prepared according to the procedure described below for the serine/threonine kinase microarray assay, omitting the addition of PKA or HepG2 lysate. After centrifugation the supernatants were removed and the pellets formed were dissolved in 0.01% NaOH (which allows complete dissolution of kaempferol at concentrations above 1 mM). The amounts of precipitated kaempferol were compared against a standard curve of kaempferol in 0.01% NaOH and the concentrations prepared corrected for this loss. K-3G was entirely soluble at all concentrations tested.

Serine/threonine kinase microarrays

Microarray experiments were performed using serine/threonine kinase (STK) PamChip peptide arrays on a PamStation12 instrument (PamGene, 's Hertogenbosch, the Netherlands). Per STK PamChip array 144 peptides are immobilized; the peptides represent 15-amino-acid sequences of which 13 residues are derived from known putative phosphorylation sites in human proteins (including 3 control peptides, i.e. one artificial sequence and two pre-phosphorylated sequences). The peptides are immobilized on a porous three-dimensional carrier through which the reagents are repeatedly pumped up and down. The experiments were conducted according to the manufacturer's instructions as follows. To prevent aspecific antibody binding the arrays were incubated with 2% bovine serum albumin (BSA) solution that was pumped through the carrier material for 30 pumping cycles (30 seconds per cycle) prior to loading the samples. Sample mixes were prepared containing 2 µg recombinant human full length PKA (Cat# 14-440, Merck Millipore) or 6 µg of the HepG2 lysate (total protein) per array in protein kinase buffer (PamGene), containing the test substances added from a 200 times concentrated stock solution in DMSO (0.5% final concentration of DMSO), and the primary antibody against phosphoserine/-threonine. The sample mixes were incubated on ice for 15 minutes prior to the addition of 100 µM ATP. Subsequently, the sample mixes were centrifuged at 20,000 rcf for 5 minutes at 4 °C to remove possible

antibody-aggregates. After aspiration of the BSA-solution and washing of the arrays with PK-Buffer (PamGene) three times for two cycles, the sample mixes were loaded onto the arrays and pumped for 60 pumping cycles at 30 °C. After aspiration of the incubation mixture and washing of the arrays three times with PBST-buffer (PamGene), 0.25 µg Alexa Fluor® 647 Donkey anti-rabbit IgG (Cat# 406414, BioLegend, San Diego, CA, USA) in antibody buffer (PamGene) were applied and incubated for 30 cycles. After aspiration of the solution containing the detection antibody the arrays were washed with PBST-buffer three times for two cycles. Subsequently, images were taken using the Cy5 channel of the CCD camera in the PamStation12. Images were analyzed by BioNavigator software (PamGene). The fluorescence intensities of the spots were corrected for background fluorescence adjacent to the spots and expressed as arbitrary relative fluorescence units (RFUs). The respective solvent control values were used as lowest concentrations in concentration response curves. To facilitate the comparison of the uninhibited phosphorylation patterns of PKA and HepG2 cell lysate the corrected relative fluorescence units (cRFUs) were derived as follows. The mean fluorescence of all peptides was calculated for every array (hereafter called the array mean). Subsequently, the mean of all array means was calculated (hereafter called the overall mean). A correction factor for every array was derived by dividing the overall mean by the respective array mean. To derive the cRFUs, the fluorescence of each peptide was scaled by multiplying the peptide's RFU with the correction factor for the respective array. For data analysis BioNavigator, Microsoft Excel (Version 14.0.7106.5003; Microsoft Corporation) and IBM SPSS Statistics (Version 22, International Business Machines Corporation) were used.

Results

Figure 6.1 shows that under solvent control conditions the recombinant PKA and the lysate of HepG2 cells both phosphorylate a range of substrates on the microarrays; both samples give very similar patterns of phosphorylation. PKA phosphorylated a total of 61 substrates and HepG2 lysate 66 substrates on the arrays with a cRFU value above 50. Figure 6.2 shows exemplary concentration-response curves of the effect of kaempferol, K-3G and staurosporine on the phosphorylation of three substrates of PKA and HepG2 lysate. The substrates for which concentration-response curves are presented are derived from the human proteins cAMP response element-binding protein (CREB1), gamma-aminobutyric acid receptor subunit beta-2 (GBRB2) and nuclear factor NF-kappa-B p105 subunit (NFKB1) respectively. The CREB1-derived substrate contains two serine residues, and the GBRB2- and NFKB1-derived substrates contain two serine residues and one threonine residue each. From Figure 6.2 it can be seen that addition of increasing concentrations of the flavonoid kaempferol and its conjugate K-3G, as well

as of the broad-spectrum protein kinase inhibitor staurosporine, result in a reduction of the phosphorylation activity of the samples in a concentration dependent manner. UPLC analysis revealed that the concentration of kaempferol decreased by around 10% during incubation and that the concentration of K-3G did not change, indicating that the flavonoid compounds were reasonably and fully stable during incubation, respectively. The chromatograms also revealed that K-3G stocks contained 0.8% kaempferol aglycone, and that during incubation with HepG2 lysate, which potentially contains β-glucuronidases that can deconjugate K-3G, no additional aglycone was released. Concentration-response curves and data of all substrates on the microarray can be found in the Supplemental Data (see Supplemental Figures 6.1 and 6.2, and Supplemental Tables 6.1 and 6.2). To compare the inhibitory potencies of the tested compounds the IC_{50} values (i.e. the concentration causing a half-maximal inhibition) were derived for all kinase substrates on the array for which the concentration response curves of all three test compounds had a coefficient of determination (R^2-value) of > 0.7. Figure 6.3 shows histograms of the IC_{50} values for the inhibition of PKA and HepG2 lysate by the three test compounds; the mean IC_{50} values are given in the figure. Glucuronidation caused a small reduction of the inhibitory potency of kaempferol as is apparent from the higher mean IC_{50} values of K-3G than of kaempferol. While for PKA the mean IC_{50} value of K-3G was around 16 times higher than that of kaempferol, glucuronidation reduced the potency of kaempferol to inhibit the phosphorylation activity of HepG2 lysate only around 3.5 times.

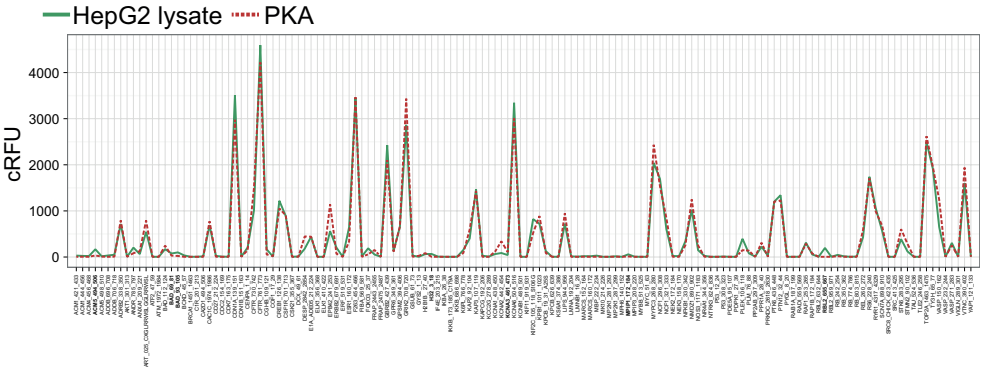

Figure 6.1 The phosphorylation patterns of HepG2 lysate and PKA show very high similarities. To facilitate comparison of the patterns the corrected relative fluorescence units (cRFU) were calculated as described in the Materials and Methods section and the fluorescence intensities of both samples were matched. The high similarities between the patterns indicate that PKA is a major constituent of HepG2 lysate.

This implies that, despite the apparent similarities in the substrate selectivity of PKA and HepG2 lysate, the relative inhibitory potency of kaempferol and K-3G differed between PKA and the HepG2 lysate. Kaempferol had lower IC_{50} values for recombinant PKA than for the HepG2 lysate (on average 36.5 μM and 80.5 μM, respectively), while K-3G had higher IC_{50} values for recombinant PKA than for HepG2 lysate (on average 594

µM and 290 µM, respectively). Staurosporine was the most potent inhibitor of the three compounds tested with an average IC_{50} of 149 nM for PKA and 1.25 µM for HepG2 lysate.

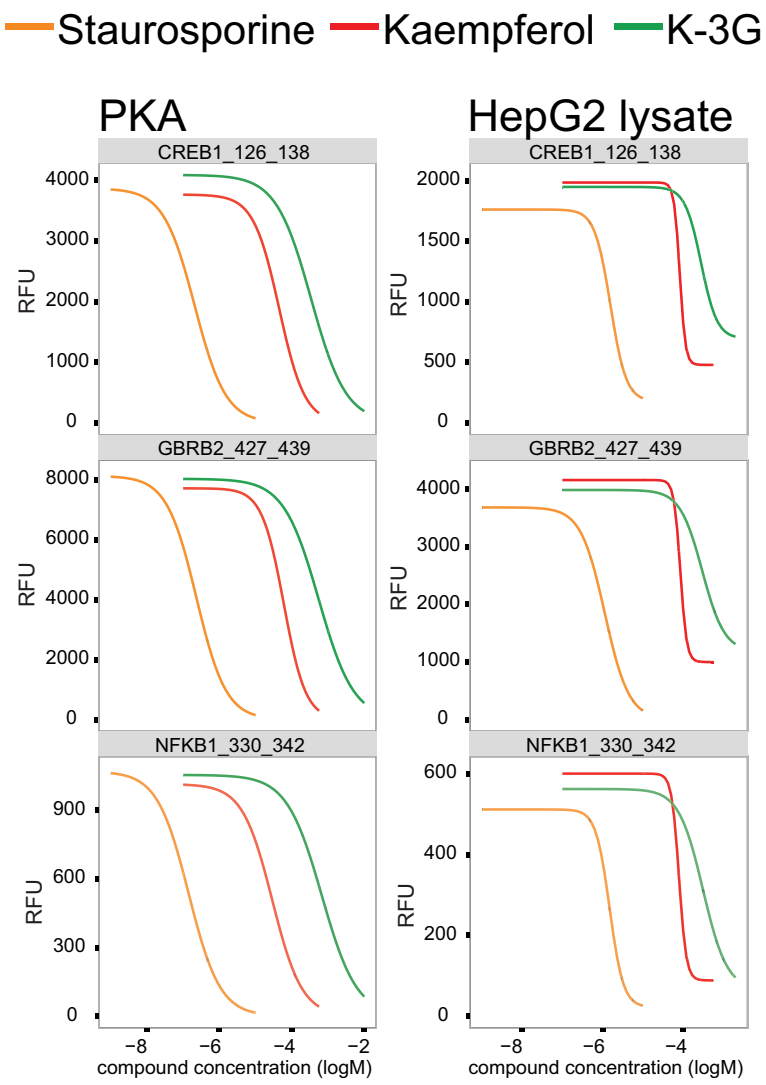

Figure 6.2 Kaempferol, K-3G and staurosporine inhibit the phosphorylation activity of PKA and HepG2 lysate in a concentration-dependent manner with different potencies (IC_{50} values). The inhibition of phosphorylation activity is shown for three exemplary substrates, which are derived from cAMP response element-binding protein (CREB1), gamma-aminobutyric acid receptor subunit beta-2 (GBRB2), and nuclear factor NF-kappa-B p105 subunit (NFKB1), respectively. The numbers in the substrate abbreviations given in the figure denote the position of the first and last amino acids of the substrate sequence in the protein of origin. Kaempferol and K-3G completely inhibit PKA at the highest concentrations tested. While there is remaining phosphorylation activity in the HepG2 lysate at the highest concentrations of kaempferol and K-3G, the shape of their concentration-response curves reveal that they have reached a maximum inhibitory effect.

