Mechanisms of toxic action of the flavonoid quercetin and its phase II metabolites

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Abstract

During and after absorption in the intestine, quercetin is extensively metabolised by the phase II biotransformation system. Because the biological activity of flavonoids is dependent on the number and position of free hydroxyl groups, a first objective of this thesis was to investigate the consequences of phase II metabolism of quercetin for its biological activity. For this purpose, a set of analysis methods comprising HPLC-DAD, LC-MS and ^{1}H NMR proved to be a useful tool in the identification of the phase II metabolite pattern of quercetin in various biological systems. These studies showed that the 3'- and 4'-hydroxyl groups of quercetin, (catechol hydroxyl groups) were important targets for methylation, sulfation and glucuronidation. Methylation of a catechol hydroxyl group of quercetin proved to decrease the pH-dependent radical scavenging capacity of the compound, both by increasing its pK_a for deprotonation and by decreasing its electron-donating properties. Methylation of a catechol hydroxyl group had a similar effect as replacement of the hydroxyl group by a hydrogen atom. Regarding the pro-oxidant properties of quercetin, methylation of a catechol hydroxyl group of quercetin did not eliminate the pro-oxidant chemistry of quercetin, reflected in the formation of covalent adducts with glutathione upon oxidation of quercetin by horseradish peroxidase. However, methylated quercetin proved to form only 42% of the level of DNA adducts in exposed cells as compared to a similar amount of unconjugated quercetin, indicating that methylation of quercetin attenuates also this biological reactivity towards DNA.

A second objective of this thesis was to obtain more insight into the possible toxic effects of quercetin by studying various mechanisms that might be relevant in the context of carcinogenesis. Quercetin appeared to have a biphasic effect on the proliferation of cancer cell lines expressing the estrogen receptor (ER). The stimulation of cancer cell proliferation was ER-dependent and appeared to occur at concentrations that are physiologically relevant in humans. With respect to the pro-oxidant activity of quercetin, peroxidase- and tyrosinase-type oxidative enzyme activity did not play a major role in the intracellular formation of covalent adducts of quercetin with DNA and protein, indicating that the formation of covalent adducts of quercetin with cellular macromolecules might also be relevant in cell types lacking oxidative enzyme activity. Furthermore, the covalent quercetin DNA adducts were of transient nature, which may either eliminate or attenuate the adverse effects of covalent DNA adduct formation. The studies presented in this thesis provided indications for the dualistic character of quercetin, regarding its role in the process of cancer development.

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1

General introduction, objectives and outline

1.1 From healing red peppers to polyphenol research

Since the 18th century, the potency of a nutrient, later called vitamin C, to cure scurvy has been known, and the Hungarian scientist Albert von Szent-Györgyi was the first to identify the chemical structure of the compound by demonstrating its similarity to hexuronic acid (*1, 2*). He later re-baptized this compound ascorbic acid. The Hungarian town Szeged, where he worked at that time, was the centre of the red pepper industries. In 1934, Szent- Györgyi demonstrated this vegetable to be a rich source of ascorbic acid by developing a method to isolate the crystalline ascorbic acid from this source (*3*). In these days, Szent-Györgyi was asked by a colleague, who suffered from a serious haemorrhagic diathesis (increased permeability or fragility of the capillary wall), for a large amount of pure ascorbic acid to cure the disease. However, the crystalline substance was not available in sufficient amounts and therefore, Szent-Györgyi sent him red peppers to eat. As by miracle, his colleague was cured. Later on, the scientist tried to obtain the same therapeutic effect as observed for whole red peppers by using pure ascorbic acid, but he was unsuccessful. This convinced him that red peppers had to contain other substances that contributed to their therapeutic effects (*4*).

His earlier studies on cell respiration had provided Szent-Györgyi with the insight that ascorbic acid protected plant tissues from oxidative damage resulting from a reversible interaction with a peroxidase-like enzyme. He demonstrated that a third substance, having a polyphenol structure, played an important role in this protective effect. His idea was that the peroxidase oxidized the polyphenol to a quinone metabolite, which subsequently oxidized ascorbic acid by taking up both its hydrogen atoms (*5*). He showed that this polyphenol belonged to a large group of yellow phenol-benzol-γ-pyran plant dyes (including flavones, flavonols and flavanones). It became clear that the members of this group of plant dyes possessed great biological activity. Based on his experience that the therapeutic effects of red peppers were larger than the therapeutic effects of a corresponding amount of pure ascorbic acid, he proposed that, possibly by interactions with ascorbic acid, these polyphenol-like dyes might also repair damaged mammalian tissues by influencing the permeability and resistance of capillary blood vessels. He was of the opinion that these substances represented a promising group of compounds with therapeutic potential against a variety of diseases. Because his experiments showed that certain members of this group of plant dyes possessed vitamin-like properties, he called the polyphenols vitamin P (*4*). In 1937, the importance of these findings was officially recognized when he was awarded Nobel Prize of Medicine for this pioneering work on vitamin C and vitamin P.

"I regret that I must conclude with many questions asked and none answered, but I hope to leave the reader with the impression that flavonoids represent one of the most exciting, broad, and hopeful fields of biological inquiry and I am glad to close on such an optimistic note". These are the words the Nobel-Prize winning scientist closed a lecture with in 1955 (*6*). And he was right, because his findings on the biological activity of polyphenols were the start of decades of research on polyphenols.

1.2 Flavonoids and quercetin

Polyphenols have a multitude of biological functions in the plant, including signalling, fertility, and protection against UV-light and phytopathogens (*7*). Polyphenols show a great diversity of structures, ranging from rather simple molecules (monomers and oligomers) to polymers (*8*). They can be divided into four major classes, according to the nature of their carbon skeleton, i.e. phenolic acids, flavonoids, stilbenes and lignans.

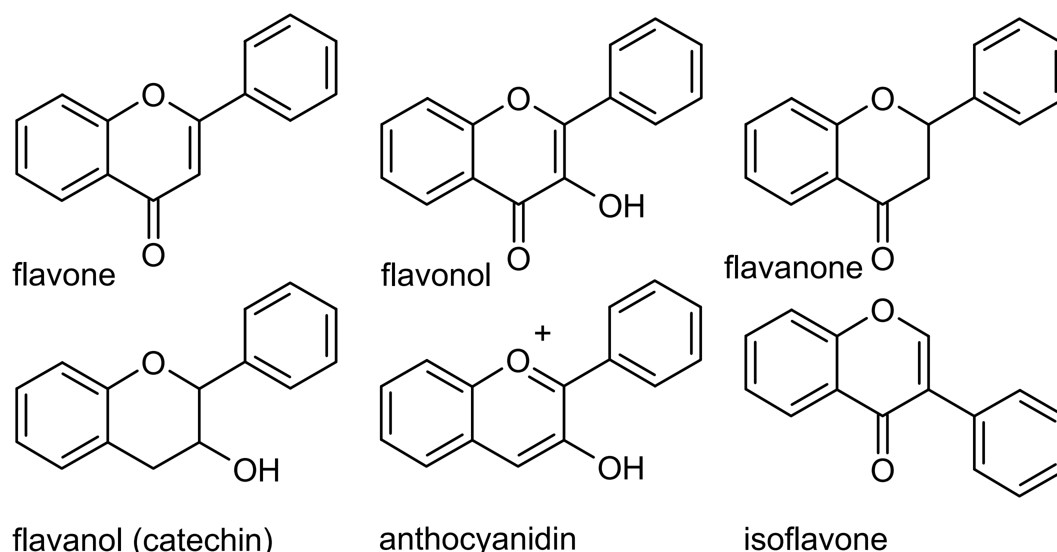

Figure 1 Structures of the major classes of flavonoids.

Flavonoids (Figure 1) are the most abundant polyphenols in our diet (*9*). In the plant, they are products from the shikimic acid pathway (*10*). They consist of a three-ring structure and can be divided into several classes, depending on the degree of oxidation of the heterocyclic ring (also called ring C): flavones, flavonols, isoflavones, anthocyanins, flavanols, and flavanones (*9*). These major classes of flavonoids are depicted in Figure 1. In addition to the findings of Szent-Györgyi on the beneficial effects of vitamin P, the interest in the effects of flavonoids

on health was enhanced by the so-called French Paradox. The central question behind this French Paradox is why a high consumption of saturated fatty acids does not lead to a high coronary heart disease risk in Southern France (*11*). Although there is currently some controversy on the real causes for the lower disease risk, the high consumption of red wine, containing relatively large amounts of flavonoids, was proposed as a possible explanation for the paradox (*12*).

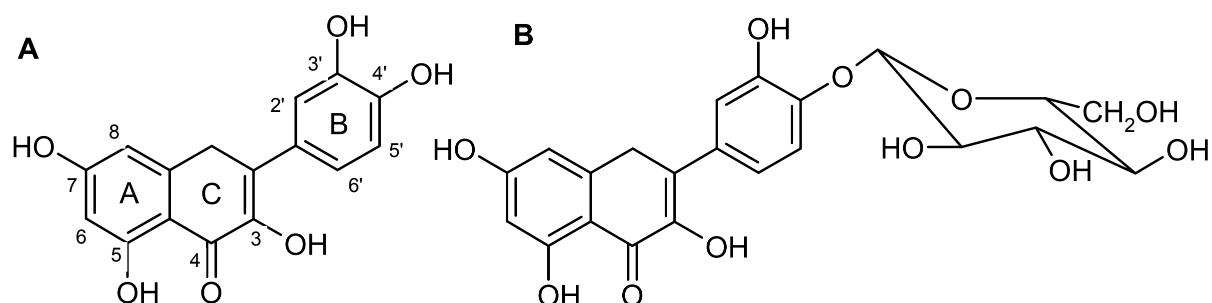

Figure 2 Structure of A) quercetin with the numbering of its carbon atoms and rings, and of B) quercetin-4'-β-D-glucoside.

Table 1 Quercetin content of various vegetables, fruits and beverages (*16, 17*).

Vegetables and fruits[1)]		Beverages[2)]	
Vegetables		*Wine*	
Onion	347	Red Bordeaux	4.1
Kale	110	Red Chianti	16
Broccoli	30	White Bordeaux	<0.5
Lettuce	14		
Tomato	8		
Fruits		*Tea*	
Apple	6	Black	10-25
Strawberry	8.6	Green	13-24
Grape, white	12		
Grape, black	15		

[1)] in mg/kg of fresh edible part; [2)] in mg/L

Quercetin (Figure 2A) is the main flavonol present in our diet (*9*). Because of its interesting chemical and biological properties (*10*), quercetin has been one of the most studied flavonoids. Quercetin occurs mainly as glycoside (*13*) in a wide variety of fruits, vegetables and beverages. In plants, different types of sugars, including glucose, galactose, rhamnose, rutinose or xylose, are bound to the hydroxyl groups of the aglycon by glycosidic bonds. The

major targets for glycosidation are the 3-, 7- and 4'-hydroxyl groups (*14*). As an example, Figure 2B shows the structure of quercetin-*4'-β-D*-glucoside. Table 1 provides an overview of the quercetin content of a number of plant-based products. According to the data of the Seven Countries Study, the average daily intake of flavonoids varies from approximately 3 mg/day in West-Finland to 68 mg/day in Japan. Quercetin represents 40 to 100% of this total flavonoid intake (*15*).

2. Beneficial health effects of quercetin, with special emphasis on cancer

2.1 Introduction

Due to several thousand years of experience, man has become convinced of the role for plant foods in maintenance of health. Although conclusions from recently published epidemiological studies on the influence of fruit and/or vegetable intake on the risk for various forms of cancer point at the absence of relevant effects (*18, 19*), the majority of studies performed before 1996 on this subject have provided evidence that a high consumption of fruits and vegetables is associated with a lower risk of many epithelial cancers (*20*). Although the reasons for the differences in effects between recent and earlier studies may currently be a matter of debate (*21*), the putative beneficial health effects of fruits and vegetables, together with the finding that fruits, vegetables and various beverages contain considerable amounts of flavonoids (*16, 22*), have focussed the attention on the beneficial health effects of flavonoids.