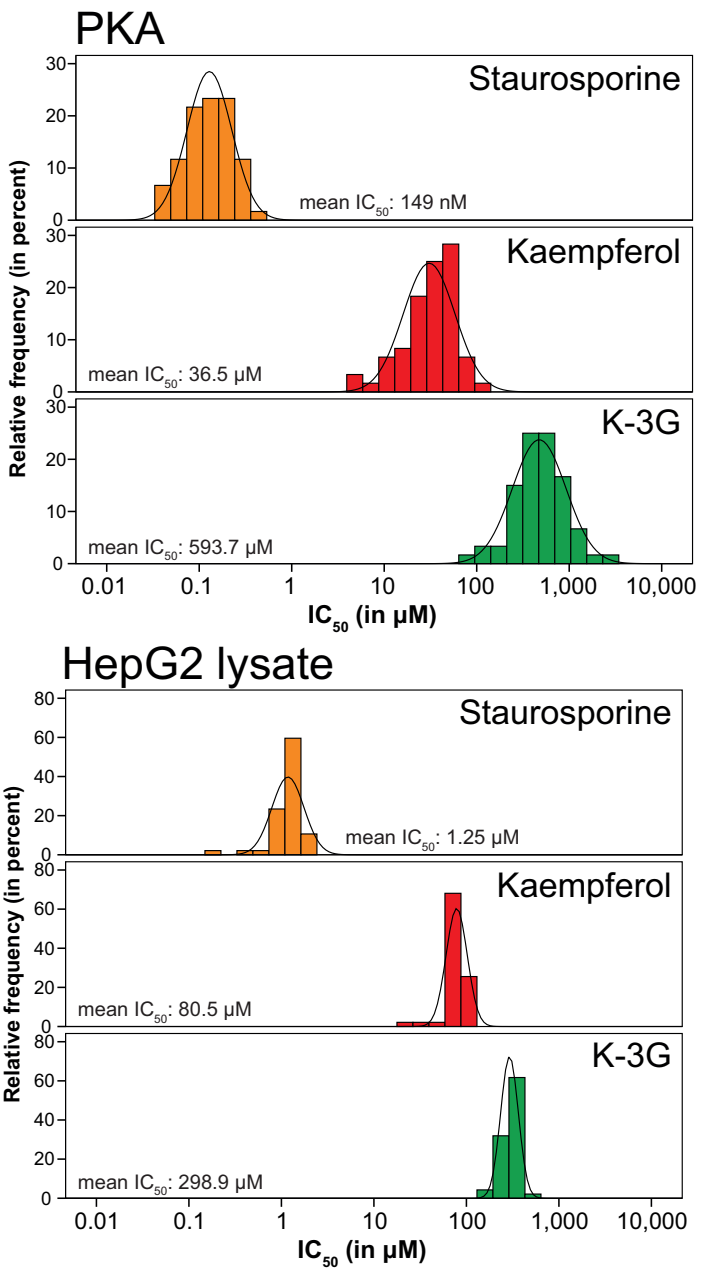

Figure 6.3 Kaempferol, K-3G and staurosporine inhibit the phosphorylation activity of PKA and HepG2 lysate with different potencies (IC_{50} values). The figure shows histograms of the IC_{50} values for concentration-response curves where all tested compounds in either PKA or HepG2 lysate had an R^2 value of >0.7. The frequency distribution of the IC_{50} values is expressed in percent of the total number of IC_{50} values per compound. Mean values are given in the figure. Glucuronidation reduces the potency of kaempferol to inhibit the phosphorylation activity of HepG2 lysate to a lower extent than the potency to inhibit the phosphorylation activity of recombinant PKA.

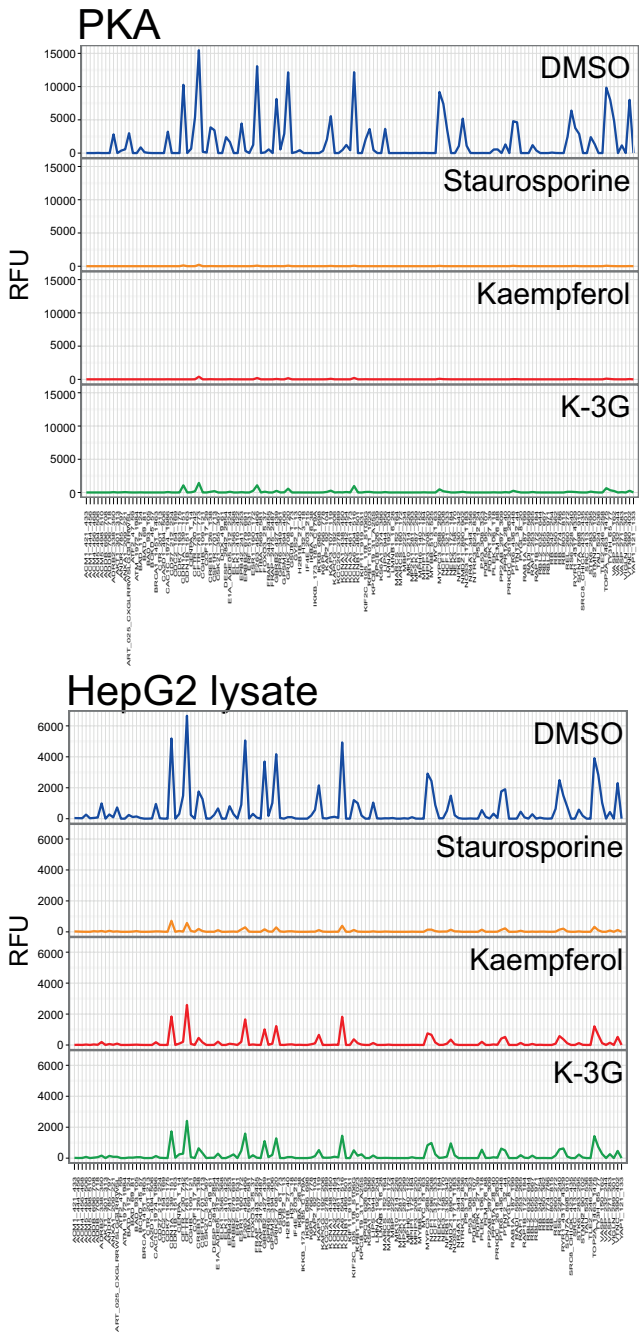

Figure 6.4 Staurosporine, kaempferol and K-3G have different inhibitory effects on the phosphorylation activity of PKA and HepG2 cell lysate. At their highest concentrations tested (i.e. 10 µM staurosporine, 566 µM kaempferol and 10 mM K-3G) all test compounds strongly inhibit the phosphorylation activity of PKA. While staurosporine causes complete inhibition of phosphorylation activity of HepG2 lysate, kaempferol and K-3G only partially inhibit the phosphorylation activity of HepG2 lysate, suggesting that kaempferol and K-3G do not inhibit all protein kinases present in the lysate.

Figure 6.4 shows that at the highest concentration of staurosporine tested (i.e. 10 μM) the phosphorylation activity of PKA and HepG2 lysate were completely inhibited. This is in line with reports that staurosporine has a very broad kinase specificity [29, 30]. Results from a literature search on the kinase inhibitory potential of kaempferol reveals that kaempferol is also able to inhibit a wide range of protein kinases (see Table 6.1). The flavonoid kaempferol and its conjugate K-3G caused a near complete inhibition of PKA activity at the highest concentration tested (i.e. 566 μM and 10 mM, respectively) while at the same maximal concentrations the phosphorylation activity of the HepG2 lysate was only partially inhibited. This partial inhibition indicates that there are also other active kinases present in the cell lysate which are inhibited by staurosporine but not by kaempferol and K-3G. This is in line with the results presented in Figure 6.2 which show that at the highest concentrations of kaempferol and K-3G tested in the HepG2 cell lysate the inhibitory effect levels off before reaching complete inhibition of phosphorylation activity.

Discussion

The objective of this study was to characterize the effect of flavonoid glucuronidation on the inhibition of protein kinases by the dietary flavonoid kaempferol. To this end the effects of kaempferol and its major plasma metabolite K-3G, as well as of the positive control staurosporine on the phosphorylation activity of PKA and of a lysate from HepG2 cells on 141 putative phosphorylation sites derived from human proteins were characterized. Both samples caused very similar patterns of phosphorylation on the microarrays (Figure 6.1). Due to the very high similarity between patterns of phosphorylation it appears that PKA is the most active kinase in the HepG2 lysate; PKA is reported to be present [31-33] and active [34, 35] in HepG2 cells. Further, the HepG2 cell line is derived from a human hepatocellular carcinoma and it is reported that PKA is overexpressed in many types of cancer [36-38]. Overexpression of PKA is associated with deregulation of the cell cycle and increased cellular proliferation [39] and PKA is therefore increasingly targeted in cancer therapy [40]. PKA phosphorylated nearly half of the substrates present on the microarrays, this high number of substrates can be brought into perspective when considering that some kinases are reported to be able to phosphorylate up to hundreds of different substrates [21, 41].

At the highest concentrations of kaempferol and K-3G tested, which both completely inhibited PKA, comparable patterns of remaining phosphorylation activity in HepG2 lysate are observed (Figure 6.4). This suggests that both compounds target the same kinases in the lysate and that glucuronidation therefore does not affect the specificity of kaempferol for kinase inhibition. The data show that kaempferol and K-3G are inhibitors of PKA, but only partially inhibited the phosphorylation activity of the HepG2

lysate. This remaining phosphorylation activity in the cell lysate indicates that there are other active kinases than PKA present in the lysate which are apparently less sensitive to inhibition by the flavonoid compounds.

An important observation is that staurosporine and kaempferol had lower average IC_{50} values for the inhibition of phosphorylation reactions of PKA than of the HepG2 lysate, while K-3G had a higher average IC_{50} value for PKA than for the HepG2 lysate. This is a surprising observation considering that PKA appears to be a major constituent of the HepG2 lysate and that kaempferol and K-3G are structurally similar compounds. The rather complex composition of the HepG2 lysate as compared to the PKA sample might, at least in part, explain the observed differences in inhibition between PKA and HepG2 lysate. A variety of positive and negative feedback mechanisms exist in cells that can amplify or reduce the activity and effect of a given kinase [42-46], some of which might also be functioning in the cell lysate used. The HepG2 lysate contains a range of different protein kinases, phosphatases and other functional enzymes which can alter the phosphorylation state and activity of each other; differential influence on their activity by the test compounds could affect the final phosphorylation of the substrates on the microarrays. The lower average IC_{50} of K-3G for HepG2 lysate than for PKA is very noteworthy as K-3G is the main form of kaempferol in plasma, and the experiments with cell lysates are closer to physiological conditions than the experiments with a recombinant kinase. As K-3G was not deconjugated to the aglycone during incubation with the HepG2 lysate this can be excluded as a reason for the lower IC_{50} values of K-3G in the cell lysate than with PKA.

The average IC_{50}s of the flavonoids observed (i.e. for PKA 36.5 μM (kaempferol) and 594 μM (K-3G) and for HepG2 cell lysate 80.5 μM (kaempferol) and 299 μM (K-3G)) are higher than expected plasma concentrations of flavonoids, which usually do not exceed the lower μM range [47]. It shall be noted that also the positive control staurosporine had unexpectedly high IC_{50} values in this assay. The reported IC_{50} values of staurosporine for protein kinases are generally in the low nM range [48-51], while in the assay employed they were around 100 to 1,000 fold higher than these reported values (i.e. 149 nM for PKA and 1.25 μM for HepG2 lysate). Also kaempferol is reported to be able to inhibit various protein kinases at lower concentrations in other assays (see Table 6.1), and K-3G is therefore also likely to inhibit protein kinase activity at lower concentrations in other assays. The unexpectedly high IC_{50}s seem to reflect an intrinsic and systematic methodological deviation of the used array methodology from other assays to study protein kinase activity. Therefore, in the present study the results obtained were interpreted in a relative way by comparing the effect of the aglycone to that of its glucuronidated metabolite.

Considering that K-3G retains substantial inhibitory potency relative to the aglycone (i.e. only around 3.5 times lower in HepG2 lysate), not only the capability of a given tissue to deconjugate and accumulate the aglycone, but also to take up and accumulate K-3G itself, can be expected to greatly affect the likelihood of an inhibitory effect on intracellular protein kinases to be observed in vivo. In addition, intracellular formation of K-3G from kaempferol will only have a small effect on its inhibitory potential. While many signaling cascades are initiated at the cell membrane by G-protein coupled receptors and receptor tyrosine kinases, the protein kinases in the signaling cascades that are potential targets of the flavonoid (conjugates) are present within the intracellular space [52]. An exception are ecto-protein kinases, for example ecto-PKA that is reported to promote the formation of oligomeric amyloid β-peptide assemblies in the pathogenesis of Alzheimer disease that are active in the extracellular space [53-55].

In conclusion, our results show that kaempferol partially inhibited the phosphorylation activity of HepG2 lysate. The partial inhibition appears to be primarily due to inhibition of PKA in the lysate while other protein kinases remained active at the concentrations of kaempferol tested. This partial inhibitory activity did not appear to be affected by glucuronidation. Glucuronidation caused only a small reduction in the intrinsic potency of kaempferol to inhibit the phosphorylation activity of PKA or of the kinases present in the HepG2 cell lysate. Especially in the context of the HepG2 cell lysate, which has a closer resemblance to the intracellular composition of signaling molecules, the reduction in intrinsic inhibitory potency was small. The data imply that K-3G does not necessarily need to be deconjugated to the aglycone for a potential inhibitory effect on protein kinases.

Supplemental data

Supplemental Figures 6.1 - 6.2 and Supplemental Tables 6.1 - 6.2 can be downloaded from: https://goo.gl/jd7kBJ (case sensitive)

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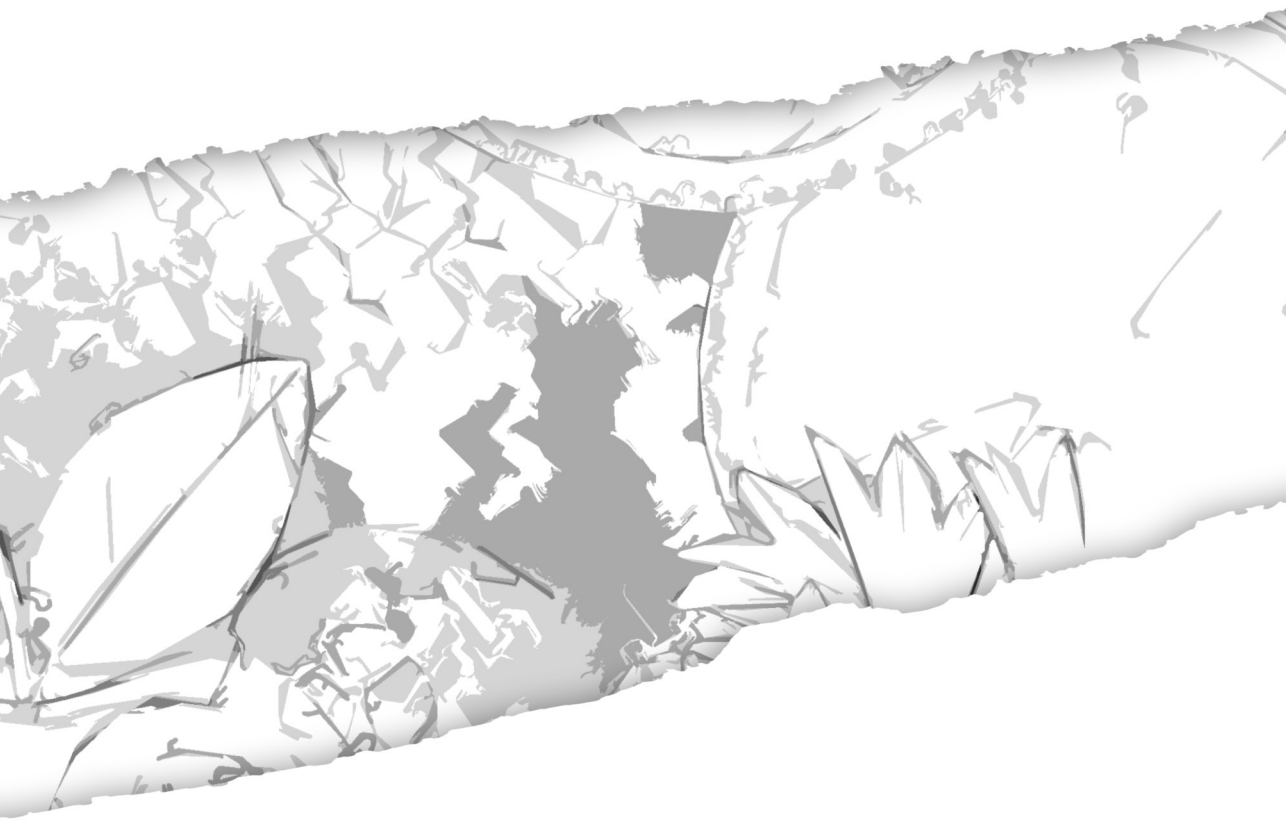
Chapter 7

General discussion



General discussion

Flavonoid consumption is reported to cause a wide range of health effects, such as the prevention of cardiovascular diseases [1, 2], neurodegenerative diseases [3], and diabetes [4, 5]. These effects are often ascribed to the activity of the parent flavonoid aglycones even though these forms of the flavonoids generally have a low systemic bioavailability [6, 7]. Upon intake, flavonoids are extensively metabolized and are present in the systemic circulation nearly exclusively as conjugates (see **Chapter 1**). In spite of this, the vast majority of in vitro studies conducted to elucidate the mechanisms of action behind the biological effects of flavonoids have been conducted with the aglycone forms of flavonoids and not with the conjugated forms that are present in the systemic circulation after consumption. Conjugation alters the physico-chemical properties of flavonoids and it is widely accepted that this can affect their biological activity. The question has been raised if studies using flavonoid aglycones and glycosides instead of the flavonoid conjugates found in the systemic circulation after ingestion can adequately predict the mechanisms of action of flavonoids in vivo [8-10].

To further the understanding of the relevance of flavonoid conjugates for human health, the aim of this thesis was to study the effect of conjugation on the biological activity of selected flavonoids towards different endpoints relevant for human health. Conjugation with glucuronic acid was taken as the model type of conjugation because this modification is generally observed to be the main metabolic conjugation reaction for flavonoids in man [11]. **Chapter 2** provides an overview of research published until early 2012 that compares the activity of conjugated forms of flavonoids with their respective aglycones in various in vitro assays. The overview clearly reveals that metabolic conjugation can affect the biological activity of flavonoids in different ways; conjugation can increase, decrease, inverse or not affect the biological effects, depending on the type and position of conjugation, the endpoint studied and the assay system used. It is concluded that the effect of conjugation has to be studied on a case-by-case basis. An updated overview of literature on this topic published since 2012 is presented as part of this chapter.

Some of the main factors hampering research on the biological activity of flavonoid conjugates are their generally low commercial availability, and the high prices of available conjugates. Descriptions of the chemical synthesis of flavonoid conjugates can be found in literature [12-19], showing that the chemical synthesis of these conjugates can be rather complex and requires specific knowledge and equipment. Another strategy to obtain these metabolites is the biosynthesis of conjugates using microsomes, S9 fractions, recombinant enzymes, or cell cultures [20-36]. In **Chapter 3**, a convenient and versatile method for the preparation of metabolically relevant flavonoid conjugates in sufficient quantities for in vitro assays is described. The chapter further presents a strategy to characterize the conjugates by LC-MS and ^{1}H-NMR using MetIDB, a publicly

accessible database of predicted and experimental ^{1}H-NMR spectra of flavonoids. Using the described method, only a few micrograms of sample are needed for the identification. For some samples, the described automated strategy for identification resulted in more than one possible flavonoid metabolite that matched the spectral characteristics, which could be easily solved by manual examination of the spectra. Using the described method of biosynthesis and identification, sufficient amounts of well-defined flavonoid conjugates for in vitro bioassays can be generated relatively inexpensively. A major advantage of the method described is its versatility, as different flavonoids can be used as substrates. In addition, as different cell lines and different enzymes produce different conjugates, cells derived from different tissues and species, as well as selected enzymes produced by recombinant techniques can be used [20-36], potentially yielding different metabolites.

The next step of the thesis was to characterize the influence of conjugation on the biological activity of flavonoids. It is suggested that flavonoids exert their biological activity at least in part by targeting nuclear receptors [37]. Nuclear receptors are reported to be the second largest group of drug targets among FDA approved pharmaceuticals [38], which underlines the relevance of nuclear receptors for human health. The two main types of nuclear receptors are type I and type II nuclear receptors. The main differences between these types of nuclear receptors are the localization of the inactive receptor in the cell, and the nature of the dimerizing partner. Inactive type I nuclear receptors are located in the cytosol and upon activation they homodimerize, while inactive type II nuclear receptors are present in the nucleus, heterodimerized with retinoid X receptor [37]. Upon activation by an agonistic ligand, the ligand binding domains (LBDs) of the receptors undergo a conformational change leading to dismissal of co-repressors and recruitment of co-activators. Flavonoids have been reported to interact with a variety of different nuclear receptors, and the interaction of flavonoids and their conjugates with nuclear receptors may provide a means to study the consequences of conjugation on the biological effects of flavonoids. The influence of conjugation on the interaction of flavonoids with different nuclear receptors was investigated in **Chapters 4 and 5** of the present thesis.

Chapter 4 describes the effects of quercetin, kaempferol, and their major plasma conjugates quercetin-3-*O*-glucuronide (Q-3G) and kaempferol-3-*O*-glucuronide (K-3G) on different endpoints related to peroxisome proliferator-activated receptor (PPAR)-γ, a type II nuclear receptor. PPAR-γ activation is reported to have positive health effects related to adipogenesis, insulin resistance and inflammation [39]. As consumption of diets rich in flavonoids are associated with a reduced risk for diabetes [40], stimulating effects of flavonoids on PPAR-γ might at least in part contribute to the mode of action behind these effects. The results obtained show that the flavonoid aglycones increased PPAR-γ mediated gene expression in a stably transfected reporter gene cell line and

that glucuronidation diminished their effect. Since this reduction may either be due to a decrease in the intrinsic ability of the conjugates to activate PPAR-γ-mediated gene expression as compared to the aglycones, or due to a relatively lower cellular uptake of the conjugates than the aglycones, the intrinsic activity of the test compounds to activate PPAR-γ was studied using a novel microarray technique. In this microarray technique, the ligand-induced interactions of nuclear receptor-LBDs with154 different binding motifs derived from 66 different nuclear receptor coregulators can be characterized in a cell-free environment. In this system it was demonstrated that unlike the known PPAR-γ agonist rosiglitazone, neither the flavonoid aglycones nor the conjugates are agonistic ligands of the PPAR-γ receptor. This absence of an effect suggests that the increased reporter gene expression caused by the flavonoids are not mediated by agonism of the PPAR-γ LBD. It was found that the increases in reporter gene expression in the reporter cells were accompanied by increased PPAR-γ receptor-mRNA expression, and quercetin was found to synergistically increase the effect of rosiglitazone in the reporter gene assay. Glucuronidation reduced the activity of the flavonoid aglycones to increase PPAR-γ receptor-mRNA expression. As the lower activities of flavonoid conjugates were observed in a cellular assay system, lower cellular uptake of flavonoid glucuronides than the aglycones might contribute to these differences. The uptake of flavonoid glucuronides is generally considered to be lower than that of flavonoid aglycones, as the hydrophilic glucuronides have to entirely rely on active transport, while many flavonoid aglycones are thought to be able to passively diffuse into cells [41]. As PPAR-γ is a type II nuclear receptor, possible ligands would not only have to be taken up into the cytosol, but also reach the nucleus. It has been reported for HepG2 and T47D cells that quercetin accumulates in cellular structures, most importantly the nucleus and mitochondria [42]. Interestingly, it has been reported that known PPAR-γ ligands rely on a cellular transport mechanism mediated by fatty acid-binding protein 1 (also known as liver-type fatty acid-binding protein) to direct the ligands to PPAR-γ [43]. Nevertheless, even though this indicates that at least quercetin aglycone should be able to reach PPAR-γ, it appears that the mode of action behind the increased reporter gene expression is different from normal agonist activity. The observed increased reporter gene expression is suggested to be caused by increased PPAR-γ receptor expression, as indicated by increased PPAR-γ receptor-mRNA expression, and agonistic effects of endogenous PPAR-γ ligands or ligand-independent mechanisms. As discussed in **Chapter 4**, it is important to note that flavonoids also affected the expression of PPAR-γ in other cell types and also in experimental animals [44-46]. Apart from the relevance for energy metabolism, PPAR-γ is also reported to play a role in the cell cycle of malignant cells. In malignant cells in vitro, increased PPAR-γ expression and its activation is reported to cause cell cycle arrest [47, 48]; in A549 lung cancer cells plasma metabolites of quercetin are shown to increase PPAR-γ expression which is suggested to be associated with the co-observed

cell cycle arrest [49]. This is in line with reports that PPAR-γ expression is lower in tumor tissue in lung cancer than in healthy tissue, and that lower PPAR-γ receptor-mRNA expression is associated with a worse prognosis [50, 51]. Altogether, quercetin might have beneficial effects for human health by increasing PPAR-γ levels.