In a large number of *in vitro* studies, flavonoids have been characterised as good antioxidants (*14, 23-29*). Structure-antioxidant activity relationships on flavonoids have identified a number of structural elements in the flavonoid structure that contribute to the antioxidant activity of the whole molecule. These include a C_2=C_3-double bond, a C_4-keto group, a C_3-hydroxyl group and an *ortho*-diphenolic structure, also called catechol group, in the B-ring (*14*). Quercetin possesses all these structural elements (see Figure 2A), and the potent ability of quercetin to scavenge reactive oxygen species (*30-32*), singlet oxygen (*33*), in addition to radicals of different origin (*34, 35*) has been confirmed in various *in vitro* systems. However, it is still not clear whether the antioxidant activity seen *in vitro*, results in beneficial health effects *in vivo*. In the last decades, many claims on the beneficial health effects of quercetin have been stated, including protection against various forms of cancer, cardiovascular diseases and neurodegenerative diseases (*10, 36*). Furthermore, anti-inflammatory, antibacterial and muscle-relaxating activities have been ascribed to quercetin (*37-40*). The

following paragraphs will discuss the scientific evidence for the health claims associated with quercetin, with special emphasis on the presumed protecting effects of quercetin against cancer.

2.2 *In vitro* evidence for the protecting effects of quercetin against cancer

Scientific evidence for the claim that quercetin may have protecting effects against cancer are to a significant extent based on *in vitro* studies. One of the reasons for this claim is the finding that quercetin is a potent antioxidant *in vitro* (*14*), and may as such cause decreased levels of oxidative stress, known to be involved in the development of certain cancers (*41, 42*).
A second mechanism that may play a role in protection against cancer is the ability of quercetin to modulate the metabolism of carcinogens, through inhibition and/or induction of enzymes involved in the biotransformation of these carcinogens (*43, 44*). Flavonoids, including quercetin, are so-called bi-functional inducers, indicating that they induce both phase I and phase II biotransformation enzyme activities at the level of gene expression (*45, 46*). For example, quercetin is known to stimulate the Electrophile Responsive Element (EpRE) (*46*), present as enhancer in the promoter region of certain genes encoding for phase II enzymes (*47*). Furthermore, modulation of the activity of biotransformation enzymes can occur at the level of the proteins themselves. Indeed, quercetin is known as a potent competitive inhibitor of certain cytochromes P450 (*48-50*) and sulfotransferases (*51-53*).
A third line of evidence supporting the health claims on protection against cancer is the large number of studies reporting inhibiting effects of quercetin on the proliferation of cells from cancer cell lines *in vitro*. An overview of such *in vitro* studies presently available is presented in Table 2. Quercetin has an inhibiting effect on the proliferation of a broad range of cell lines, with IC_{50} values ranging from 0.1 pM to 45 μM, depending on the cell type and experimental conditions used (Table 2). Several mechanisms for the antiproliferative effect of quercetin have been proposed, including the induction of DNA strand breakage, cell cycle arrest, and/or apoptosis (*54-56*). The ability of quercetin to modulate the activity of enzymes involved in signal transduction, and cell growth and development, including phosphatidyl-inositol-3-kinase, protein kinase C and protein tyrosine kinase (*57, 58*) might play a role in these effects. Furthermore, the inhibiting effect of quercetin on cell proliferation has been linked to its interaction with type II estrogen binding sites (EBS) (*59-61*). In support of this, the sensitivity of cells from the human colorectal cancer cell line HT29 for the

antiproliferative effect of quercetin appeared to correlate with the number of type II EBS per cell (*62*).

Table 2 Summary of studies on the inhibiting effect of quercetin on cell proliferation.

Cell line	Exposure time	Assay	IC_{50} (μM)[1)]	Reference
Breast				
MCF7	3 d	[^{3}H]-thymidine incorporation	15	(*65*)
MDA-MB-435	48 h	[^{3}H]-thymidine incorporation	32	(*66*)
MDA-MB-231	6 d	MTT	$2.4x10^{-6}$	(*67*)
MCF-7			$4.4x10^{-6}$	
T47D			$0.1x10^{-6}$	
Colon				
HT-29	48 h	Cell counting	13	(*68*)
Caco-2	48 h	Cell counting	45	(*56*)
HT29			53	
IEC-6			40	
HT-29	96 h	Cell counting	2.5	(*62*)
WiDr			0.06	
Colo-201			3.1	
LS-174T			0.7	
Leukemia				
TGBC11-TKB	72 h	Alamar blue reduction	5.9	(*69*)
ALL	16 h	[^{3}H]-thymidine incorporation	2	(*70*)
AML			3	
Ovary				
OVCA-433	72 h	Cell counting	10	(*71*)
Prostate				
PC3	48 h	Cell counting	45	(*72*)
Skin				
OCM-1	48 h	Cell counting	18	(*73*)
A549	72 h	Alamar blue reduction	1	(*69*)
B16-4A5			10	
CCRF-HSB-2			7.2	
TGBC11-TKB			5.9	
A431	72 h	Cell counting	21	(*74*)

[1)] IC_{50}-values presented were either given or estimated from the presented data.

Although the majority of publications point at the inhibitory effect of quercetin on cell proliferation, experiments with the oral squamous cell line SCC-25 showed a biphasic effect of quercetin on cell proliferation (*63*). At concentrations up to 10 μM, quercetin stimulated cell proliferation, whereas at higher quercetin concentrations, cell proliferation was inhibited. Altogether, the data on effects of quercetin on cell proliferation obtained from *in vitro* experiments suggest that quercetin may exert a dualistic effect on cell proliferation. It is unclear under what conditions either of the effects –stimulation or inhibition- prevails. In addition, quercetin was shown to inhibit the differentiation of Caco-2 cells, which is also an

effect opposite to what would be expected from a compound with anti-carcinogenic potential (*64*).

2.3 Epidemiological studies

Besides *in* vitro studies, the available epidemiological studies may provide additional support for the beneficial health effects of quercetin. So far, epidemiological studies focussing on the relationship between flavonoid intake and health effects have been performed using databases in which dietary intake was linked to health effects (*75-79*). From data on fruit and vegetable intake, flavonoid intakes were calculated based on the mean flavonoid content of dietary components as determined by Hertog *et al.* in 1992 (*16*). Although there is rather consistent evidence from various studies that there is an inverse association between flavonoid intake and the risk for cardiovascular diseases (recently reviewed by Arts and Hollman (*80*)), the epidemiological evidence for protecting effects of flavonoids against cancer is not unequivocal.

A number of epidemiological studies have investigated the association between flavonoid intake and the incidence of a variety of cancers (*75-79*), as recently reviewed by Arts and Hollman (*80*). Significant associations were found only for lung cancer and colorectal cancer. In two Finnish studies, i.e. the Finnish Mobile Clinic Health Examination Survey (*78*) and the Alpha-Tocopherol, Beta-Carotene Cancer Prevention Study (*77*), significant inverse associations were found between flavonoid intake and lung cancer risk. However, in the ATBC-prevention study, a borderline positive association was found for colorectal cancer (*77*).

Thus, similar to the set of available *in vitro* proliferation studies of quercetin, epidemiological studies on associations between flavonoid intake and cancer risk do not provide consistent evidence for protecting effects of flavonoids against cancer.

3. Risks associated with the consumption of quercetin

3.1 Introduction

As a consequence of the supposed beneficial health effects of quercetin, which were derived merely from *in vitro* and epidemiological studies (see Section 2), manufacturers have applied quercetin as ingredient of functional foods and food supplements. In health shops and on the Internet, a large number of quercetin supplements is sold as a cure and/or preventive agent for a large variety of physical discomforts, including hay fever, cataract, bruises, prostate

problems, but also cardiovascular diseases and cancer. The recommended dosages amount up to 1 gram per day. This development may increase the daily dose 15 to 1000-fold as compared to the daily intake of quercetin from a regular Western diet (*15*).

Already around 1500, the Swiss scientist Paracelsus was aware of the concept of dose: "*Alle Dinge sind Gift und nichts ist ohn' Gift; allein die Dosis macht dass ein Ding kein Gift ist*" ("all things are poison and not without poison; only the dose makes a thing not a poison"). In view of this concept of dose, it may be envisaged that the significant increase in the daily dose of quercetin may entail a health risk. Together with the fact that direct scientific evidence for the beneficial health effects of quercetin is rather poor, the above described marketing trend asks for a thorough investigation of the possible adverse health effects of increased consumption of quercetin.

3.2 Genotoxicity of quercetin

For several decades, the genotoxic effects of quercetin have been studied in a large variety of *in vitro* test systems. Quercetin is mutagenic in bacterial systems including the Ames-test, even without metabolic activation (*81-84*). However, the genotoxic effects of quercetin are less evident in mammalian cells. Certain authors have reported the induction of chromosomal aberrations in Chinese hamster V79 and ovary cells (*83, 85*). Furthermore, the induction of single strand breaks and point mutations in the DNA have been reported in mouse lymphoma L5178Y cells (*86*). The latter result, however, could not be confirmed in Chinese hamster V79 cells, nor could the induction of sister chromatid exchanges. It was suggested that quercetin exhibits a mutagenic response in mammalian cells by causing gross chromosomal lesions (*87*). In support of this, the clastogenic activity of quercetin has been reported in various cell types including Chinese hamster fibroblasts and ovary cells and human fibroblasts (*85, 88*).

Nevertheless, in bacterial test systems, an obvious structure-activity relationship for the mutagenic potential of flavonoids, including quercetin was found. The mutagenic activity of flavonoids proved to be dependent on:

1) the presence of a C_3-OH group;
2) the presence of a C_4-keto group;
3) the planarity of the flavonoid, associated with the presence of a C_2=C_3 double bond, which allows the conjugation of the lone electron pairs on the oxygen of the C_4-keto-group with those in the ring system;

4) the presence of a structure permitting the C_3-OH proton to tautomerize to a C_3-keto-group, which requires the presence of a C_5-OH group, because by the formation of a strong intramolecular hydrogen bond with the C_4-keto group, the C_5-OH prevents the C_3-OH proton from forming such a bond with the C_4-keto-group;
5) the presence of an *ortho*-3',4'-dihydroxygroup in the B-ring allowing the formation of quinone-type metabolites. When this so-called catechol group is present in the parent molecule, the compound is mutagenic even without metabolic activation (*81-83*).
6) In contrast to mammalian cells, the presence of the C_7-OH group is not a prerequisite for genotoxic effects of quercetin in bacterial test systems (*83*).

The differences in the structure-activity relationship of flavonoids for mutagenic effects between bacterial and mammalian test systems suggest that the mechanisms underlying these effects are not comparable. In support of this, the inability of quercetin to induce sister chromatid exchanges and point mutations in mammalian cells and the fact that the clastogenic effects of quercetin appeared to be abolished in the presence of a metabolic activation system, may explain the negative outcome of the majority of carcinogenicity studies on quercetin in mammals (*87*). The structural requirements for genotoxic effects of quercetin in bacteria have suggested the formation of quinone-type metabolites as intermediates in the mechanism underlying the mutagenic effects of quercetin (*81*).

3.3 Quinone chemistry of quercetin

For various compounds, including polyaromatic hydrocarbons and estrogens, metabolic activation leading to the formation of a catechol group (*ortho*-dihydroxybenzene group) is thought to be involved in the mechanism underlying the carcinogenic effects of these compounds (*89-92*). Catechol groups are prone to redox-cycling, leading to the formation of reactive oxygen species (ROS), known to be involved in a variety of pathological processes, including carcinogenesis, neurodegenerative and cardiovascular diseases, and aging (*41, 93-95*). Furthermore, the relatively easy oxidation of catechol groups can lead to the formation of quinone or quinone methide-type metabolites, which, due to their electrophilic character, may form covalent adducts with cellular macromolecules, including DNA, RNA and protein. As such, they may disturb cellular processes (*92, 96*).

Even without metabolic activation, quercetin already possesses a catechol group in the B-ring. Pro-oxidant effects of quercetin leading to the generation of *ortho*-semi-quinone anion radicals (SQ) and ROS have been demonstrated, especially in the presence of transition

metals (*23, 97, 98*). In addition, the quinone/quinone methide chemistry of quercetin has been thoroughly investigated using the glutathione trapping method. Oxidation of quercetin by horseradish peroxidase or tyrosinase in the presence of glutathione leads to the formation of two major metabolites, identified as 6- and 8-glutathionyl quercetin (*99, 100*). The fact that addition of glutathione had occurred in the A-ring was explained by the mechanism depicted in Figure 3 (*100*). Oxidation of the catechol group of quercetin to the *ortho*-quinone is followed by swift isomerization to *para*-quinone methide intermediates. Although quantum-mechanical calculations indicated that the *para*-quinone methides are thermodynamically less stable than the *o*-quinone, their electrophilic reactivity towards glutathione is much higher. Therefore, the swift reaction of the quinone methides with glutathione shifts the equilibrium between the isomers in favour of formation of these *para*-quinone methides and their C_6- and C_8-glutathione adducts.