Different protein kinase signaling pathways are implied in the expression of PPAR-γ [52], and the transcriptional activity of PPAR-γ can be affected by its phosphorylation state [53]. Also other post-transcriptional modifications, i.e. sumoylation and acetylation, are reported to affect the transcriptional activity of PPAR-γ [54-56]. As flavonoids are reported to affect many protein kinases [57], the mechanism underlying the observed effects might also be mediated by an effect on protein kinases. The influence of flavonoid conjugation on their effects on protein kinase activities was described in **Chapter 6** and will be discussed further below.

To further study the influence of glucuronidation on the effects of flavonoids on nuclear receptors, the effects of isoflavones and their main plasma glucuronides on the type I nuclear receptors estrogen receptor (ER)α and ERβ were studied and described in **Chapter 5** of this thesis. Isoflavones are known to have phyto-estrogenic properties and are reported to act as agonistic ligands on ERα and ERβ due to their structural resemblance to 17β-estradiol (E2). The intake of soy isoflavones is associated with beneficial but also adverse health effects, which might in part be caused by the activation of ERα and ERβ. ERα and ERβ have different biological functions; activation of ERα promotes cell proliferation and activation of ERβ promotes apoptosis [35]. To study the influence of glucuronidation on the intrinsic estrogenic effects of isoflavones, the agonistic activity of the two main dietary isoflavones daidzein and genistein, and their major plasma conjugates daidzein-7-O-glucuronide (D-7G) and genistein-7-O-glucuronide (G-7G) on the activation of ERα- and ERβ-LBD were studied. To this end the effects of the selected isoflavones and their conjugates on ERα- and ERβ-LBD interactions with 154 different binding motifs derived from 66 different nuclear receptor coregulators were studied in a cell-free assay system. The tested isoflavone compounds were less potent than E2 (around 5 to 1,580 times for the aglycones) but modulated the LBD-coregulator interactions in a manner similar to E2. Genistein had lower average half-maximal effect concentrations (EC_{50}) than daidzein for both receptor subtypes, and it had a strong preference for ERβ-LBD activation over ERα-LBD, while daidzein had a slight preference for ERα-LBD activation over ERβ-LBD. The glucuronides of daidzein and genistein were 8 to 4,400 times less potent than the respective aglycones to induce ERα-LBD and ERβ-LBD – coregulator interactions. Glucuronidation changed the preferential activation of genistein from ERβ to ERα and increased the preferential activation of daidzein for ERα. These changes in receptor subtype preferences are of special importance because of the different biological functions the receptor subtypes have. An increased preference for ERα over ERβ activation upon glucuronidation may also shift the biological effect of the

isoflavones from apoptosis to cell proliferation. Especially for ERα-positive tumors this effect of glucuronidation may be unfavorable.

Apart from D-7G and G-7G, the major plasma conjugates of daidzein and genistein, there is another metabolite that is known for its estrogenic activity. *S*-equol is a microbial metabolite of daidzein which is reported to have a higher bioavailability and slower clearance than daidzein and genistein [58], and to be a more potent inducer of ERs than daidzein and genistein [58, 59]. *S*-equol is reported to preferentially bind to ERβ over ERα [59-62]. This metabolite, however, is only produced by 20-35% of the Western adult population and 50-55% of the Asian adult population [63-66]. Equol-producing individuals are reported to benefit more from isoflavone ingestion than non-producers [67], which implies that certain biological effects of isoflavone ingestion are enhanced by the production of equol. It is of interest to note that most animal species are reported to produce equol [67], which indicates that studies in laboratory animals might overestimate the effect of isoflavone ingestion when compared to the general human population. Like genistein and daidzein, also equol undergoes extensive metabolic conjugation during uptake and is predominantly found in plasma as equol-7-*O*-glucuronide [68-70]. No reports on the effects of glucuronidation on the biological activity of equol have been published as yet, and this remains an interesting topic for future research.

As mentioned above, another suggested mode of action behind flavonoid activity is the modulation of protein kinase activities [57]. Protein kinases are of high pharmacological relevance and, notably, most protein kinase inhibitors with clinical approval are used for cancer treatment [71, 72]. As protein kinases are involved in a wide range of physiological processes by controlling signaling cascades and regulating protein functions, modulation of their activities can have a wide range of biological effects. Flavonoid aglycones are reported to be capable of inhibiting a wide range of protein kinases in vitro [57, 73, 74]; the effect of their circulating conjugated metabolites, however, is generally not known. In **Chapter 6** of this thesis the flavonol kaempferol and its major plasma conjugate K-3G were selected as model compounds to study the effect of flavonoid conjugation on the potential to inhibit protein kinase activity. To that end the effects of kaempferol and its metabolite K-3G on the phosphorylation activity of recombinant protein kinase A (PKA) and of a cell lysate prepared from the hepatocellular carcinoma cell line HepG2 were compared using a microarray platform that determines the phosphorylation of 141 putative serine/threonine phosphorylation sites derived from human proteins. PKA is an interesting and relevant target as overexpression of PKA is associated with deregulation of the cell cycle and increased cellular proliferation [75], and PKA is increasingly targeted in cancer therapy [76]. Flavonoids are reported to cause cell cycle arrest in cell lines with a deregulated cell cycle [77-79]; this induction of cell cycle arrest might at least in part be mediated by inhibitory effects on the underlying deregulated

signaling pathways. The results described in **Chapter 6** reveal that glucuronidation reduces the inhibitory potency of kaempferol on the phosphorylation activity of PKA and HepG2 lysate on average about 16- and 3.5-fold, respectively. It was shown that the remaining inhibitory activity of K-3G is not caused by deconjugation to the aglycone. The fact that the glucuronide appears to be only a few times less potent than kaempferol implies that K-3G does not necessarily need to be deconjugated to the aglycone to exert an inhibitory effect on protein kinases. Furthermore, the results show that kaempferol and K-3G, unlike the broad-specificity protein kinase inhibitor staurosporine, do not appear to inhibit all protein kinases present in the HepG2 lysate to a similar extent, indicating that kaempferol selectively targets protein kinases. The results also revealed that glucuronidation does not affect this kinase target selectivity.

Most protein kinase activity is restricted to the intracellular space, meaning that flavonoids have to be taken up into the cells to exhibit inhibitory effects on protein kinases. An up-and-coming field of protein kinase research focuses on ecto-protein kinases; these are protein kinases that, given high enough ATP concentrations, are active in the extracellular space [80-82]. Interestingly, the activity of these ecto-protein kinases is reported to be involved in a variety of diseases [83]; ecto-PKA activity, for example, is reported to promote the formation of oligomeric amyloid β-peptide assemblies in the pathogenesis of Alzheimer disease [81]. Flavonoid conjugates with a low cellular uptake can therefore potentially exert inhibitory effects on ecto-protein kinases of relevance in human diseases.

Altogether, the results obtained in the present thesis support the conclusion that glucuronidation of flavonoids does not necessarily abolish their activity and that flavonoid glucuronides may be biologically active themselves, albeit at higher concentrations than the parent aglycones. As plasma concentrations of flavonoid conjugates are far higher than the concentrations of their parent aglycones, which are often only detectable in trace amounts or absent from human plasma, flavonoid glucuronides may be relevant for the biological effects of flavonoids. The observation that flavonoid conjugates may retain biological activity is in line with the conclusion that emerged from the literature overview presented in **Chapter 2** which is based on literature published until early 2012. In the following section, an updated literature overview is presented that covers literature published between early 2012 and late 2015.

Updated literature overview

Since the completion of the literature overview presented in **Chapter 2**, a number of new publications reporting on the biological activities of flavonoid aglycones and conjugates became available. In the following section an overview of this literature is presented.

Amelioration of oxidative stress

Flavonoids are known for their strong anti-oxidant activities in vitro. However, it is now commonly agreed that this antioxidant activity does not contribute to the modes of action of the flavonoids in vivo, most importantly due to the relatively low concentrations of circulating flavonoid metabolites that can be found in plasma [84, 85]. Despite of this, some recently published articles report on the protective effects of (-)-epicatechin, quercetin, and their conjugates against oxidative stress in vitro (see Table 7.1). The radical scavenging activity of (-)-epicatechin, as well as its protection of HUVEC from H_2O_2 induced oxidative stress can be affected by conjugation. The activity on both of these endpoints are not affected by glucuronidation, while methylation or sulfation, as well as glucuronidation and methylation together are reported to reduce the activity. Methylation or glucuronidation are reported to not affect the reduction of H_2O_2 induced heme oxygenase-gene expression by (-)-epicatechin in HUVEC, while glucuronidation and methylation together reduced the activity.

Table 7.1 Recent studies (i.e. 2012 until present) on protective effects of quercetin and its conjugates (G = glucuronidated; M = methylated; S = sulfated) against oxidative stress in vitro (studies published before the year 2012 are included in the review in Chapter 2 of this thesis [89])

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(-)-Epicatechin					
Superoxide radical scavenging	=	↓	↓	(G + M)[b]: ↓ 4'M-(-)-EC > 3'M-(-)-EC	[86]
Reduction of H_2O_2 induced oxidative stress in HUVEC	=	↓	↓	(G + M)[b]: ↓ 3'M-(-)-EC > 4'M-(-)-EC	[86]
Reduction of H_2O_2 induced heme oxygenase-gene expression	=	=	↓	(G + M)[b]: ↓ 3'M-(-)-EC = 4'M-(-)-EC EC-4'-S increased heme oxygenase-gene expression	[86]
Quercetin					
Protection of H9c2 cardiomyocytes against H_2O_2	↓	=	-		[90]
Reduction of 6OH-dopamine induced H_2O_2 production in Neuro-2a neuroblastoma	↓	-	-	Cellular uptake of Q-3G lower than of quercetin	[88]
Reduction of TBHP induced oxidative stress in HT-22 cells (murine hippocampus)	↓	-	-		[91]

a↓, the respective conjugate(s) have a lower activity than the aglycone; =, the respective conjugates have an equal activity to the aglycone; -, no data available; A > B, conjugate A is more active than conjugate B. *b* The conjugate is conjugated at two positions to different moieties. *Abbreviations* HUVEC: human umbilical vein endothelial cells, TBHP: tert-Butyl hydroperoxide, NADPH: Nicotinamide adenine dinucleotide phosphate.

In contrast, sulfation is reported to further increase of H_2O_2 induced heme oxygenase-gene expression in HUVEC[86]. Glucuronidation is reported to reduce the ability of quercetin to protect cells from oxidative stress in vitro, while methylation is reported to not affect the activity of quercetin on the protection of H9c2 cardiomyocytes against hydrogen peroxide. The protective effects reported in these studies do not necessarily depend on antioxidant activity of the flavonoids tested but can be secondary to, for example, activation of Nrf2, a transcription factor regulating the expression of proteins with antioxidant activities, or protein kinase signaling [87]. The lower cellular uptake of Q-3G compared to quercetin [88] is likely to explain at least part of the reported lower activity of the conjugate on the 6OH-dopamine induced H_2O_2 production in Neuro-2a neuroblastoma cells.

Anti-inflammatory effects

Inflammation is a response to tissue damage, pathogens and chemical irritation; it is initiated by the migration of immune cells from blood vessels to the tissue, ultimately causing the recruitment of inflammatory cells and the release of reactive oxygen species (ROS), and pro-inflammatory cytokines [92, 93]. In the case of acute inflammation, the body returns to normal homeostasis after the insult to the system has been dealt with. Chronic inflammation results if these inflammatory responses do not subside, which can inflict damage. Chronic inflammation is regarded as a risk factor for many diseases, such as metabolic disorders and cardiovascular diseases [94]. Flavonoids are reported to have anti-inflammatory effects [95-97] and it is of interest to note that protein kinases play a fundamental role in inflammation [98-100]. In recent in vitro studies, glucuronidation is reported to reduce the anti-inflammatory effect of quercetin in macrophages (see Table 7.2). Interestingly, Q-3G is reported to accumulate in macrophage-derived foam cells in human atherosclerotic lesions, but not in normal aorta [101]. In human brain, Q-3G was detected by an anti-Q-3G antibody in the epithelial cells of the choroid plexus; in fresh infarcts, Q-3G was localized in the cytoplasm of the cortical neurons, and in recent infarcts Q-3G appeared to be localized in foamy macrophages in the necrotic core [102]. Upon Q-3G exposure, murine RAW264 macrophages and MG6 microglia are reported to accumulate Q-3G and quercetin, as well as further conjugate quercetin to methylated quercetin [101-103]. When RAW264 macrophages were stimulated with LPS, intracellular levels of Q-3G and quercetin were higher than in cells not stimulated, and no methylated conjugate was detected [101-103]. Ishisaka et al. proposed that Q-3G is deconjugated extracellularly before diffusing into the cells [103]. Human neutrophils, which are another type of phagocytes, are reported to be able to deconjugate quercetin and luteolin glucuronides and thereby release the aglycone, especially in sites of inflammation [104, 105]. These results underline that in sites of inflammation, the availability of flavonoid aglycones, which, at least in the case of quercetin have a higher anti-inflammatory activity than Q-3G, is higher than in non-inflamed tissues.

Table 7.2 Recent studies (i.e. 2012 until present) on anti-inflammatory effects of quercetin and its conjugates (G = glucuronidated; M = methylated; S = sulfated; studies published before the year 2012 are included in the review in Chapter 2 of this thesis [89])

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
Quercetin					
Ex vivo mouse peritoneal macrophages (treated with and w/o LPS), decreased pro-/anti-inflammatory cytokine secretion	↓	-	-		[106]
Reduction of LPS-stimulated inflammatory response in RAW264	↓	-	-		[102]

a↓, the respective conjugate(*s*) have a lower activity than the aglycone; -, no data available. *Abbreviation*: LPS: Lipopolysaccharide

Cardioprotective effects

The cardioprotective health effects of flavonoids have been studied extensively [107]. Recent in vitro and ex vivo studies report that in most instances glucuronidation reduces the activity of flavonoids to affect endpoints related to vasorelaxation and cardioprotection, while for few endpoints there was no difference to the respective aglycone observed (see Table 7.3). Isorhamnetin is reported to be equally active as quercetin in vitro and ex vivo [108]. Sulfation is generally reported to reduce the activity of flavonoids on different endpoints, though one study reports that Q-3'S had an inverse activity compared to quercetin and increased levels of the vasoconstrictor endothelin-1 in HUVEC [109]. The inhibitory effect of Q-3G on NADPH oxidase activity in vascular smooth muscle cells is reported to be partially inhibited by the addition of the beta-glucuronidase inhibitor saccharolactone [110], indicating that Q-3G is at least partially deconjugated to the aglycone in this assay system which can explain part of the observed activity of Q-3G. It has further been shown in experimental animals in vivo and ex vivo, that the vasorelaxant effects induced by quercetin ingestion and intravenous Q-3G administration depend on the deconjugation of the glucuronide to its aglycone form [111-113]. In humans, vasorelaxation after quercetin consumption was correlated with Q-3G plasma concentration when corrected for the beta-glucuronidase activity in plasma of the test subjects [114]. Altogether, while for certain endpoints it is reported that also the circulating conjugates can exert cardioprotective effects, the deconjugation and release of the respective aglycones appears to contribute significantly to the cardioprotective effects, most importantly the vasorelaxant activity of flavonoids. In addition to these direct effects, polyphenol rich foods are reported to promote reduction of dietary nitrite to nitric oxide in the stomach which is suggested to positively affect plasma nitric oxide levels [115] and might partially contribute to the vasorelaxant effects of dietary flavonoids.

Table 7.3 Recent studies (i.e. 2012 until present) on the effects of flavonoids and their conjugates (G = glucuronidated; M = methylated; S = sulfated) on vasorelaxation and cardioprotection-related endpoints (studies published before the year 2012 are included in the review in Chapter 2 of this thesis [89])

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
Genistein					
Induction of paraoxonase 1 transactivation in Huh7 based reporter gene assay	↓	-	↓	G-4',7'diS: ↓ Luciferase activity as readout	[116]
Hesperetin					
Increased NO release, reduced $O2^{\bullet-}$ release, reduced NADPH oxidase activity in HUVEC	↓/=	-	-	H-7G only slightly less active than hesperetin	[117]
in vivo rats, intravenous injection of compounds, reduction of systolic blood pressure	↓	-	-	H-7G > H-3'G	[118]
ICAM-1 gene expression in primary rat endothelial cells	↓	-	-	H-7G > H-3'G	[118]
MCP-1 gene expression in primary rat endothelial cells	↓/=	-	-	H-7G > H-3'G	[118]
Quercetin					
Reduction of eNOS protein and mRNA expression in HUVEC	↓	-	↓	(G + M): ↓	[109]
Reduction of ET-1 protein expression in HUVEC	↓	-	↑	Q-3'S increased ET-1 expression (G + M)[b]: ↓	[109]
Inhibition of NADPH oxidase activity in vascular smooth muscle cells	↓	↓	-	The effect of the glucuronide was partially inhibited by saccharolactone	[110]
Reduction of HOCl induced constriction of mouse aortic rings	↓	=	-		[108]
Reduction of NOS activity of mouse abdominal aortas	-	=	-		[108]
Increased phosphorylation of eNOS, AMPK and ACC in HAEC following serum starvation	=	=	-	Effect of Q-3G not tested on eNOS phosphorylation	[108]
Reduction of IL-1β-stimulated iNOS protein and gene expression, nitrite production, IκBα phosphorylation, NF-κB activation, iNOS promoter activity and increase of insulin secretion of RINm5F β-cells	↓	-	↓	(G + M)[b]: ↓	[119]
Reduction of palmitate induced ROS production and associated inflammation, restoring membrane potential (ΔΨm), PI3K signaling, and NO excretion in HUVEC	=	-	-		[120]
Resveratrol					
Increased eNOS activity and NO release in endothelial EA.hy926 cells	↓	-	↓	R-diS: ↓	[121]

a↓, the respective conjugate(s) have a lower activity than the aglycone; =, the respective conjugates have an equal activity to the aglycone; -, no data available; A > B, conjugate A is more active than conjugate B. *b* The conjugate is conjugated at two positions to different moieties. *Abbreviations* ICAM-1: intercellular adhesion molecule-1, MCP-1: monocyte chemoattractant protein-1, eNOS: endothelial nitric oxide synthase, AMPK: adenosine monophosphate-activated protein kinase, ACC: acetyl-CoA carboxylase, HAEC: human aortic endothelial cells endothelin-1, IL-1β: interleukin-1β, iNOS: inducible nitric oxide synthase, NF-κB: nuclear factor-κB, IκBα: inhibitor of nuclear factor-κB alpha,

Chemoprevention

Dietary flavonoids, as well as other polyphenols and phenolic acids are reported to exert chemopreventive effects via a wide range of mechanisms in vitro and in experimental animals in vivo at high doses [122, 123] but, as already outlined in **Chapter 2** of this thesis, there is much debate about the relevance of these findings for humans.

In a recent study by Yamazaki et al., Q-3G was equally active as quercetin to downregulate β2-adrenergic receptor signaling, thereby reducing 4-OH-estradiol and noradrenaline-induced DNA damage in MCF-10A cells as indicated by reduced γ-H2AX, which is a marker for DNA double-strand breaks, and apurinic sites, as well as cell invasiveness and matrix metalloproteinase (MMP) activity [124]. Interestingly, these effects were already observed at concentrations of 0.1 μM and below.

Transfer over the blood-brain barrier and neurobiological effects

For flavonoids and their conjugates to exert organ specific effects, flavonoids need to be able to reach the respective target organs. One particularly well protected target tissue is the brain, which is protected by the blood-brain barrier that serves as a selectively permeable barrier separating the blood from the cerebrospinal fluid. In an in vitro model, the transfer of different flavonoids and their conjugates over the blood-brain barrier was assessed [125] (see Table 7.4). Interestingly, methylation increased the transfer of the flavan-3-ols (+)-catechin and (-)-epicatechin, and glucuronidation

Table 7.4 Recent studies (i.e. 2012 until present) on the effects of flavonoids and their conjugates (G = glucuronidated; M = methylated; S = sulfated) on neurobiological endpoints and their transport over the blood-brain barrier (BBB) (studies published before the year 2012 are included in the review in Chapter 2 of this thesis [89]).

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(+)-Catechin					
In vitro transport over BBB (hCMEC/D3 cells)	-	↑	-		[125]
(-)-Epicatechin					
In vitro transport over BBB (hCMEC/D3 cells)	-	↑	-	3'M-(-)-EC = 4'M-(-)-EC	[125]
Quercetin					
In vitro transport over BBB (hCMEC/D3 cells)	↑	-	-		[125]
Inhibition of MAO-A activity in murine brain mitochondria	-	↓	-	3'M-Q > 4'M-Q	[126]

a↓, the respective conjugate(s) have a lower activity than the aglycone; =, the respective conjugates have an equal activity to the aglycone; ↑, the respective conjugates have a higher activity than the aglycone; -, no data available; A > B, conjugate A is more active than conjugate B.

increased the transfer of quercetin. The transfer over the blood-brain barrier is essential for flavonoids to exert neurobiological effects. As has been described above, Q-3G could be detected in human brains, and it can also be deconjugated and subsequently conjugated to

isorhamnetin [102], which, in vitro, had a lower inhibitory activity on monoamine oxidase A (MAO-A) in brain mitochondria than quercetin [126]. Also in the brains of experimental rats, Q-3G could be detected after ingestion of quercetin [127], and neurological effects, as assessed by the forced swimming test, indicate that flavonoids can have behavioral effects that suggest biological activities past the blood-brain barrier [128-130]. In vitro, Q-3G inhibits amyloid β-peptide generation in primary Tg2576 corticohippocampal neurons, which is a neuropathological hallmark of Alzheimer's disease. The effect of quercetin, however, was not assessed in this study so no comparison between the activity of quercetin and Q-3G can be made [127].