Additional studies showed that both 6- and 8-glutathionyl quercetin isomerise under physiological conditions (*100, 101*). This indicates that the formation of these adducts is reversible. This may in theory generate possibilities for transport of reactive quinone metabolites of quercetin to sites different from their site of formation. Cysteine is preferred as thiol scavenging agent of quercetin quinone metabolites over glutathione and N-acetylcysteine. However, preferential scavenging by cysteine over glutathione is not expected to occur in biological systems because physiological concentrations of glutathione are considerably higher than those of cysteine, which appears to shift the balance in favour of glutathionyl adduct formation (*101*).

The findings discussed above were obtained using cell-free *in vitro* systems. Nevertheless, the formation of these glutathione conjugates of quercetin was also demonstrated in a tyrosinase-rich *in vitro* cellular model consisting of B16F10 mouse melanoma cells, indicating that the quinone chemistry of quercetin may also be physiologically relevant in a cellular system (*102*). In support of these results, evidence was found for the formation of covalent adducts of quercetin with cellular protein and DNA during exposure of various cell types to quercetin (*103*).

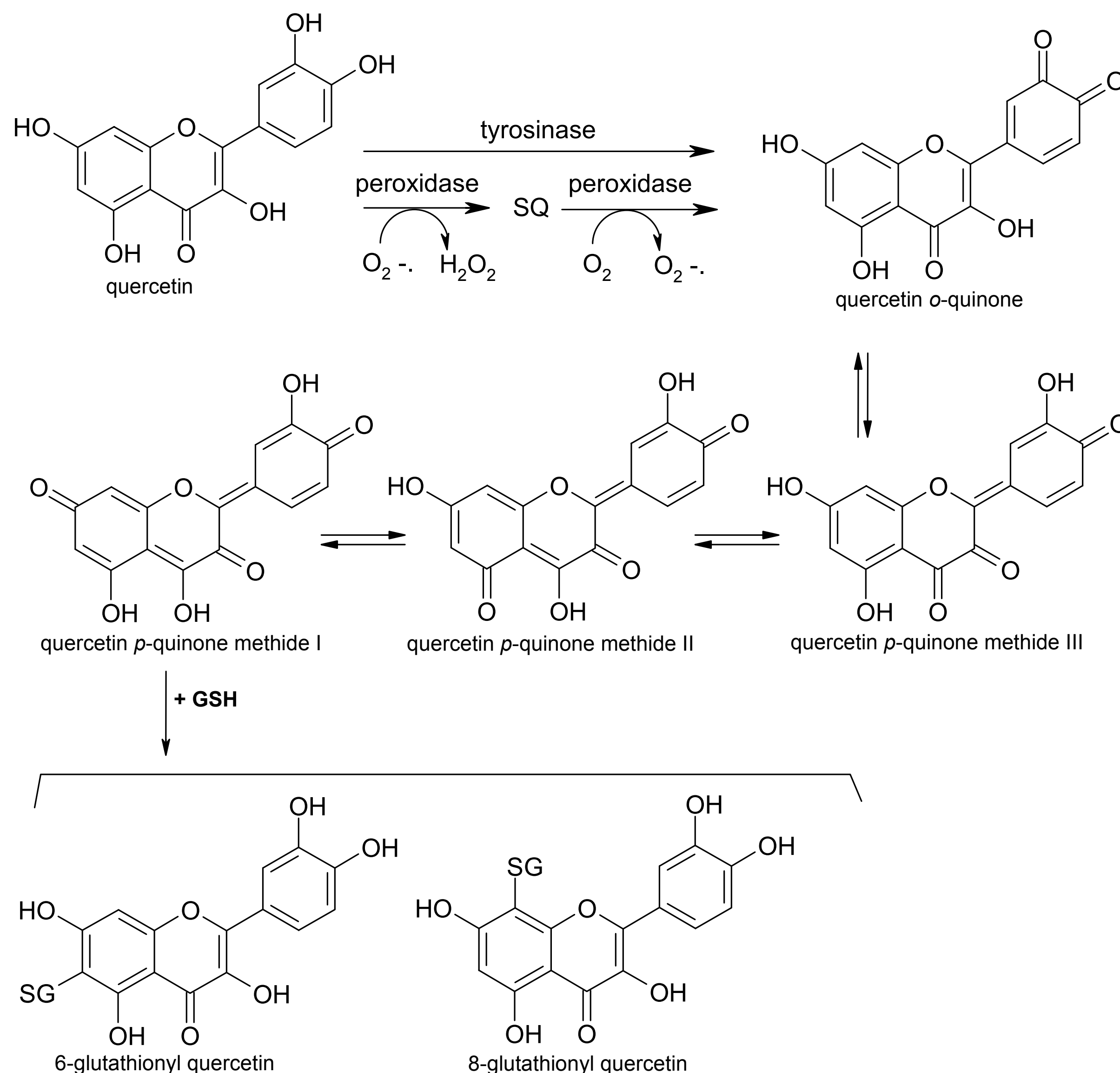

Figure 3 Hypothetical mechanism underlying the formation of 6- and 8-glutathionyl quercetin during the oxidation of quercetin by tyrosinase in the presence of glutathione (*100*). SQ = ortho-semi-quinone anion radical.

3.4 *In vivo* animal studies with quercetin

Although quercetin is genotoxic in bacterial and to a lesser extent in mammalian *in vitro* genotoxicity tests (*81-86*), the putative carcinogenic effects of quercetin have been a matter of debate for over 25 years. A large number of studies has been published, of which the global design and results are summarised in Tables 3, 4, and 5. The tables summarise studies showing respectively no effects (Table 3), protecting effects (Table 4) or stimulating effects (Table 5) of quercetin on the process of carcinogenesis, either or not in the presence of cancer initiating model compounds, such as for example azoxymethane, methylcholanthrene and 7,

Table 3 Summary of *in vivo* studies studying carcinogenic effects of quercetin, in which quercetin did not affect tumor incidences.

Study setup	**Animal**	**Time**	**Amount of quercetin in the diet** [1)]	**Presence initiator/promotor**	**End point(s)**	**Effects of quercetin**	**Reference**
Supplementation	F344 rats (m/f)	540 d	0.1%	-	tumor incidence	No effects	(*108*)
Supplementation	F344 rats (m/f)	64 w	0.1% or 0.2%	-	tumor incidence	No effects	(*109*)
Supplementation	ACI rats (m)	1) 540 d 2) 850 d	1) 1 or 5% 2) 10%	-	tumor incidence	No effects	(*110*)
Role in initiation and/or promotion of 2-stage bladder carcinogenesis +/- promoter/initiator N-butyl-N-(4-hydroxybutyl)-nitrosamine (BHBN)	F344 rats (m)	1) 29 w 2) 33 w	5%	1) Initiator BHBN 0.01% in diet 2) Promotor BHBN 0.001% in diet	histological examination bladder	No initiating nor promoting activity quercetin in 2-stage carcinogenesis of urinary bladder	(*111*)
Supplementation +/- initiation with phenobarbital (PB)	ACI rats (m)	2 w	50 or 500 mg/kg bw /day (IP)	Initiator PB 0.05% in diet	tumor incidence genotoxicity in primary hepatocytes	No effects	(*112*)

[1)] unless adminstered otherwise

Table 4 Summary of *in vivo* studies studying carcinogenic effects of quercetin, in which quercetin reduced tumor incidences.

Study setup	Animal	Time	Amount of quercetin in the diet [1)]	Presence inducer/promotor	End point(s)	Effects of quercetin	Reference
Supplementation	F344 and DuCrj rats (m/f)	112w	1.25% or 5%	-	Examination luminar surfaces of intestines and urinary bladder	Highest dose -Hyperplasic polyps in cecum ↑ (m) [2)] -Foci of cellular alteration liver ↓(f) -Fibroadenomas mammary gland ↓	(*113*)
Supplementation +/- induction with azoxymethane (AOM)	CF1 mice (m/f)	6 w	2% Q	Inducer 5 mg/kg bw, 7 mg/kg bw, and 4 times 10 mg/kg bw (s.c.) for 6 consecutive weeks	Focal areas of dysplasia (FAD) in colonic mucosa	Number and size FAD ↓	(*114*)
Supplementation +/- induction with azoxymethane (AOM)	F344 rats (m)	5 w	500 ppm	Inducer 15 mg/kg bw AOM (s.c.) once per week for 3 weeks	Aberrant crypt foci (ACF)	Number of ACF 48% ↓	(*115*)
Supplementation +/- induction with azoxymethane (AOM)	CF1 mice (f)	9 w	0.5%, 2% or 5% with 5% or 20% corn oil	Inducer 10 mg/kg bw AOM per week for 6 weeks	Tumor incidence focal areas of dysplasia (FAD)	In high fat diet, FAD ↓	(*116*)
Supplementation +/- induction with azoxymethane (AOM)	F344 rats (f)	5 w	2%	Inducer 1 mg/rat AOM (IP) once a week for 3 weeks	Aberrant crypt foci	Total number of ACFs and number of ACFs with >2 crypts ↓	(*117*)
Supplementation +/- induction with dimethylbenza-anthracene (DMBA) or nitrosomethylurea (NMU)	SD rats (f)	21 w	2% or 5%	Inducers: 65 mg/kg bw DMBA single dose (intragastric administration) or 50 mg/kg NMU single dose (IV)	mammary tumors	-DMBA-tumors ↓ dose-dependently; -time to first palpable tumor ↑; -idem for NMU, but weaker effect	(*118*)

[1)] unless administered otherwise
[2)] In contrast with the other parameters measured in this study, this parameter did not point at a protecting effect of quercetin against cancer.

Table 5 Summary of *in vivo* studies studying carcinogenic effects of quercetin, in which quercetin increased tumor incidences.

Study setup	Animal	Time	Amount if quercetin in the diet[1)]	Presence initiator/promotor	End point(s)	Effects of quercetin	Reference
Supplementation	NwAlbino rats (m/f)	58 w	0.1%	-	Tumor incidence	Intestinal tumors↑ Urinary bladder tumors↑	(*119*)
Supplementation	SD- and F344 rats (f)	139 w	1) SD: 0.5% 2) F344: 1%		Hepatic tumors	1) SD: hepatoma ↑ 2) F344: Biliary adenoma ↑	(*120*)
Supplementation	Male and female F344/N rats	2 y	1000-40000 ppm	-	Tumor incidence	After 2 years, males: -renal tubule hyperplasia and neoplasia (adenomas, adenocarcinomas; benign) ↑ -chronic nephropathy -incidence/severity of parathyroid gland hyperplasia↑ dose-related; Females: -Fibroadenomas mammary gland ↓ dose-related	(*104*)
Supplementation +/- initiation with croton oil (CO)	Golden hamster (m/f)	733 d 709 d	1) 10% 2) 1%	1) - 2) Promotor croton oil 1% in diet	tumor incidence	1) forestomach papillomas and cortical adenomas ↑ (n.s.)[2)] 2) forestomach papillomas/papillomatoses (n.s)[2)]	(*121*)
Supplementation +/- induction with azoxymethane (AOM) or dimethylbenza-anthracene (DMBA)	F344 rats (1-2: m) or SD rats (3: f)	1) 46w 2) 10w 3)100d	1) 16.8 and 33.8 g/kg 2) 30 g/kg 3) 20 g/kg	1) 30 mg/kg bw AOM (s.c.) 2) 15 mg/kg bw AOM (s.c.) 3) 12 mg DMBA (diet)	1) Tumor incidence colon 2) Aberrant crypt foci (ACF) 3) Mammary tumors	1) Adenocarcinomas: Incidence and Multiplicity ↑ 2) No effect on ACF 3) Mammary tumors ↓	(*122*)
Supplementation +/- induction with methylcholanthene (MCA)	C57Bl/6 mice (f)	420 d	1) 0-20 mg (i.m) or; 2) 0.1% or 10%	Inducer Ad 1) and 2): 0.1 mg or 1.0 mg inducer MCA (intramuscular)	Subcutaneous sarcomas at site of MCA injection	1) -latency ↓ -lung metastasis ↑ 2) latency ↓	(*123*)

[1)] unless administered otherwise; [2)] n.s. = not significant.