Cytotoxicity

There are many types of lethal and non-lethal biological effects of flavonoids on cells that can be considered cytotoxic effects. Very commonly, the term cytotoxicity is used to describe effects that cause parts of a population of cells to enter apoptosis or necrosis, and there are a variety of stimuli that can cause this condition [131]. Many assays used to study general cytotoxic effects actually measure a surrogate endpoint to estimate the amount of living cells in a population of exposed cells relative to a control population on non-exposed cells. A recently published study describes the effect of conjugation on the cytotoxicity of flavonoids to different cell lines [132] (see Table 7.5). It shall be noted that while the author refers to the observed effects as antiproliferative effects, the experimental setup as described in the article does not actually allow the distinction between antiproliferative effects and other forms of cytotoxicity; therefore the results are here referred to as cytotoxic effects. Cell death caused by exposure of cells to exceedingly high concentrations of flavonoids should not necessarily be considered a relevant biological effect in vivo, but it can compromise the results obtained from in vitro experiments where the exposure conditions are not well characterized.

Inhibition of sulfotransferases

Flavonoids are known inhibitors of sulfotransferases [21, 133-135], and conjugation is reported to reduce the activity of quercetin to inhibit sulfotransferases (see Table 7.6). Methylation, sulfation and glucuronidation are reported to reduce the activity of quercetin to inhibit sulfotransferases in human liver S9, while methylation does not affect the activity of quercetin to inhibit sulfotransferases in human intestinal S9. The activity of quercetin to inhibit sulfotransferases in HepG2 cells is reported to be reduced by sulfation and abolished by glucuronidation, which is likely to be due to the very low cellular uptake of the glucuronides [134].

The data presented in this updated literature overview are in line with the conclusions drawn from the literature overview presented in **Chapter 2**. The reviewed literature shows that the effect of conjugation on the biological activity of flavonoids depends on the type and position of conjugation, the endpoint studied and the assay system used.

The results presented in this thesis are in line with this conclusion, as it is shown that glucuronidation often reduces but not necessarily abolishes the biological activity flavonoids. As further research is needed to elucidate the role of conjugation in the biological activity of flavonoids, in the following section recommendations for further research are described.

Table 7.5 Recent studies (i.e. 2012 until present) on cytotoxic effects of flavonoids and their different conjugates (G = glucuronidated; M = methylated; S = sulfated; studies published before the year 2012 are included in the review in Chapter 2 of this thesis [89]).

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
(-)-Epicatechin					
cytotoxicity	-	=/↑	=/↑	Results differ by cell type: HFF-1 (-)-EC not cytotoxic Caco2 (-)-EC not cytotoxic MCF7 (-)-EC not cytotoxic, 4'M-(-)-EC > 3'M-(-)-EC Bxpc-3 (-)-EC not cytotoxic, 3'M-(-)-EC > 4'M-(-)-EC	[132]
Quercetin					
cytotoxicity	↓	=/↓	↓	Results differ by cell type: HFF-1 4'M-Q > 3'M-Q Caco2 4'M-Q > 3'M-Q MCF7 4'M-Q > 3'M-Q Bxpc-3 4'M-Q > 3'M-Q	[132]

a↓, the respective conjugate(s) have a lower activity than the aglycone; =, the respective conjugates have an equal activity to the aglycone;↑, the respective conjugates have a higher activity than the aglycone; -, no data available; A > B, conjugate A is more active than conjugate B.

Table 7.6 Recent studies (i.e. 2012 until present) on the inhibition of sulfotransferases by quercetin and its different conjugates (G = glucuronidated; M = methylated; S = sulfated; studies published before the year 2012 are included in the review in Chapter 2 of this thesis [89])

Flavonoid	G[a]	M[a]	S[a]	Comment[a]	References
Quercetin					
Inhibition of sulfotransferase activity in human liver S9	↓	↓	↓	Q-7G > Q-3G	[134]
Inhibition of sulfotransferase activity in HepG2 cells	↓	-	↓	Both Q-7G and Q-3G were inactive and taken up poorly.	[134]
Inhibition of sulfotransferase activity in human intestinal S9	-	=	-		[134]

a↓, the respective conjugate(s) have a lower activity than the aglycone; =, the respective conjugates have an equal activity to the aglycone; A > B, conjugate A is more active than conjugate B.

Future perspectives

Based on the results of this thesis and other scientific literature reporting on the effects of conjugation on the biological activity of flavonoids several recommendations and perspectives for future research can be formulated. These recommendations include methodological considerations for studying the biological activity of flavonoids and their conjugates, the relevance of the gut microbiome for flavonoid bioactivity, as well as considerations regarding the pharmacokinetics and pharmacodynamics of flavonoids in vivo.

Methodological considerations

When studying the biological activity of flavonoids and their conjugates, there are many factors that need to be taken into consideration but are often overlooked. These aspects include the stability of the flavonoids in in vitro assays, the cellular uptake and metabolism of flavonoids in cellular assays, as well as a range of inherent physico-chemical properties of flavonoids that can interfere with various assay systems. In the following section these methodological considerations are discussed in more detail.

Flavonoid stability

An important aspect that can affect the outcomes of in vitro experiments with flavonoids and that requires more attention in future work as it is not always adequately addressed by researchers, is that flavonoids and their conjugates can be susceptible to auto-oxidation. Some flavonoid aglycones are reported to have half-lives of only a few minutes under common cell culture conditions [136]. It is reported that conjugation can affect the stability of flavonoids [137, 138]. Especially glucuronidation is considered to reduce the auto-oxidation of flavonoids, thereby increasing their stability and half-life. In addition, flavonoid stability may vary with the culture conditions applied, depending on e.g. the pH and the presence of proteins or antioxidants (e.g. ascorbic acid) [136, 139-141]. When studying the biological effects of flavonoids in vitro, it is crucial to assess and ensure the stability of the compounds under the experimental conditions to avoid false negative results.

Cellular uptake

An important factor that needs to be taken into consideration in cellular assays is the cellular uptake of flavonoids and their conjugates. The uptake of flavonoid glucuronides is generally considered to be lower than that of flavonoid aglycones. Flavonoid conjugates, though to a certain extent also their respective aglycones, are reported to be taken up by organic anion transporters [142, 143] and the uptake is affected by type and position of conjugation (see **Chapter 2**); many flavonoid aglycones are suggested to also be able to passively diffuse into cells causing a higher cellular uptake. It is of importance to note

that many commonly used cell lines differ from their tissues of origin in the expression of transport proteins [144], which hinders the extrapolation of in vitro results to the in vivo situation. Lower observed activity especially of flavonoid glucuronides than the respective aglycones in cellular assays can at least in part be caused by lower uptake of the conjugates.

Metabolism

Another aspect that needs to be considered in cell based assays is the potential metabolic activity of the cells used. Cell cultures used for in vitro experiments can be derived from different tissues of different species which each have their own expression of transport proteins and metabolic enzymes. Certain cell lines can deconjugate flavonoid glucuronides to yield their respective aglycones [145], and some cell lines can also conjugate flavonoid aglycones and conjugates [146-148]. This is illustrated by the results from **Chapter 3** in which the biosynthesis of flavonoid conjugates using cell lines is described. Different cell models appear to produce different flavonoid metabolite patterns [20]. This indicates that for a selected cell model the metabolic fate of the flavonoid compounds needs to be determined to ensure that the compound studied is in its intended form.

Experimental artefacts

Many common experimental assay systems can be susceptible to a wide range of experimental artefacts caused by different physico-chemical properties of flavonoids and their conjugates. These effects need to be taken into account when conducting experiments with flavonoids but are often overlooked. A first example of interferences of flavonoids and their conjugates with biological assays is based on their interaction with peroxidases. Flavonoids can inhibit peroxidase activity [149-151] and this can cause false results in different experimental assays, such as commonly used assays for the quantification of free fatty acids and triglycerides [152]. Figure 7.1 shows an example of the interference of flavonoids with a colorimetric assay for the quantification of free fatty acids (FFAs).

Further, flavonoids can interfere with assays that rely on fluorescence readouts through fluorescence quenching. Examples of assays that are affected by the fluorescence quenching of flavonoids are assays based on time-resolved fluorescence energy transfer (TR-FRET) for the determination of nuclear receptor - coregulator interactions (data not shown) or assays that use fluorogenic reaction products as readout for MMP activity. Figure 7.2 gives an example of the interference of flavonoids with a fluorimetric assay for the quantification of MMP activity.

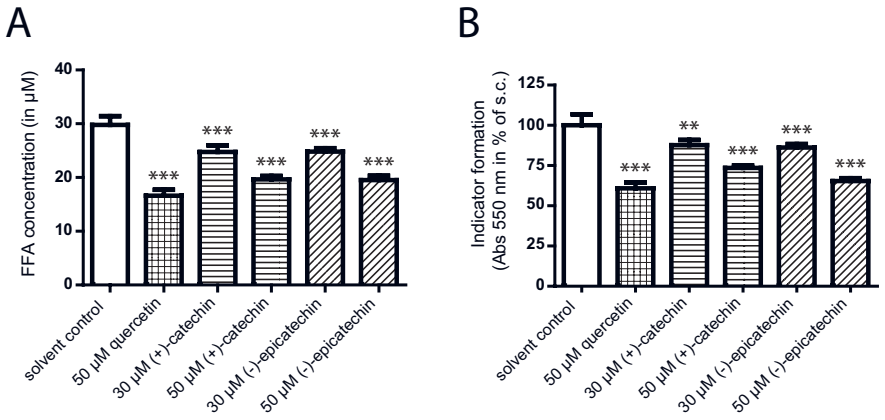


Figure 7.1. Flavonoids interfere with the Wako NEFA-HR kit (Wako Diagnostics, Richmond, VA, USA) for the quantification of FFAs. (A) The effects of flavonoids on free fatty acids in cell culture medium were tested according to the manufacturer's instructions. The flavonoids appear to reduce FFA concentrations. However, in a control experiment (B), it was shown that the test compounds interfere with the assay system. The effect of the tested flavonoids on the formation of the colorimetric indicator used for the quantification of FFAs was tested using an equal amount of FFA standard (Linoleic Acid-Oleic Acid-Albumin, Sigma-Aldrich, St. Louis, MO, USA; catalogue # L9655) in each incubation. The tested compounds were shown to interfere with the assay system and inhibited the formation of the colorimetric indicator resulting in apparently lower FFA concentrations. This interference is likely to be due to the inhibition of peroxidase activity [152]. The results suggest that the measured apparent reduction of FFAs in cell culture medium is to a significant extent due to this experimental artefact. Statistically significant difference from solvent control: ** $p > 0.01$; *** $p > 0.001$.

Another way by which flavonoids can interfere with experimental assays is through autofluorescence. An example of this interference is the autofluorescence of flavonols like quercetin and kaempferol in the same spectral range as the commonly used fluorophore fluorescein isothiocyanate (FITC; excitation 495 nm, emission 519 nm). Figure 7.3 shows that autofluorescence of kaempferol can cause false-positive results in STK PamChips. Further examples of interference of flavonoids with experimental assays can be found in literature. Flavonoids are reported to interfere with assays determining MAO-A activity [153] through their antioxidant activity. In addition, flavonoids are reported to interfere with fluorescent intracellular probes used in flow cytometry through different mechanisms [154]. Flavonoids and their conjugates can also have post-translational effects on luciferase, which is a common reporter protein used in reporter gene assays, increasing its stability, and thereby causing false positive results [155, 156]. Moreover, flavonoids can reduce tetrazolium salts thereby affecting assays that determine mitochondrial reduction of tetrazolium salt as an indication of cell viability, like the commonly used MTT assay [157]. Certain flavonoids are also reported to lead to the generation of H_2O_2 in cell culture media [158]. It appears that some effects ascribed to flavonoids in current literature are actually the result of these confounding activities of flavonoids.

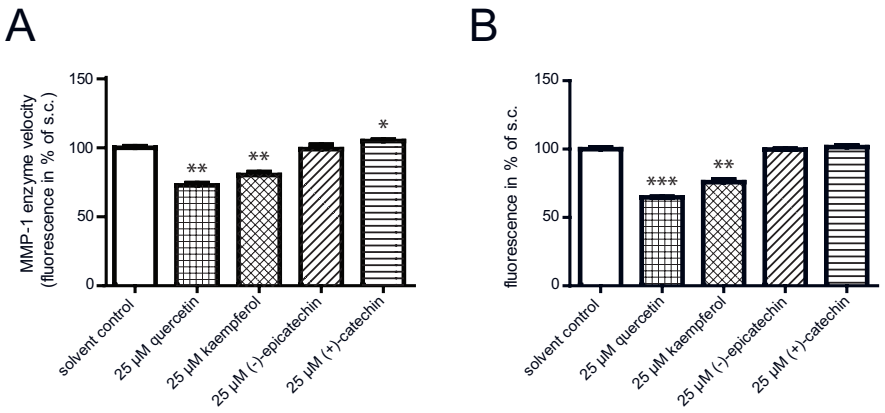


Figure 7.2 Flavonoids interfere with the MMP-1 fluorimetric drug discovery kit (Enzo Life Sciences, Raamsdonksveer, the Netherlands; catalogue # BML-AK405-001) for the quantification of MMP-1 activity. (A) The inhibitory activity of different flavonoids on MMP-1 activity was tested according to the manufacturer's instructions. Quercetin and kaempferol appear to inhibit MMP-1 activity. However, in a control experiment (B), it was shown that quercetin and kaempferol interfere with the assay system. The effect of the test compounds on the fluorescence of an equal amount of reference standard (OmniMMP® fluorogenic control, Enzo Life Sciences; catalogue # BML-P127) for the fluorogenic reaction product of the OmniMMP® fluorogenic substrate was tested. Quercetin and kaempferol were shown to interfere with the assay system and reduced the fluorescence of the fluorogenic reaction product that is used for the quantification of MMP-1 activity through fluorescence quenching, resulting in lower fluorescence in spite of equal concentrations of fluorogenic reaction product. The results suggest that the measured apparent inhibition of MMP-1 activity is to a significant extent to due to fluorescence quenching. Statistically significant difference from solvent control: * $p > 0.05$; ** $p > 0.01$; *** $p > 0.001$.

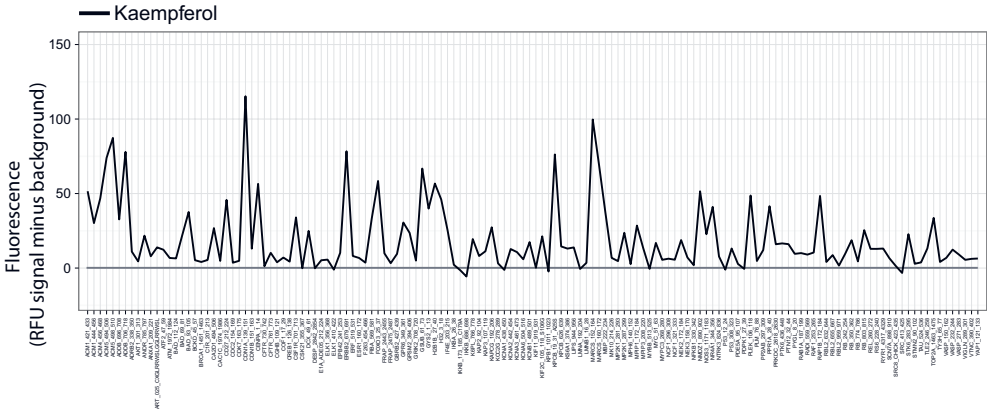


Figure 7.3 The autofluorescence of kaempferol on STK PamChip causes false positive signals. Experiments were conducted as described in Chapter 6 in the absence of protein kinase and fluorescent antibody; signals were determined using the FITC channel of the PamStation 12.

Relevance of the microbiome

There are indications that the gut microbiome might play an important role in the health effects associated with flavonoid consumption. The microbial metabolism of flavonoids and the biological activity of these microbial metabolites, as well as the effects of flavonoids on the microbiome are described in this section.

Microbial metabolites of flavonoids

Apart from the conjugated flavonoid metabolites that are subject of this thesis, there are small microbial breakdown products of flavonoids formed in the intestine by the gut microbiome that can be taken up and enter the systemic circulation, and might contribute to the biological effects associated with the ingestion of flavonoids. It should be noted that polyphenols are not the sole source of phenolic acids that can be detected in plasma and urine after ingestion of polyphenol rich foods, as certain foods contain phenolic compounds alongside polyphenols [159-161]. While only a limited amount of research has been performed on the health effects of phenolic acids, there are indications that certain phenolic acids, e.g. caffeic acid, 3,4-dihydroxybenzoic acid, and p-coumaric acid, exert anti-inflammatory effects [162-172]. These phenolic compounds are also subject to phase II conjugation during uptake, and it has been reported that their reduction of TNF-α secretion in THP-1 monocytes is affected by conjugation [173]. Some phenolic acids can have a higher bioavailability and higher plasma concentrations than the polyphenols [174, 175] and should be considered in future studies on the biological effects of flavonoids and their metabolites.

Effects on the microbiota

Flavonoids are hypothesized to further affect human health by exerting direct effects on the gut microbiota. Flavonoid ingestion is reported to affect the composition of the gut microbiota and these changes are suggested to have positive health effects [176-180]. High polyphenol intake is often reported to cause increased counts of Bifidobacteria and Lactobacilli, and decreased counts of Clostridia and Bacteroides [181-186]. Lactobacilli and Bifidobacteria are reported to prevent the growth of pathogenic organisms [187] and stimulate the production of beneficial organic acids such as lactate and acetate [188]. Some flavonoids are reported to further cause Bifidobacteria to excrete anti-inflammatory substances, [189] as well as increase secretion of lactic and acetic acids [190], which can further be converted to butyrate by Firmicutes [191]. Flavonoids themselves can be cleaved into short-chain fatty acids, such as acetate, butyrate and proprionate [192] which are suggested to have positive health effects [193-195] and inhibit the production of pro-inflammatory cytokines in vitro [196, 197]. Bifidobacteria are further associated with positive effects, amongst others on the immune system [198, 199] and the potential to lower plasma cholesterol levels [200]. The class Clostridia, however, contains various pathogens which are amongst others associated with colitis

and in experimental animals increased numbers of colonic tumors [201]. It is expected that the effects of flavonoids on the gut microbiome and subsequently health will become increasingly important in future research.

Pharmacokinetics in humans

Another topic of interest for future research is based on the consideration that cells from different tissues are differently capable of accumulating and metabolizing flavonoids [20, 202]. Thus, instead of focusing on the concentrations and conjugation state of flavonoids in plasma in vivo and cell culture medium in vitro, the intracellular concentrations and conjugation state of flavonoids ought to be of higher relevance for effects on any intracellular targets. A better understanding of the pharmacokinetics of flavonoids in humans, determining their kinetics not only in plasma but also in cells and tissues, is needed to be able to translate results from in vitro research to an in vivo situation.

The role of the endothelium

An important aspect for future research related to the pharmacokinetics of flavonoids is the elucidation of the role of the endothelium as interface between blood and other tissues. It is hypothesized that endothelial cells can deconjugate flavonoid glucuronides, thereby supplying the adjacent tissues with flavonoid aglycones, also those tissues that would otherwise have a low capacity to take up flavonoid conjugates [113]. This hypothesis is supported by in vivo research showing that the amount of quercetin aglycones in porcine organs is not correlated to the tissue-specific activities of beta-glucuronidase, suggesting that the aglycones are supplied by the adjacent endothelial cells [203].

Physiologically-based pharmacokinetic and pharmacodynamic (PBPK) models

As information on the distribution and effects of flavonoids in human tissues are difficult to obtain, refined PBPK models might prove to be a useful tool in the study of flavonoid modes of actions. Models are needed that not only describe concentrations and types of flavonoids in plasma but also in tissues, and also describe the microbial metabolites formed. Ultimately, an ideal PBPK model should also incorporate information on flavonoid-induced differential metabolism through e.g. the inhibition of sulfotransferases, the activation of Nrf2 [204], or the effect of co-administration of different flavonoids [205].

Conclusions

The research presented in this thesis describes the effect of conjugation on the biological activity of selected flavonoids towards different endpoints relevant for human health. Based on the results obtained, it can be concluded that glucuronidation does not eliminate the biological activity; the extent to which the activity of the flavonoid aglycone is reduced by glucuronidation varies greatly, depending on the flavonoid and endpoint studied. Many issues, including the role of and interaction with the microbiome and specific target tissues remain to be elucidated.

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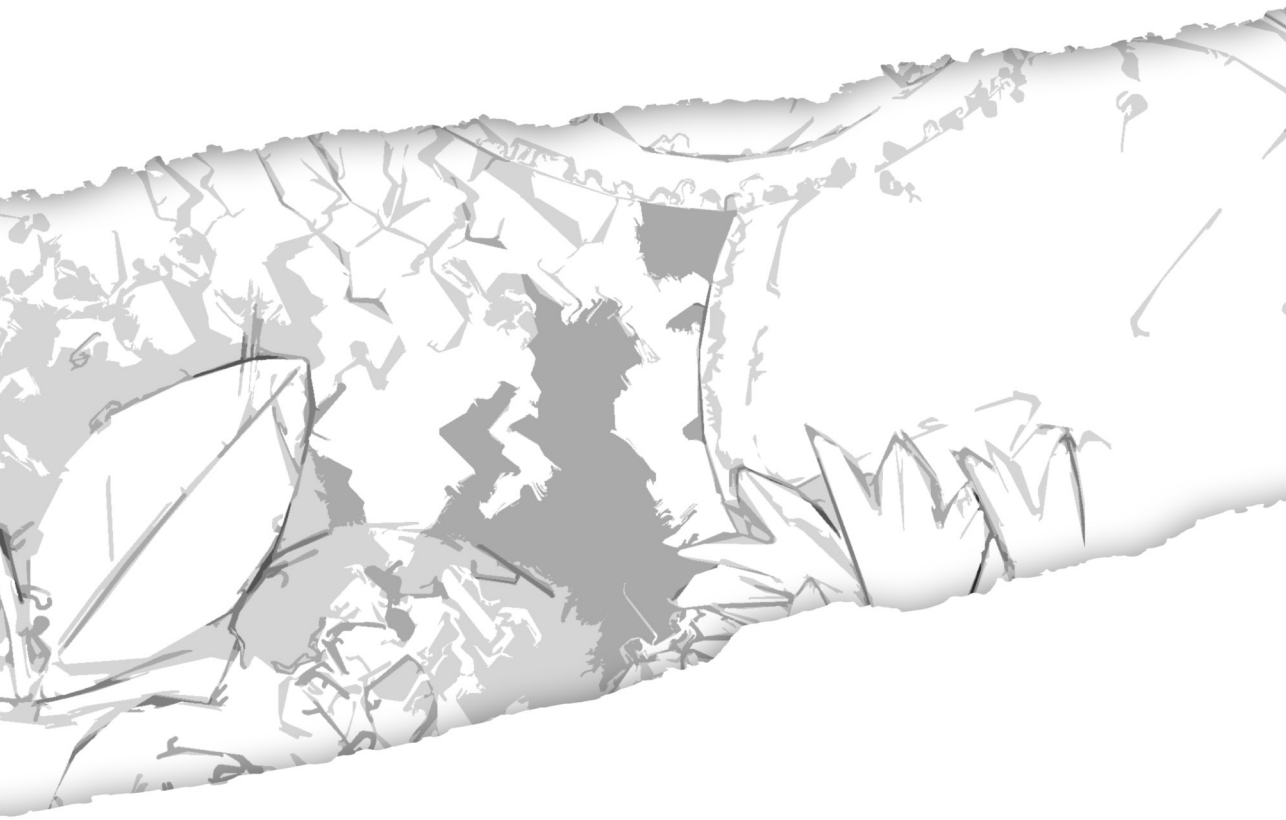

Summary

Flavonoid consumption is often correlated with a wide range of health effects, such as the prevention of cardiovascular diseases, neurodegenerative diseases, and diabetes. These effects are usually ascribed to the activity of the parent flavonoid aglycones, even though these forms of the flavonoids generally have a low systemic bioavailability. During uptake, flavonoids undergo phase II metabolism and are present in the systemic circulation nearly exclusively as conjugated metabolites (Chapter 1). The aim of this thesis was to study the effect of conjugation on the biological activity of selected flavonoids towards different endpoints relevant for human health. To this end, conjugation with glucuronic acid was taken as the model type of conjugation because this modification is generally observed to be the most important metabolic conjugation reaction for flavonoids in man.