12-dimethylbenza*[α]*anthracene. As can be derived from the results presented, the available data are contradictory, which may be due to differences in animal models and experimental designs used. Therefore, the American Food and Drug Administration (FDA) nominated quercetin for toxicity and carcinogenicity studies in the rat, also because of its wide distribution in foods.

As a consequence, in 1991, the National Toxicology Program (NTP) published a 2-year carcinogenicity study on quercetin, under the direction of the National Institute for Environmental Health Sciences (NIEHS), in compliance with NTP laboratory health and safety requirements. Under the conditions of these 2-year feeding studies, there was evidence for "some carcinogenic activity" of quercetin in male F344/N rats based on an increased incidence of renal tubule cell adenomas. There was no evidence of carcinogenic activity of quercetin in female F344/N rats given the same diet (*104, 105*). Because the abnormalities were observed in exposed male rats only, the suspicion was raised that the carcinogenic effects seen might be related to other toxic effects induced by the quercetin exposure.

Indeed, analogous to chemically induced nephropathy and carcinogenicity induced by other carcinogens in male rats only (*106*), α_{2u}-globulin nephropathy has been suggested to play a role in quercetin-induced renal carcinogenicity in male rats (*107*). At present, α_{2u}-globulin nephropathy and its associated carcinogenicity represent the best characterised non-genotoxic carcinogenic mechanism known. It is caused by the reversible binding of the chemical or of its metabolites to this abundant male rat-specific protein. The chemical-protein complex is less easily degraded than free α_{2u}-globulin by renal lysosomal enzymes, leading to accumulation of α_{2u}-globulin-chemical complexes, cell death and compensatory proliferation. Upon chronic exposure to such chemicals, there is a marked increase in cell proliferation, promoting the development of renal neoplasms. Chemicals that cause α_{2u}-globulin nephropathy have structures that promote the formation of a hydrogen bond with a specific amino acid situated deep in the pocket of α_{2u}-globulin, thereby preventing the rapid digestion of the chemical-protein complex by lysosomal enzymes (*106*). Because α_{2u}-globulin is produced in much lower quantities in female rats as it is in humans, and because no evidence for carcinogenic activity of quercetin was observed in female F344/N rats, it is still questionable whether quercetin might be carcinogenic in man. In the absence of convincing epidemiological data obtained from studies focusing on the compound quercetin itself and not

on food stuffs containing quercetin, no well-funded evaluation of the carcinogenicity of quercetin to man can be made.

4. Bioavailability of quercetin

4.1 Plasma concentrations and tissue distribution

Table 6 shows an overview of reported plasma concentrations of quercetin, for various species and different types of diets. Plasma concentrations in man are in the nanomolar range and can increase up to the micromolar range upon supplementation with quercetin at a dose of 1 g/day for 4 weeks (Table 6). Tissue distribution studies in rats showed that the highest accumulation of quercetin plus metabolites took place in the lungs, amounting to approximately 4 to 15 nmol/g wet tissue depending on the quercetin content of the diet (*124*). The lowest concentrations were detected in brain, white fat and spleen. In pig, the highest quercetin concentrations were detected in liver (6 nmol/g tissue) and kidney (3 nmol/g tissue), whereas brain, heart and spleen showed the lowest concentrations. Pig lung was not analysed (*124*). Bioavailability studies performed with quercetin have recently been reviewed and showed that the rate of elimination of quercetin metabolites is relatively slow, with half-lives ranging from 11 to 28 hours. This could favour accumulation in plasma with repeated intakes (*125*).

Table 6 Plasma concentrations of quercetin.

Species	Dose Source	Plasma concentration[1)]	Reference
Human	Overnight fasting	50-80 nM	(*126, 127*)
Human	64 mg quercetin single dose fried onions	650 nM after 2.9 hours	(*128*)
Human	11 mg/day quercetin for 28 days blueberry/apple juice	6-12 nM	(*129*)
Human	1 g/day quercetin for 28 days supplement	1005 nM	(*130*)
Rat	0.1 % quercetin for 11 weeks 1% quercetin for 11 weeks diet	23 μM 108 μM	(*124*)
Pig	500 mg/kg bw for 3 days diet	1.3 μM	(*124*)

[1)] The plasma concentration represents the sum of all quercetin forms present in plasma.

4.2 Phase II metabolism of quercetin

Most of the plasma concentrations presented in Table 6 were obtained from plasma samples that were treated with a β-glucuronidase and/or sulfatase preparation before analysis, resulting in the hydrolysis of glucuronide and sulfate conjugates (*124, 126, 128-130*). The majority of quercetin circulates in plasma as glucuronide or sulfate conjugates of quercetin or 3'-O-methylquercetin (also called isorhamnetin). Table 7 shows a specification of metabolites found in human (*131*) or rat plasma (*132, 133*).

Table 7 Phase II metabolite pattern of quercetin as determined in rat (*133*) or human (*131*) plasma.

Conjugated form of quercetin (%)[1)]	Rat	Human
Methylated	0	0
Sulfated	13	35
Methylated + sulfated	79	0
Glucuronidated	5	46
Methylated + glucuronidated	4	19

[1)] Percentages are expressed relative to the sum of all quercetin forms present in plasma.

According to these data, the biotransformation reactions of quercetin include mainly phase II metabolic reactions, i.e. methylation by catechol-O-methyltransferase (COMT), sulfation by sulfotransferases (ST) and glucuronidation by UDP-glucuronyltransferases (UGT) (*134*).

COMT methylates quercetin at one of the catechol hydroxyl groups, resulting in the formation of 3'-O- and 4'-O-methylquercetin, also called isorhamnetin and tamarixetin, respectively (*135*). In rats, hepatocytes and kidney cells contain considerable cytosolic COMT activities, whereas enterocytes and lung cells contain a fraction of this activity (*136, 137*). It is remarkable that 4'-O-methylquercetin is almost not found in blood plasma, whereas it is certainly produced in the liver. This may be the result of selective permeability of hepatocyte membranes for one of either forms of quercetin, resulting in the preferential excretion of 4'-O-methylquercetin to the bile. In addition, cytochrome P450 may be responsible for selective demethylation of 4'-O-methylquercetin (*136, 138*).

Cytosolic STs catalyse the sulfation of the hydroxyl groups of quercetin. The ST family comprises various ST enzymes with varying substrate specificities and regioselectivity. Quercetin is especially sulfated by human ST1A1 and 1A3, which are highly expressed in the liver and the intestine respectively. To a smaller extent, ST1E1 and 2A1 also contribute to the

biotransformation of quercetin, both in the liver and the intestine (*139, 140*). Details on the regioselectivity of the sulfation of quercetin by these isoforms have not been described.
Microsomal UGTs catalyse the addition of a glucuronic acid moiety to the hydroxyl groups of quercetin. Four different glucuronides were detected during the glucuronidation of quercetin using microsomal preparations (*141, 142*), identified as 7-, 3-, 3'- and 4'-glucuronosyl quercetin (*141*). The main UGT isoforms responsible for these reactions are UGT1A1 and 1A8, which are abundant in the intestine, whereas the isoform involved in the glucuronidation of quercetin in the liver is UGT1A9 (*141*).

4.3 Fate of quercetin along the gastro-intestinal tract

Because the diet is the primary source of quercetin for humans, the fate of dietary forms of quercetin, mainly quercetin glycosides (*13*) in the major compartments of the gastro-intestinal tract will be discussed in some more detail.

4.3.1 Oral cavity

Recently, Walle and co-workers have demonstrated that quercetin glucosides are hydrolyzed in the human oral cavity by both bacteria and enzyme activity present in the cytosol of damaged epithelial cells, thereby delivering the biologically more active aglycon to the surface of the epithelial cells (*143*). In addition, human oral bacterial isolates also appeared to hydrolyze rutin (quercetin-3-O-rhamnoglucoside), to the aglycon (*144*). These studies illustrate that although quercetin occurs mainly as glycoside in the diet, the aglycon may already be formed to some extent in the oral cavity.

4.3.2 Stomach

Although to date, absorption of dietary forms of quercetin by the human stomach could not be demonstrated (*145*), the rat stomach appeared to be able to absorb quercetin aglycon, in contrast with quercetin glycosides (*146*).

4.3.3 Small intestine

4.3.3.1 Uptake in the small intestine

Because of the hydrophilic character of quercetin glycosides, passive diffusion of the glycosides across the cell membrane of the enterocyte is unlikely to occur. The small intestine is the site of various mechanisms that allow the absorption of quercetin either as its aglycon or

bound to certain types of sugar moieties. Studies with human ileostomy volunteers showed that quercetin aglycon is absorbed with different efficiencies, depending on the quercetin source (*147*). For example, the absorption of quercetin aglycon was approximately 52% from fried onions (containing mainly quercetin-4'-glucoside and quercetin-3,4'-diglucoside), 17% from rutin (quercetin-3-O-rhamnoglucoside) and 24% from quercetin aglycon (*147*). Additional studies demonstrated that conjugation of quercetin to especially a glucose molecule enhances the absorption from the small intestine as compared with the aglycon and conjugation to a rhamnoglucose molecule (like in rutin) considerably decreases the rate and efficiency of absorption. Two different mechanisms may contribute to the intestinal absorption of quercetin and its glycosides, and may provide an explanation for the fact that quercetin glucosides are absorbed faster, result in a higher plasma peak concentration of total quercetin and are more bioavailable than quercetin aglycone or rutin (*148-150*).

A first route that could facilitate absorption of quercetin glycosides from the small intestine involves the luminal deglycosylation of quercetin glycosides by β-glucosidases. This process liberates the aglycone, capable of crossing the enterocyte membrane by passive diffusion. Lactase Phlorizin Hydrolase (LPH) is a β-glucosidase with specific activity towards flavonoid glycosides. Its is situated in the apical membrane of the small intestine (the luminal side of the brush border membrane (*145, 151*). The fact that the absorption of quercetin glucosides in an *in situ* rat small intestine perfusion model was inhibited by a specific LPH inhibitor provided direct evidence for the involvement of LPH in the intestinal absorption of quercetin glucosides (*152*).

A second possible route of absorption of quercetin glycosides may be the involvement of the sodium-dependent glucose transporter-1 (SGLT-1). After first indications for the enhancement of carrier-mediated galactose transport by quercetin glucosides via a sodium-dependent pathway, suggesting the interaction of the glucosides with SGLT-1 (*153*), studies with cell lines transfected with SGLT-1 confirmed that quercetin-4'-glucoside is transported by SGLT-1 across the apical membrane of enterocytes (*154*). These results were corroborated by experiments with rat jejunum tissue in Ussing chambers, clearly indicating a role of SGLT-1 in mucosal uptake of quercetin-3-O-glucoside (*155*).

After the uptake of the flavonoid glucoside into the enterocyte by interaction with SGLT-1, the glucoside may be hydrolysed by intracellular cytosolic β-glucosidase activity (*145, 151*). In support of this possibility, small intestinal cell extracts appeared to hydrolyze quercetin glycosides. Remarkably, the β-glucosidase activity present in the cell homogenates showed

preferential deconjugation of especially quercetin-4'-glucoside, the quercetin form mainly present in onions, and hardly any activity towards rutin (*156, 157*), providing a possible explanation for the relatively high absorption of quercetin from onion, but not of quercetin present in the form of rutin (*147*).

Considering the knowledge available to date, it was concluded that both the LPH and the SGLT-1 play a role in the absorption of quercetin glucosides and that the regioselectivity of the glucosidation is an important determinant of the route of absorption: i.e. quercetin-3-glucoside utilises almost exclusively the LPH hydrolytic pathway, whereas for quercetin-4'-glucoside, uptake by SGLT-1 followed by hydrolysis by intracellular β-glucosidases, as well as hydrolysis by LPH followed by absorption of the aglycone by passive diffusion into the enterocytes are possible routes of uptake into the enterocyte (*158*).

Although SGLT-1 and LPH are generally recognized as important contributors to the absorption of quercetin glucosides, other studies have suggested the involvement of other transporter proteins present in the small intestine in the bioavailability of these dietary components, i.e. Glucose Transporter Isoform 2 (GLUT2) (*155*) and Multidrug Resistance Protein 2 (MRP2) (*159, 160*). In addition, the involvement of Breast Cancer Resistance Protein 1 (Bcrp1/Abcg2) was reported to limit the net intestinal uptake of quercetin in rats. This transporter protein is situated at the apical side of the enterocyte and appears to mediate the efflux of quercetin glucuronides formed intracellular back into the intestinal lumen (*160*).