A review of scientific literature published until early 2012 reveals that metabolic conjugation can affect the biological activity of flavonoids in different ways (Chapter 2). Conjugation can increase, decrease, inverse or not affect the biological activity, depending on the flavonoid, the type and position of conjugation, the endpoint studied, and the assay system used. Based on the literature reviewed it is concluded that the effect of conjugation has to be studied on a case-by-case basis.

As the research on the biological activity of biologically relevant flavonoid conjugates is often hampered by the generally low commercial availability and high prices of these conjugates, a simple and versatile method for the biosynthesis of metabolically relevant flavonoid conjugates is described (Chapter 3). Using this method, relevant conjugates can be prepared from different flavonoid substrates in sufficient quantities for in vitro bioassays. Further, an efficient strategy for the identification of these flavonoid conjugates by LC-MS and ^{1}H-NMR using MetIDB (Metabolite Identification Database), a publicly accessible database of predicted and experimental ^{1}H-NMR spectra of flavonoids, is presented.

To study the effect of conjugation on the biological activities of flavonoids, in the subsequent chapters of the thesis several different assay systems and endpoints were used to study the activity of different flavonoids and their conjugates (Chapters 4 to 6). In Chapter 4 the effects of quercetin, kaempferol, and their main plasma conjugates quercetin-3-*O*-glucuronide and kaempferol-3-*O*-glucuronide (K-3G) on different endpoints related to peroxisome proliferator-activated receptor (PPAR)-γ were studied. PPAR-γ activation is reported to have positive health effects related to adipogenesis, insulin resistance and inflammation. The presented results show that the flavonoid aglycones increased PPAR-γ mediated gene expression in a stably transfected reporter gene cell line, and that glucuronidation diminished this effect. These observed increases

in reporter gene expression were accompanied by increased PPAR-γ receptor-mRNA expression upon exposure to kaempferol, an effect that was also reduced by glucuronidation. Using the cell-free Microarray Assay for Real-time Coregulator-Nuclear receptor Interaction (MARCoNI) it was demonstrated that, unlike the known PPAR-γ agonist rosiglitazone, neither the flavonoid aglycones nor the conjugates are agonistic ligands of the PPAR-γ receptor. Supporting the hypothesis that the tested compounds have a different mode of action from normal LBD agonism, quercetin appeared to synergistically increase the effect of rosiglitazone in the reporter gene assay. The modes of action behind the observed effects remain to be elucidated and might include effects on protein kinase activities affecting expression of the PPAR-γ receptor, or post-transcriptional modifications of PPAR-γ.

Another type of nuclear receptor known to be targeted by certain flavonoids are the estrogen receptor (ER)α- and ERβ. ERs are the main targets of estrogenic compounds, and upon their activation different transcriptional responses with opposite effects on cell proliferation and apoptosis are elicited; ERα activation stimulates cell proliferation, while ERβ activation causes apoptosis and reduces ERα mediated induction of cell proliferation. Using the MARCoNI assay, the intrinsic estrogenic effects of the two main dietary isoflavones daidzein and genistein, and their plasma conjugates daidzein-7-*O*-glucuronide and genistein-7-*O*-glucuronide on the ligand induced coregulator binding of ERα- and ERβ-LBD were studied and compared to the effect of the positive control 17β-estradiol (E2). The results show that the tested isoflavone compounds are less potent agonists of ERα- and ERβ-LBD than E2, although they modulate the LBD-coregulator interactions in a manner similar to E2. Genistein is shown to be a more potent agonist than daidzein for both receptor subtypes. While in the MARCoNI assay genistein had a strong preference for ERβ-LBD activation over ERα-LBD activation, daidzein had a slight preference for ERα-LBD activation over ERβ-LBD activation. Glucuronidation reduced the intrinsic agonistic activities of both daidzein and genistein to induce ERα-LBD and ERβ-LBD - coregulator interactions and increased their average half maximal effective concentrations (EC_{50}s) by 8 to 4,400 times. The results presented further show that glucuronidation changed the preferential activation of genistein from ERβ-LBD to ERα-LBD and increased the preferential activation of daidzein for ERα-LBD; this is of special interest given that ERβ activation, which is counteracting the possible adverse effects of ERα activation, is considered one of the supposedly beneficial modes of action of isoflavones.

Many flavonoids are reported to be inhibitors of protein kinases. To study the effect of conjugation on the inhibition of serine/threonine protein kinases by flavonoids, kaempferol and its main plasma conjugate K-3G were selected as model compounds (Chapter 6). Protein kinases are involved in a wide range of physiological processes by controlling signaling cascades and regulating protein functions; modulation of

their activities can have a wide range of biological effects. The inhibitory effects of kaempferol, K-3G, and the broad-specificity protein kinase inhibitor staurosporine on the phosphorylation activity of recombinant protein kinase A (PKA) and of a lysate prepared from the hepatocellular carcinoma cell line HepG2 were studied using a microarray platform that determines the phosphorylation of 141 putative serine/threonine phosphorylation sites derived from human proteins. The results reveal that glucuronidation reduces the intrinsic potency of kaempferol to inhibit the phosphorylation activity of PKA and HepG2 lysate on average about 16 and 3.5 times, respectively. It is shown that the inhibitory activity of K-3G in the experiments conducted was not caused by deconjugation to the aglycone. Furthermore, the results show that kaempferol and K-3G, unlike the broad-specificity protein kinase inhibitor staurosporine, did not appear to inhibit all protein kinases present in the HepG2 lysate to a similar extent, indicating that kaempferol selectively targets protein kinases, a characteristic that appeared not to be affected by glucuronidation. The fact that K-3G appeared to be only a few times less potent than kaempferol implies that K-3G does not necessarily need to be deconjugated to the aglycone to exert potential inhibitory effects on protein kinases.

The results obtained in the present thesis support the conclusion that glucuronidation of flavonoids does not necessarily abolish their activity and that flavonoid glucuronides may be biologically active themselves, albeit at higher concentrations than the parent aglycones (Chapter 7). In line with the conclusions from the earlier review presented in Chapter 2, an updated literature review on the effect of conjugation on the biological activity of flavonoids concludes that that the effect of conjugation on the biological activity of flavonoids depends on the type and position of conjugation, the endpoint studied and the assay system used. Based on the results described and the literature reviewed in this thesis, several recommendations and perspectives for future research are formulated. Several methodological considerations are formulated that need to be taken into account when studying the biological activity of flavonoids and their conjugates to avoid confounding results. Further, the relevance of the gut microbiome for flavonoid bioactivity is highlighted, and considerations regarding the pharmacokinetics and pharmacodynamics of flavonoids in vivo are formulated. Altogether, it can be concluded that circulating flavonoid conjugates may exert biological activities themselves, and that understanding these is a prerequisite to successfully elucidate the mechanisms of action behind the biological activities linked to flavonoid consumption.

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Karsten

About the author

Karsten Beekmann was born on 13 June 1983 in Bremen, Germany. In 2002, he finished secondary school at the Gymnasium Osterholz-Scharmbeck. After one year of community service, he continued his education with a BA in Biology & English Language and Literature at the University of Vechta, Germany. Following graduation from his Bachelor's degree, Karsten moved to the Netherlands to follow the MSc programs Nutrition and Health, and Food Safety at Wageningen University. During these studies, Karsten spent 6 months at the Department of Pathology at the New York Medical College, NY, USA, for his MSc thesis research in Food Toxicology, and wrote his MSc thesis in Food Law at the Law and Governance group at Wageningen University. Karsten concluded his studies with an internship at RIKILT Institute of Food Safety in Wageningen. After graduation from his MSc degrees, he started his PhD research described in this thesis at the Division of Toxicology at Wageningen University. During his PhD, Karsten followed a postgraduate education program and registered as toxicologist-in-training, to become European Registered Toxicologist after graduation. Karsten is currently working as Assistant Professor in Systems Toxicology at the Division of Toxicology at Wageningen University, researching the role of the gut microbiota for toxicology.

List of Publications

Hoek-van den Hil, E. F., K. Beekmann, J. Keijer, P. C. H. Hollman, I. M. C. M. Rietjens and E. M. van Schothorst, *Interference of flavonoids with enzymatic assays for the determination of free fatty acid and triglyceride levels*. Analytical and Bioanalytical Chemistry, 2011. **402**(3): p. 1389-1392.

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Beekmann, K., L. Rubió, L. H. J. De Haan, L. Actis-Goretta, B. Van Der Burg, P. J. Van Bladeren and I. M. C. M. Rietjens, *The effect of quercetin and kaempferol aglycones and glucuronides on peroxisome proliferator-activated receptor-gamma (PPAR-γ)*. Food and Function, 2015. **6**(4): 1098-1107.

Beekmann, K., L. H. J. de Haan, L. Actis-Goretta, R. Houtman, P. J. van Bladeren and I. M. C. M. Rietjens, *The effect of glucuronidation on isoflavone induced estrogen receptor (ER)α and ERβ mediated coregulator interactions.* The Journal of Steroid Biochemistry and Molecular Biology, 2015. **154**: 245-253.

Beekmann, K., L. H. J. de Haan, L. Actis-Goretta, P. J. van Bladeren and I. M. C. M. Rietjens, *Effect of Glucuronidation on the Potential of Kaempferol To Inhibit Serine/Threonine Protein Kinases*. Journal of Agricultural and Food Chemistry, 2016. **64**(6): 1256-1263.

Beekmann, K., V. V. Mihaleva, I. M. C. M. Rietjens and J. Vervoort, *Flavonoid conjugate biosynthesis and identification using the Metabolite Identification Database (MetIDB)*. In preparation.

Overview of completed training activities

Discipline specific courses

Risk Assessment	Postgraduate Education in Toxicology (PET)	2011
Molecular Toxicology	PET	2011
Immunotoxicology	PET	2012
Ecotoxicology	Division of Toxicology, Wageningen University (WU-TOX)	2012
Organ Toxicology	PET	2013
Mutagenesis and Carcinogenesis	PET	2013

Meetings and conferences

International Conference on Polyphenols and Health	Sitges, Spain	2011
32nd Annual meeting of the Netherlands Society of Toxicology (NVT)	Zeist, the Netherlands	2011
35th Annual meeting of the Netherlands Society of Toxicology (NVT)	Veldhoven, the Netherlands	2014
54th Annual Meeting of the Society of Toxicology	San Diego, CA, USA	2015

General courses

VLAG PhD week	VLAG, Wageningen	2011
Laboratory Animal Science	PET	2011
Project and Time Management	Wageningen Graduate Schools (WGS)	2012
Communication in Interdisciplinary Research	WGS	2013

Optional activities

Preparation of PhD research proposal	WU-TOX	2010
Organizing PhD trip Italy & Switzerland	WU-TOX	2011
PhD trip England	WU-TOX	2013
General Toxicology	WU-TOX	2013
Attending scientific presentations	WU-TOX	2010-2015

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