4.3.3.2 Metabolism by the small intestine

Besides various forms of glycosidase activity present in the small intestine (*145, 151*), the small intestine contains a large variety of microflora catalysing ring fission reactions, leading to the breakdown of the backbone structure of quercetin and to the formation of smaller molecules, including hydroxyphenyl acetic acids (*161*).

Furthermore, after absorption of quercetin in the enterocyte, quercetin is extensively converted to methylated, sulfated and glucuronidated metabolites within the enterocyte, which could be derived from experiments using perfused small intestine segments or intestinal microsomal preparations (*141, 162*). Due to the presence of COMT activity in small intestinal cell homogenates (*137*), methylation of quercetin will probably also take place during the absorption process through the intestinal wall. Figure 4 presents a schematic overview of the processes involved in absorption and metabolism of dietary quercetin in the intestine, with quercetin-4'-glucoside as an example.

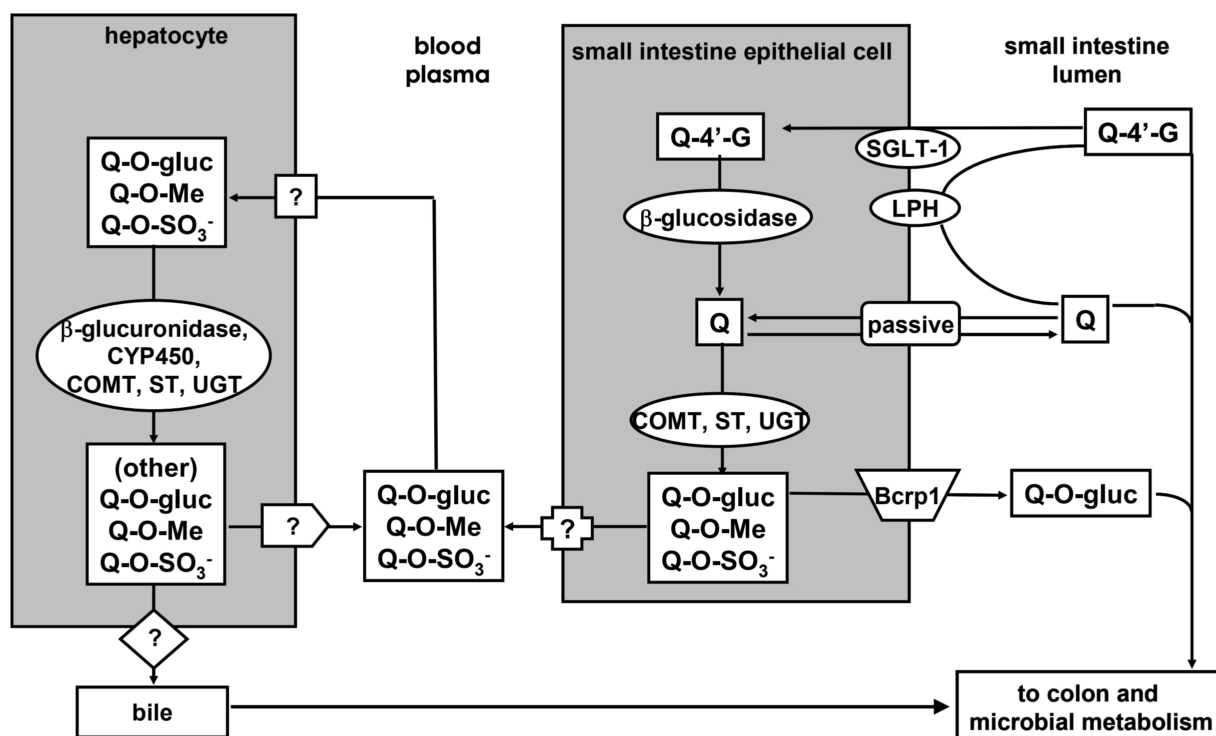

Figure 4 Overview of processes involved in the absorption and metabolism of quercetin-4'-glucoside (Q-4'-G). For explanation, see text.

4.3.4 Colon

Quercetin glycosides that have not been processed and/or absorbed by the small intestine may be transported into the colon. The colonic microflora expresses considerable α,L-rhamnosidase and β,D-glucosidase activity, allowing efficient deglycosylation of quercetin glycosides. In addition to deglycosylation reactions, the microflora present in the colon is also capable of other metabolic reactions including fission of the A- and/or B-ring of quercetin, leading to the formation of lower molecular weight phenolics, including phloroglucinol, 3,4-dihydroxyphenylacetic acid and 3-methyl-4-hydroxybenoic acid (*14, 163*). Due to the presence of microflora with β,D-glucuronidase activity, quercetin glucuronides excreted via the bile can be deglucuronidated, thereby liberating the aglycon and providing possibilities for enterohepatic cycling (*163*). In the colon, both the aglycon and the degradation products of quercetin can be absorbed into the blood stream or excreted via the feces. After absorption by the cells of the colon, quercetin and/or its degradation products may be glucuronidated and to

a smaller extent also methylated by the UGT and COMT activities present in the colonic mucosa (*137*).

4.3.5 Liver

After absorption into the blood by the small intestine or the colon, quercetin phase II conjugates may reach the liver via the portal vein. The liver is generally considered the major metabolising organ in the body. It metabolises flavonoids, including quercetin, to glucuronidated, sulfated and methylated conjugates (*137, 141, 164, 165*). Furthermore, an *in vitro* hepatic model, the cell line HepG2, has been shown to express endogenous β-glucuronidase activity. The cells appeared to absorb and turnover quercetin-7-O- and quercetin-3-O-glucuronide, leading to the formation of methylated glucuronides and quercetin-3'-O-sulfate (*166*). This suggests that, although quercetin reaches the liver as phase II conjugates, intracellular deconjugation and exposure of hepatocytes to the aglycon followed by hepatic phase II conjugation may occur. After processing by hepatocytes, the phase II conjugates of quercetin are excreted into the bile or back into the blood plasma in a process possibly mediated by Multi-Drug Resistant Protein 2 (MRP2) (*166*).

4.3.6 Other organs involved in quercetin metabolism

In addition to the gastro-intestinal tract and the liver, different organs have been reported to possess metabolic activity towards flavonoids. For example, the kidney of rats expresses considerable UGT and COMT activity (*137*). Furthermore, the lung may be an important organ in the metabolism of flavonoids and quercetin in particular. As discussed above, rat lung accumulates considerable amounts of quercetin (*124*). Therefore, the metabolic activity of the lung for quercetin may have consequences for the biological activity of this compound. Both in rats and humans, 30 to 62% of intravenously administered quercetin appeared to be excreted as $^{14}CO_2$ (*167, 168*). In these experiments, quercetin was labelled at the C_4-position. However, although these experiments illustrate that quercetin may be extensively metabolised to smaller molecules, they do not provide any information on the fate of the A- and B-ring. Regarding phase II biotransformation activities, rat lung appears to metabolise flavonoids to glucuronidated and methylated phase II conjugates, at rates respectively comparable to or approximately 20 times lower than the corresponding rates observed in the liver (*137*).

5. Objective and outline of this thesis

Over the course of the years, a large number of beneficial health effects have been ascribed to quercetin, including protection against various diseases and the process of aging. However, the scientific evidence for these health claims, merely based on epidemiologic studies and *in vitro* studies (see Section 2) can be qualified as insufficient. Epidemiological evidence for health effects of fruit- and vegetable components, including flavonoids, derived from associations between dietary intakes and health effects may be considered weak and indirect, because it involves complex matrices comprising thousands of different bioactive compounds. There is a lot of uncertainty about whether the effects seen can be ascribed to a particular nutrient.

Furthermore, besides the traditional difficulties regarding the extrapolation of *in vitro* data obtained at high doses to physiologically relevant conditions representative for the human *in vivo* situation, the *in vitro* studies with quercetin have mostly been performed with the aglycon, whereas bioavailability studies have shown that this is not always the quercetin form present *in vivo*. Internal exposure to dietary quercetin includes exposure to mainly quercetin glycoside conjugates in the pre-absorption phase and a mixture of glucuronide- , sulfate- and methyl conjugates in the post absorption phase (see Section 4). These conjugates of quercetin are known to have altered biological activities as compared to the aglycon, because the number and position of free hydroxyl groups in flavonoids determines their biological activity (*14, 23*).

In addition to the insufficiencies regarding scientific evidence for putative beneficial health effects of quercetin, several reports have been published that pointed at possible damaging aspects of quercetin, including its pro-oxidant chemistry, genotoxicity and presumed cancer-inducing effects (as described in Section 3). Also in the majority of these types ofstudies, the knowledge on physiologically relevant forms of quercetin has not been taken into account.

It is behind this background that the Netherlands Organisation for Health Research and Development decided to finance the project “Benefit-risk evaluation of flavonoids in foods and their use as functional food ingredients” (project no. 014-12-012). The objective of the project was to provide the biological evidence required for the balanced benefit-risk evaluation for the use of flavonoids as functional food ingredients. To achieve the objective, five tasks have been defined, to allow well-funded research in different areas of interest. Because it is impossible to study the family of flavonoids, comprising more than 6000

members (*38*) as a whole, the model compound quercetin was selected as the subject of the studies. The tasks have been defined as follows:

1. Bioanalysis and biokinetics. The purpose of this task is the identification, characterisation and quantification of biokinetics (ADME) and first-pass biotransformation pathways of flavonoids.
2. Toxic risk assessment. The goal of this task is to define the mechanisms and cellular targets for toxicity of flavonoids. Together with task 1, this task defines the hazard characterisation required for a balanced risk assessment.
3. Health benefit assessment. The objective of this task is to define and quantify the mechanisms for beneficial health effects of flavonoids. Together with task 1, this task defines the characterisation of beneficial effects required for a balanced assessment of the beneficial effects.
4. Biomarker development. In this task, the emphasis is laid on the development of new functional biomarkers for positive and negative flavonoid-induced health effects, by means of genomics and proteomics. This may facilitate future benefit-risk assessments of functional food ingredients.
5. Human intervention study. In a human intervention study, the potential risk-reducing and beneficial health effects of supplementation by flavonoids-rich foods will be assessed, to investigate and validate the risk-benefit balances of flavonoids supplementation and the consequences of human genetic polymorphisms in this respect.

The studies described in this thesis contribute to Tasks 1 and 2. The aim of this thesis was to investigate the mechanisms of toxicity of quercetin, with special emphasis on its extensive phase II metabolism. As a start, the regioselectivity of phase II metabolism of quercetin was studied in Chapter 2. An analytical method based on HPLC-DAD, LC-MS and ^{1}NMR was developed for the identification of the phase II metabolites of quercetin and the phase II metabolite pattern of quercetin was identified in a variety of biological *in vitro* models. These studies showed that the catechol group of quercetin was an important target for phase II conjugation. Using the knowledge thus obtained on the regioselectivity of phase II metabolism of quercetin, the consequences of the catechol-*O*-methylation of quercetin for the antioxidant activity and the quinone/quinone methide chemistry of quercetin was investigated in Chapters 3 and 5 respectively.

A second objective of this thesis was to obtain more insight into possible toxic effects of quercetin by studying various mechanisms *in vitro* that might be relevant in the context of carcinogenesis. First, the quinone/quinone chemistry of quercetin in a cellular environment was investigated in Chapters 4 and 5. In Chapter 4, the role of peroxidase and/or tyrosinase-like oxidative enzyme activity in the formation of covalent adducts of quercetin with glutathione, DNA and protein was investigated. Furthermore, the stability of intracellular quercetin DNA adducts in time was studied in more detail. In addition, the consequences of catechol-*O*-methylation of quercetin for the formation of covalent adducts of quercetin with glutathione and DNA was investigated in Chapter 5.
Second, the effect of quercetin on cell proliferation was investigated in Chapter 6, using experimental conditions meeting with the instability and poor solubility of this compound. Subsequently, the involvement of the estrogen receptor in the effects of quercetin on cell proliferation and the role of quercetin as phyto-estrogen was investigated in Chapter 7. Finally, because the effect of quercetin on the proliferation of estrogen receptor-positive cells appeared to show a biphasic dose-response relationship sometimes referred to as hormesis (*169*) Chapter 8 presents a discussion on the definition of hormesis and on implications of hormetic dose-response relationships for risk assessment strategies.
Finally, Chapter 9 summarizes the results of this thesis and presents a risk-benefit evaluation of quercetin for its use as functional food ingredient or food supplement, based on the findings described in this work.

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2

Identification of 14 quercetin phase II mono- and mixed conjugates and their formation by rat and human phase II *in vitro* model systems

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Abstract

In this study, the HPLC, UV-VIS, LC-MS and ^{1}H NMR characteristics of 14 different phase II mono- and mixed conjugates of quercetin were determined, providing a useful tool in the identification of quercetin phase II metabolite patterns in various biological systems. Using these data, the phase II metabolism of quercetin by different rat and human liver and intestine *in vitro* models, including cell lines, S9 samples and hepatocytes, was investigated.

Comparison of quercetin phase II metabolism between rat and human liver and intestine cell lines, S9 and hepatocytes showed considerable variation in the nature and ratios of quercetin conjugate formation. It could be established that the intestine contributes significantly to phase II metabolism of quercetin, especially to its sulfation, that organ-dependent phase II metabolism in rat and man differ significantly and that human inter-individual variation is higher for quercetin sulfation than for glucuronidation or methylation. Furthermore, quercetin conjugation by different *in vitro* models from corresponding origin may differ significantly. The identification of the various mono- and mixed quercetin phase II conjugates revealed significant differences in phase II conjugation by a variety of *in vitro* models and lead to the conclusion that none of the *in vitro* models converted quercetin to a phase II metabolite mixture similar to the *in vivo* plasma metabolite pattern of quercetin. Altogether, the identification of a wide range of phase II metabolites of quercetin as presented in this study allows the determination of quercetin phase II biotransformation patterns, and opens the way for a better-funded assessment of the biological activity of quercetin in a variety of biological systems.

Introduction

Quercetin (Figure 1) is an important member of the class of flavonoid food components. It is abundant in fruits and vegetables, especially apples and onions, in which it is present as a glucoside (*1*). The average western daily diet contains about 16 mg of quercetin (*2*). Many epidemiological studies point at protective effects of flavonoids against cardiovascular diseases and certain forms of cancer (*3-5*). Because of the various health claims, the interest for the use of flavonoids like quercetin as functional food ingredient and/or food supplement is increasing rapidly. Use of these supplements by consumers at the indicated daily levels may increase the daily intake of quercetin 25- to 60-fold.

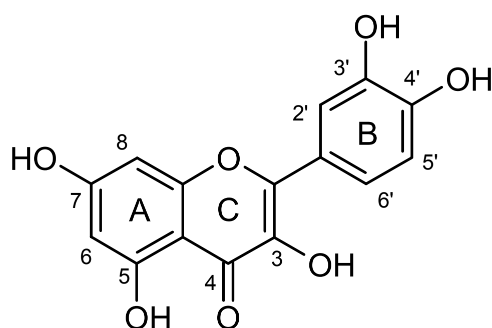

Figure 1 Structure of quercetin and numbering of relevant carbon atoms.

The scientific basis for quercetin-based health claims is a matter of considerable debate (*3-5*), and recent data even indicate a possible toxic pro-oxidative action of quercetin (*6-12*). Both the antioxidant and prooxidant potential of quercetin are related to the number and position of the free hydroxyl groups in the molecule (*9, 13*). Structural features essential for pro-oxidant action (*9, 14*) are similar to those required for efficient antioxidant action (*13*) and reveal the important role of the free hydroxyl moieties of quercetin for these biological activities. Therefore, the regioselectivity of conjugation of these hydroxyl moieties in so-called phase II reactions can be expected to modulate the biological activity of quercetin.

Naturally occurring flavonoids in plants are glycosylated (*2*), but may become deconjugated during passage across the small intestine (*15*) or by bacterial activity in the colon (*16*). *In vivo* studies in man on the bioavailability of quercetin revealed the metabolism of quercetin to methylated, glucuronidated and/or sulfated conjugates (*17-19*). Although regioselectivity of the phase II metabolism of quercetin is a generally recognized determinant of these biological activities (*20*), few of the published reports on the phase II metabolism of quercetin present details on the regioselectivity of phase II metabolism (*21, 22*), mainly because this requires the

unequivocal identification of the many different metabolites. Therefore, the objective of the present study was to identify the structures of the phase II metabolites of quercetin, using HPLC, LC-MS and ^{1}H NMR. Using the newly characterized and identified quercetin metabolites, the nature and regioselectivity of quercetin phase II metabolism in rat and human liver as well as intestine *in vitro* models, including various cell lines, S9 samples and hepatocytes, was investigated.

Materials and methods

Materials

Quercetin was obtained from Acros Organics (New Jersey, USA). 3'-O-Methylquercetin and 4'-O-methylquercetin were purchased from Indofine (Somerville, USA). Dimethylsulfoxide (DMSO) and sulfatase were obtained from Sigma (St. Louis, MO, USA). β-Glucuronidase was from Boehringer (Mannheim, Germany). DMSO-d6 was obtained from ARC Laboratories (Amsterdam, The Netherlands). Ascorbic acid, sodium dihydrogenphosphate, di-sodium hydrogenphosphate and sodium acetate were obtained from Merck (Darmstadt, Germany). HPLC-grade acetonitril was purchased from Lab-Scan Ltd. (Dublin, Ireland). HPLC-grade trifluoroacetic acid was obtained from Baker (Deventer, The Netherlands). Fetal calf serum, gentamicin and Dulbecco's MEM / F12 (1:1) Nutmix (HAM), with or without phenol red, but with 15 mM HEPES, L-glutamine and pyridoxine, and Hank's Balanced Salt Solution (HBSS) were purchased from Gibco Ltd Life Technologies (Paisley, UK).

Rat liver, rat small intestine, human liver and human small intestine microsomes were purchased from BioPredic International (Rennes, France). Rat liver S9 homogenate was obtained from BD Gentest (Woburn, Massachusetts, USA), and rat small intestine, human liver and human small intestine S9 homogenates were purchased from BioPredic International (Rennes, France). Hepatic S9 homogenates from human individuals were obtained from Cellzdirect (Tucson, Arizona, USA; individuals SD002, SD004, SD102, SD103, SD107, SD122, SD120, SD127 and SD134) and from InVitroTechnologies (Baltimore, Maryland, USA; individuals 1007, 1008, 1016, 1020). The cofactors uridine 5'-diphosphoglucuronic acid (UDPGA), S-adenosylmethionine (SAM) were obtained from Boehringer (Mannheim, Germany), whereas 3'-phosphoadenosine 5'-phosphosulfate (PAPS) was purchased from Fluka (Zwijndrecht, The Netherlands). Cofactor concentrations used in these studies were similar to those in other studies (*21, 22*) and were tested to be saturating (data not shown). Since saturating cofactor concentrations are used that were at least 100-fold higher than the

concentration of quercetin in the incubations (see below), the metabolite patterns are not influenced by the amount or concentration of the cofactors.
Human cell lines HT29 (colon carcinoma) and HepG2 (hepatocellular carcinoma) and rat cell lines DHD/K12/TRb (colon carcinoma), H4IIEwt (hepatocellular carcinoma) and IEC-6 (small intestine normal epithelium) were purchased from the American Type Culture Collection (Manassas USA), or from the European Collection of Cell Cultures (ECACC). Cryopreserved rat and human hepatocytes as well as their thawing and culture medium were purchased from BioPredic International (Rennes, France).

Methylation of quercetin

Methylation of quercetin was investigated using S9 homogenates from rat and human liver and small intestine. Incubation mixtures (final volume 200 μL) consisted of 0.1 M potassium phosphate pH 7.5, 1 mM vitamin C to stabilize quercetin (*10*), 2.5 mg/mL S9 homogenate protein and 40 μM quercetin from a 16 mM stock solution in DMSO. The reaction was started by the addition of 10 mM SAM from a 50 mM stock solution in nanopure. Duplicate samples were incubated in a 37°C water bath for 30 minutes, during which methylation of quercetin was linear in time (data not shown). To stop the reaction, one volume of ice cold methanol was added to the mixture. Samples were stored at -80°C until analysis. Quantification of quercetin was performed on the basis of the peak area in the HPLC chromatogram at 370 nm. Total metabolite area was compared to the calibration curve made for quercetin, in the assumption that the molar absorption coefficient of the phase II metabolites of quercetin and of the aglycon are similar.

Sulfation of quercetin

To study the sulfation of quercetin, the incubations were carried out similar to those described above for the methylation of quercetin, except that the reaction was started by the addition of 4 mM PAPS from a 20 mM stock solution in nanopure, instead of by the addition of SAM. The sulfation of quercetin was linear for at least 30 min under the conditions tested (data not shown).

Sulfation of methylated derivatives of quercetin

S9 homogenate from rat liver was used to study the regioselectivity of sulfation of 3'-O-methylquercetin (isorhamnetin) and 4'-O-methylquercetin (tamarixetin). Incubations were

carried out similar to those for sulfation of quercetin, with some minor modifications; protein concentration was 1 mg/mL and the substrates were added at 40 μM final concentration from a 4 mM stock solution in DMSO. The sulfation of 3'-O- and 4'-O-methylquercetin was linear for at least 30 min under the conditions tested (data not shown).

Glucuronidation of methylated derivatives of quercetin

Microsomes from rat and human liver were used to study the regioselectivity of the glucuronidation of 3'-O-methylquercetin and 4'-O-methylquercetin. Incubation mixtures (final volume 200 μL) consisted of 0.1 M Tris-HCl pH 7.5, 1 mM vitamin C, 0.05 or 0.25 mg/mL microsomal protein and 80 μM 3'-O-methylquercetin or 4'-O-methylquercetin from a 4 mM stock solution in DMSO. In incubations with 3'-O-methylquercetin, 0.25 mg/mL microsomal protein was used, whereas in incubations with 4'-O-methylquercetin, 0.05 mg/mL microsomal protein was used to obtain linear metabolite formation in time. The reaction was started by the addition of 10 mM UDPGA from a 50 mM stock solution in nanopure. Duplicate samples were incubated in a 37°C water bath for 30 minutes, a time interval during which glucuronidation of 3'-O-methylquercetin and 4'-O-methylquercetin was linear in time (data not shown). To stop the reaction, one volume of ice cold methanol was added to the mixture and the samples were frozen at -80°C until analysis by HPLC.

Enzymatic deconjugation

To confirm the nature of glucuronosyl or sulfate conjugates, samples were treated with β-glucuronidase or sulfatase/β-glucuronidase. For β-glucuronidase incubations, 10 μL sample was added to 90 μL 200 mM potassium phosphate pH 6.2 and after addition of 4 μL β-glucuronidase solution (0.8 units) the mixture was incubated for 1 h at 37°C. To hydrolyze both glucuronide and sulfate conjugates, medium samples were treated with sulfatase (containing β-glucuronidase activity). In short, 10 μL sample was added to 90 μL 0.1 M sodium acetate pH 5.2 and 4 μL sulfatase/β-glucuronidase solution (0.8 units) and incubated at 37°C for 18 hours. After the incubation, the mixtures were stored at -80°C until analysis.

Phase II metabolism of quercetin by S9 homogenates from different origin

S9 homogenates from rat and human liver and small intestine were used to study the species- and organ dependent differences in rate, type and regioselectivity of quercetin phase II

metabolism. Incubation mixtures (final volume 500 μL) consisted of 0.1 M potassium phosphate pH 7.5, 1 mM vitamin C, 1 mg/mL S9 homogenate protein, 10 mM SAM from a 50 mM stock solution in buffer, 10 mM UDPGA from a 50 mM stock solution in buffer and 4 mM PAPS from a 20 mM stock solution in buffer. The reaction was started by the addition of 40 μM quercetin from an 8 mM stock solution in DMSO. Duplicate samples were incubated in a 37°C water bath for 15 minutes. To stop the reaction one volume of ice cold methanol was added and the samples were stored at -80°C until analysis.

Cell culture

Cells were cultured in a humidified atmosphere at 37°C and 5% CO2, in 75-cm^2 culture flasks (Corning Inc., Corning, NY, USA) in Dulbecco's MEM/F12 NutMix (HAM) with phenol red, containing 15 mM HEPES, L-glutamine and pyridoxine, and supplemented with 10% fetal calf serum (FCS) and 50 μg/mL gentamicin.

Cell viability

In order to exclude cytotoxic effects during the metabolism studies, cell viability was measured using the LDH-leakage method (*23*) with some minor modifications for 96-wells plates (*24*). To this end confluent flasks were trypsinized and cell suspensions thus obtained were diluted in culture medium to $2*10^5$ cells/mL, plated in 96-wells plates (100 μL/well) (Greiner LaborTechnik, Frickenhausen, Germany) and cells were allowed to attach for 24 hours in the incubator. Then, the culture medium was removed and cells were washed with 100 μL/well HBSS after which they were exposed to exposure media. The exposure media consisted of 1 mM ascorbic acid in Dulbecco's MEM/F12 NutMix (HAM) medium, containing phenol red, 15 mM HEPES, L-glutamine and pyridoxine, but without FCS, supplemented with quercetin from a 200 times concentrated stock solutions in DMSO. Ascorbic acid was dissolved in culture medium. After sterilization using Schleicher & Schuell FP30/0.2CA-S filters (Dassel, Germany), quercetin was added. Cells were exposed to 100 μL medium with final quercetin concentrations up to 100 μM, in a humidified atmosphere at 37°C and 5% CO_2 for 24 hours.

Metabolism studies with cell lines

For metabolism studies, cells were diluted to $2*10^5$ cells/mL in culture medium and plated in 24-wells plates (0.5 mL/well) (Costar Corning Inc., Corning, NY, USA). After 24 hours of attachment at 37°C, medium was removed and cells were washed with 0.5 mL/well HBSS. Then, exposure medium was added to the cells. Exposure medium, prepared as described above, consisted of 1 mM ascorbic acid in Dulbecco's MEM/F12 NutMix (HAM) without phenol red, containing 15 mM HEPES, L-glutamine and pyridoxine, and supplemented with 40 μM quercetin from a 200 times concentrated stock solution in DMSO. Control incubations were exposed to 0.5% DMSO in medium containing ascorbic acid. Cells were exposed in duplicate and samples were taken up to 15 hours after starting the incubation at 37°C and 5% CO_2 in a humidified atmosphere. Samples were stored at -80°C until analysis.

Metabolism studies with cryopreserved rat and human hepatocytes

Rat or human cryopreserved hepatocytes were transferred immediately after thawing at 37°C into 7 mL/vial Leibovitz L15 medium with Glutamax I, supplemented with 0.6 M glucose and 10 mM HEPES, and were kept at room temperature for 2 minutes. Then, the homogenized cell suspension was centrifuged at 1000 rpm for 1 min at room temperature and the supernatant was removed. The cell pellet was gently resuspended to a final cell concentration of 1×10^6 cells/mL in Williams E medium with Glutamax I, supplemented with 100 IU/mL penicillin, 100 μg/mL streptomycin, 4 μg/mL bovine insulin and 50 μM hydrocortisone hemi-succinate, after which 1 mM vitamin C was added from a 100 mM filter-sterilized stock solution in nanopure to stabilize quercetin (*10*). Cells were plated in 96-wells plates and exposed to 40 μM quercetin from a 200 times concentrated stock solution in DMSO. Control incubations containing 0.5% DMSO were performed. Rat and human hepatocytes were incubated for respectively 2 and 6 hours at 37°C in a humidified atmosphere with 5% CO_2. During this period, phase II metabolite formation was linear in time (data not shown). Samples were stored at -80°C until HPLC analysis.

Phase II metabolism of quercetin by hepatic S9 homogenates from 13 human individuals

Hepatic S9 homogenates from 13 human individuals were used to study the interindividual differences in rate, nature and regioselectivity of quercetin phase II metabolism. Non-smoking

individuals were selected based on their medical history, showing the absence of drug or alcohol use. Incubation mixtures (final volume 200 μL) were of similar composition as those described above for the study of inter-species and inter-organ differences in phase II metabolism of quercetin. Incubation time was 30 min at 37°C.

HPLC analysis

HPLC was performed on a Waters M600 liquid chromatography system, using an Alltima C18 5U column (4.6 x 150 mm; Alltech, Breda, The Netherlands). Before injection, incubation mixtures were centrifuged for 4 minutes at 14000 rpm. In a typical run, aliquots of 10 μL of the supernatant were injected. Samples were eluted at a flow of 1 mL/min starting at 20% acetonitril in nanopure water containing 0.1% trifluoroacetic acid, going to 25% acetonitril in 15 minutes, to 35% acetonitril in 5 minutes, keeping this percentage for 15 minutes, followed by an increase to 80% acetonitril in 2 minutes, keeping this percentage for 1 minute, after which it was decreased to 0% acetonitril in 1 minute. This was kept for 1 minute, after which the column was re-equilibrated at the initial conditions. Detection was performed between 220 and 445 nm using a Waters 996 photodiode array detector. Chromatograms presented are based on detection at 370 nm. The limit of detection of this HPLC method for quantification of quercetin and its metabolites was 0.1 μM (injection volume 10 μL).

To collect the different metabolites for product identification by ^{1}H NMR, similar incubations and HPLC runs as described above were performed, except for the fact that the substrate was added three times to the incubation mixtures followed each time by 30 min incubation at 37°C to achieve a higher yield of the different conjugates. Additionally, before injecting the incubation mixture onto the HPLC column using a 50 μL injection loop, samples were concentrated by freeze-drying and dissolved in 75 μL 1 mM vitamin C in nanopure. Product peaks of successive runs were collected, pooled, freeze-dried and dissolved in DMSO-d6 for ^{1}H NMR analysis.

^{1}H NMR analysis

The different conjugates were characterized by ^{1}H NMR using a Bruker DPX 400 MHz. Sulfate conjugates of 3'-O-methylquercetin and 4'-O-methylquercetin were measured using a Bruker AMX 500 MHz NMR spectrometer. A 45° pulse angle and a 1.61 s acquisition time

were used (10200 Hz sweep width, 32 K data points). Spectra were obtained at 25°C. The data were processed using an exponential multiplication of 0.5 or 1.0 Hz and zero filling to 64 K data points. Resonances are reported relative to DMSO at 2.50 ppm.

LC-MS analysis

To identify the nature of the various metabolites, incubation samples were also analyzed by LC-MS. Mass spectrometric analysis was performed using a Finnigan MAT 95 equipment (San Jose, CA, USA) in the negative electrospray mode using a spray voltage of 4.5 kV and a capillary temperature of 180°C with nitrogen as sheath and auxillary gas. For LC, an Alltima C18 5U column (2.1 x 150 mm; Alltech, Breda, The Netherlands) was used. Samples were eluted at a flow of 0.2 mL/min, using a gradient that consisted of 0.1% acetic acid in nanopure, containing 20% acetonitril for 7 min, increasing to 35% acetonitril in 5 min, keeping this percentage for 15 min, increasing to 80% acetonitril in 2 min and finally keeping this percentage for 1 min. Then, the column was re-equilibrated at the initial conditions.

The sulfate metabolites of 3'- and 4'-O-methylquercetin were measured using a Waters/Micromass q-Tof with direct injection in negative mode. The measured masses of the four main metabolites (two sulfated 3'-O-methylquercetin and two sulfated 4'-O-methylquercetin derivatives) were all within 5 ppm of the theoretical expected mass of 395.0073 Da $C_{16}H_{11}O_{10}S$, in negative mode.

Results

Identification of mono-conjugated metabolites of quercetin

Methylation Figure 2A shows the HPLC chromatogram of the incubation of rat liver S9 homogenate with quercetin and the cofactor SAM and reveals the formation of two metabolites with retention times of 30.4 min and 30.8 min. The HPLC, UV-VIS and LC-MS characteristics of the two metabolites are given in Table 1. LC-MS analysis of these metabolite peaks revealed an m/z ratio of 315 for both peaks, confirming that the metabolites are methylated derivatives of quercetin. The metabolite with a retention time of 30.4 min co-eluted with commercially available 3'-O-methylquercetin. The metabolite with a retention time of 30.8 min co-eluted with 4'-O-methylquercetin. Table 1 also summarizes the ^{1}H NMR chemical shift values of the methylated metabolites of quercetin as well as of quercetin. Identification of the various ^{1}H NMR resonances has been achieved on the basis of chemical

shift values and the splitting patterns. Based on the changes in chemical shift values of the aromatic protons of the metabolite compared to quercetin, as described previously for the identification of the regioselectivity of the glucuronidation of quercetin (*21*), the metabolite with a retention time of 30.4 min can be identified as 3'-O-methylquercetin, also called isorhamnetin. Similarly, the metabolite eluting at a retention time of 30.8 min can be identified as 4'-O-methylquercetin, also called tamarixetin.

Sulfation Figure 2B shows the HPLC chromatogram of the incubation of rat liver S9 homogenate with quercetin and the cofactor PAPS and reveals the formation of two major metabolites with retention times of 10.1 and 16.3 min. Table 1 summarizes the HPLC, UV-VIS, LC-MS and the ^{1}H NMR chemical shift values of the two sulfated metabolites. LC-MS analysis revealed for both metabolites an m/z ratio of 381, confirming that the metabolites are mono-sulfated derivatives of quercetin. The ^{1}H NMR data (Table 1) reveal large shifts in the ^{1}H resonance frequencies of H6 and H8. It can therefore be concluded that the metabolite with a retention time of 10.1 min can be identified as quercetin-7-O-sulfate. Furthermore, based on a similar argumentation, the metabolite with a retention time of 16.3 min can be identified as quercetin-3'-O-sulfate.

Glucuronidation Figure 2C shows the HPLC chromatogram of the incubation of rat liver microsomes with quercetin and the cofactor UDPGA. The identification of mono-glucuronidated metabolites of quercetin has been performed previously (*21*). For comparison and to give a complete overview of all monoconjugated quercetin phase II metabolites, the HPLC, UV-VIS, LC-MS, and ^{1}H NMR characteristics of the monoglucuronidated metabolites of quercetin are also included in Table 1.

Table 1 HPLC, UV-VIS, LC-MS and ^{1}H NMR data of the metabolites formed in the incubation mixture of microsomes or S9 homogenates with quercetin and SAM, PAPS or UDPGA[1].

Compound	UV-VIS[2] (nm)	m/z LC-MS (neg. mode)	^{1}H-NMR (ppm) H6	H8	H5'	H2'	H6'
Quercetin	254.5- -371.0	301	6.18 J_{H6-H8}= 2.1 Hz	6.40 J_{H8-H6}= 2.1 Hz	6.87 $J_{H5'-H6'}$= 8.5 Hz	7.67 $J_{H2'-H6'}$= 2.1 Hz	7.53 $J_{H6'-H2'}$= 2.1 Hz $J_{H6'-H5'}$= 8.5 Hz
Methylation							
Metabolite t_R 30.4 min	254.5- -371.0	315	6.19 (+0.01) J_{H6-H8}= 1.7 Hz	6.48 (+0.08) J_{H8-H6}= 1.7 Hz	6.95 (+ 0.08) $J_{H5'-H6'}$= 8.4 Hz	7.75 (+0.08) $J_{H2'-H6'}$= 1.7 Hz	7.69 (+0.16) $J_{H6'-H2'}$= 1.7 Hz $J_{H6'-H5'}$= 8.4 Hz
Metabolite t_R 30.8 min	254.5- -367.4	315	6.19 (+0.01) J_{H6-H8}= 1.2 Hz	6.42 (+0.02) J_{H8-H6}= 1.2 Hz	7.09 (+0.22) $J_{H5'-H6'}$= 8.7 Hz	7.66 (-0.01) $J_{H2'-H6'}$= 1.9 Hz	7.65 (+0.12) $J_{H6'-H2'}$= 1.9 Hz $J_{H6'-H5'}$= 8.7 Hz
Sulfation							
Metabolite t_R 10.1 min	254.5- -371.0	381	6.49 (+0.31) J_{H6-H8}= 2.0 Hz	7.00 (+0.60) J_{H8-H6}= 2.0 Hz	6.89 (+0.02) $J_{H5'-H6'}$= 8.4 Hz	7.69 (+0.02) $J_{H2'-H6'}$= 2.0 Hz	7.62 (+0.09) $J_{H6'-H2'}$= 2.0 Hz $J_{H6'-H5'}$= 8.4 Hz
Metabolite t_R 16.3 min	247.4-265.1-364.3	381	6.18 (+0.00) J_{H6-H8}= 1.9 Hz	6.43 (+0.03) J_{H8-H6}= 1.9 Hz	6.98 (+0.11) $J_{H5'-H6'}$= 8.7 Hz	8.03 (+0.36) $J_{H2'-H6'}$= 1.9 Hz	7.85 (+0.32) $J_{H6'-H2'}$= 1.9 Hz $J_{H6'-H5'}$= 8.7 Hz

[1] The ppm changes of the ^{1}H resonances of the protons of the conjugates compared to the ^{1}H resonances of the protons of quercetin are put between brackets.
[2] The symbol -s- in the UV-VIS signals indicates the presence of a shoulder; the symbol - - indicates the absence of a shoulder.
[3] NMR data and identification taken from Boersma *et al.* (*21*).
[4] ^{1}H NMR identification of metabolites was hampered by instability, preventing identification of the chemical shift values.

Table 1 (continued) HPLC, UV-VIS, LC-MS and ^{1}H NMR data of the metabolites formed in the incubation mixture of microsomes or S9 homogenates with quercetin and SAM, PAPS or UDPGA[1].

Compound	UV-VIS[2] (nm)	m/z LC-MS (neg. mode)	^{1}H-NMR (ppm) H6	H8	H5'	H2'	H6'
Quercetin	254.5- -371.0	301	6.18 J_{H6-H8}= 2.1 Hz	6.40 J_{H8-H6}= 2.1 Hz	6.87 $J_{H5'-H6'}$= 8.5 Hz	7.67 $J_{H2'-H6'}$= 2.1 Hz	7.53 $J_{H6'-H2'}$= 2.1 Hz $J_{H6'-H5'}$= 8.5 Hz
Glucuronidation (21) [3]							
3-O-glucuronosyl quercetin[4] t_R 7.3 min	254.5- -354.5	477	-	-	-	-	-
7-O-glucuronosyl quercetin t_R 7.2 min	254.5- -367.4	477	6.40 (+ 0.22) J_{H6-H8}= 1.9 Hz	6.75 (+ 0.35) J_{H8-H6}= 1.9 Hz	6.89 (+ 0.02) $J_{H5'-H6'}$= 8.4 Hz	7.70 (+ 0.03) $J_{H2'-H6'}$= 1.9 Hz	7.57 (+ 0.04) $J_{H6'-H2'}$= 1.9 Hz $J_{H6'-H5'}$= 8.4 Hz
3'-O-glucuronosyl quercetin t_R 15.1 min	259.9 -s- 367.4	477	6.16 (- 0.02) n.d.	6.59 (+ 0.19) n.d.	6.96 (+ 0.09) $J_{H5'-H6'}$= 8.3 Hz	7.98 (+ 0.31) n.d.	7.90 (+ 0.37) $J_{H6'-H5'}$= 8.3 Hz n.d.
4'-O-glucuronosyl quercetin t_R 13.5 min	254.5 -s- 364.3	477	6.18 (+ 0.00) J_{H6-H8}= 1.9 Hz	6.45 (+ 0.05) J_{H8-H6}= 1.9 Hz	7.24 (+ 0.37) $J_{H5'-H6'}$= 8.7 Hz	7.70 (+ 0.03) $J_{H2'-H6'}$= 1.9 Hz	7.60 (+ 0.07) $J_{H6'-H2'}$= 1.9 Hz $J_{H6'-H5'}$= 8.7 Hz

[1] The ppm changes of the ^{1}H resonances of the protons of the conjugates compared to the ^{1}H resonances of the protons of quercetin are put between brackets.
[2] The symbol -s- in the UV-VIS signals indicates the presence of a shoulder; the symbol - - indicates the absence of a shoulder.
[3] NMR data and identification taken from Boersma *et al.* (*21*).
[4] ^{1}H NMR identification of metabolites was hampered by instability, preventing identification of the chemical shift values.

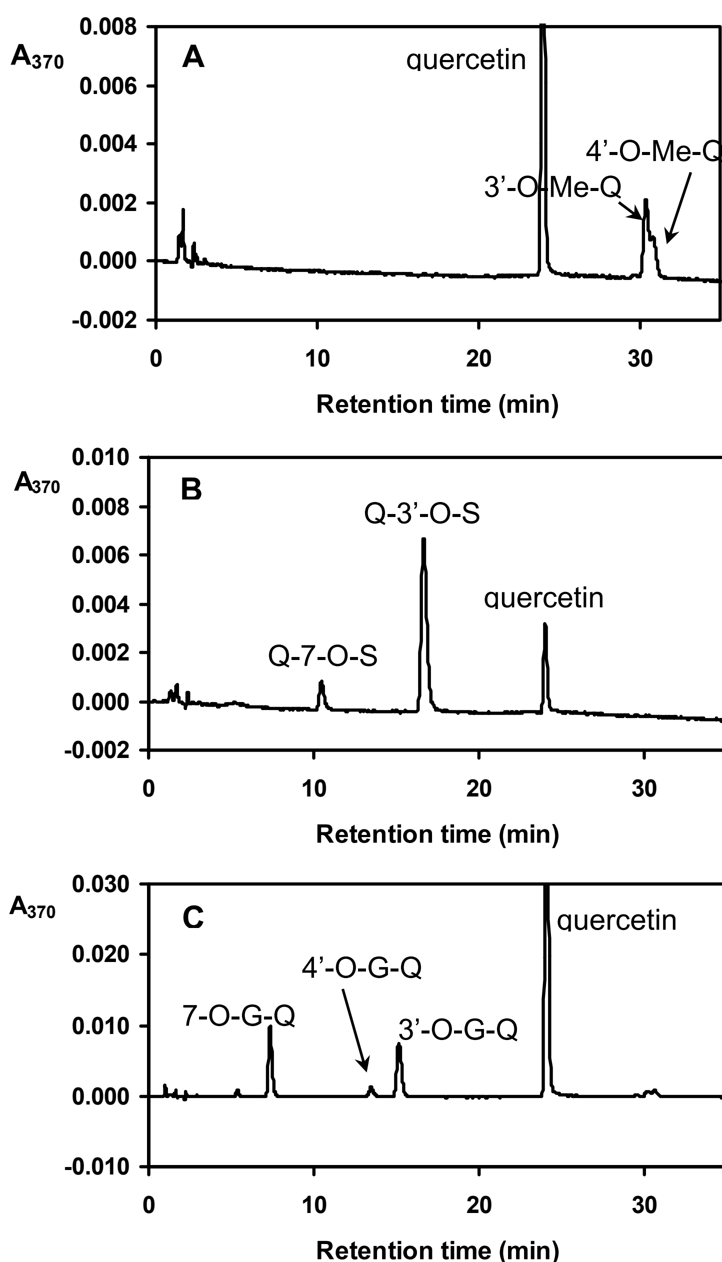

Figure 2 HPLC elution patterns of the incubation mixtures of quercetin with (A) rat liver S9 and SAM, (B) with rat liver S9 and PAPS or (C) with rat liver microsomes and UDPGA after 30-minute incubation at 37 °C. Q = quercetin, Me = methyl, S = sulfate, G = glucuronide.

Identification of sulfated metabolites of 3'-O-methylquercetin

Figure 3A shows the HPLC chromatogram of the incubation of rat liver S9 homogenate with 3'-O-methylquercetin and the cofactor PAPS and reveals the formation of two metabolites with retention times of 17.2 min and 18.1 min. Table 2 summarizes the HPLC, UV-VIS and LC-MS data of the sulfated metabolites. LC-MS analysis of these metabolites shows an m/z

ratio of 395 to both peaks, confirming that the metabolites formed are mono-sulfated metabolites of 3'-O-methylquercetin.

The ^{1}H NMR chemical shift values of the metabolite with a retention time of 18.1 min (Table 2) identify this metabolite as 3'-O-methylquercetin-7-O-sulfate.

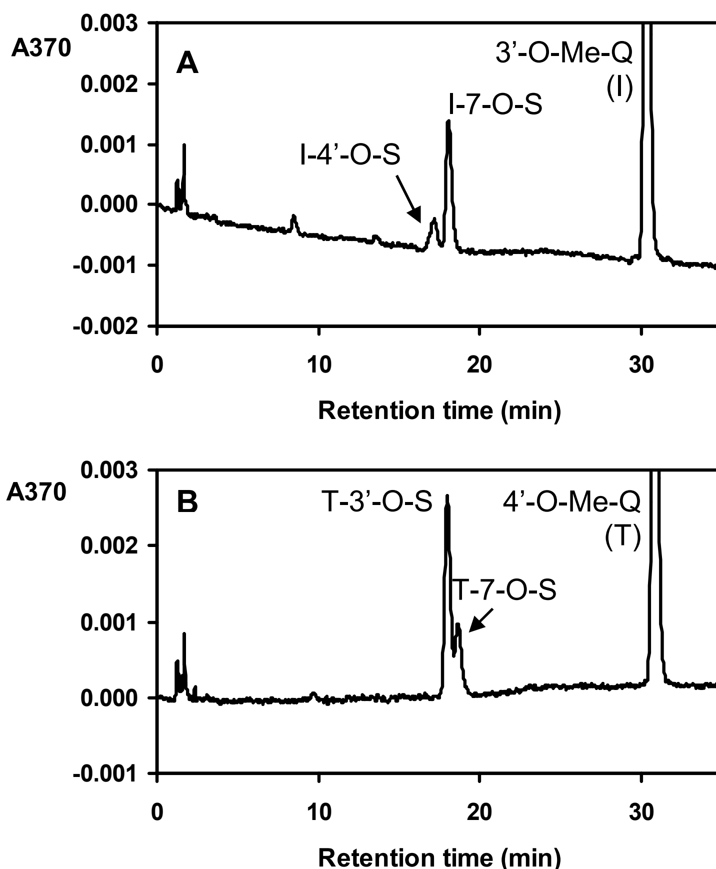

Figure 3 HPLC elution patterns of the incubation mixtures of rat liver S9 homogenate with PAPS and (A) 3'-O-methylquercetin or (B) 4'-O-methylquercetin after 30-minute incubation at 37 °C. Q = quercetin, Me = methyl, I = 3'-O-methylquercetin (isorhamnetin), T = 4'-O-methylquercetin (tamarixetin), S = sulfate.

^{1}H NMR analysis of the metabolite with a retention time of 17.2 min was hampered by instability of the compound resulting in low signal to noise ratios in the NMR spectra. However, the signals could be tentatively assigned to the aromatic protons, as shown in Table 2. Based on these assignments, a shift of + 0.68 ppm could be observed for H5' compared with the spectrum of 3'-O-methylquercetin, whereas the signals from the aromatic protons in the A-ring, H6 and H8 remained fairly unchanged. These results point at sulfation in the B-ring with the H5' proton *ortho* to

Table 2 HPLC, UV-VIS, LC-MS and ^{1}H NMR data of the metabolites formed in the incubation mixture of microsomes or S9 homogenates with 3'-O-methylquercetin and PAPS or UDPGA[1)].

Compound	UV-VIS[2)] (nm)	m/z LC-MS (M-1)	^{1}H-NMR (ppm) H6	H8	H5'	H2'	H6'
3'-O-methylquercetin	254.5- -371.0	315	6.19 $J_{H6\text{-}H8}$= 1.7 Hz	6.48 $J_{H8\text{-}H6}$= 1.7 Hz	6.95 $J_{H5'\text{-}H6'}$= 8.4 Hz	7.75 $J_{H2'\text{-}H6'}$= 1.7 Hz	7.69 $J_{H6'\text{-}H2'}$= 1.7 Hz $J_{H6'\text{-}H5'}$= 8.4 Hz
Sulfation							
Metabolite[3)] t_R 17.2 min	250.9-265.1-367.3	395	6.19 (+0.00) n.d.	6.45 (-0.03) n.d.	7.63 (+0.68) n.d.	7.75 (+0.00) n.d.	7.68 (–0.01) n.d.
Metabolite[4)] t_R 18.1min	254.5- -371.4	395	6.53 (+0.34) n.d.	7.00 (+0.52) n.d.	6.93 (-0.02) n.d.	7.77 (+0.02) n.d.	7.73 (+0.04) n.d.
Glucuronidation							
Metabolite[5)] t_R 12.1 min	254.5- -361.2	491	-	-	-	-	-
Metabolite t_R 13.5 min	254.5- - 367.4	491	6.45 (+0.26) $J_{H6\text{-}H8}$= 1.6 Hz	6.85 (+0.37) $J_{H8\text{-}H6}$= 1.6 Hz	6.96 (+0.01) $J_{H5'\text{-}H6'}$= 8.4 Hz	7.79 (+0.04) $J_{H2'\text{-}H6'}$= 1.6 Hz	7.73 (+0.04) $J_{H6'\text{-}H2'}$= 1.6 Hz $J_{H6'\text{-}H5'}$= 8.4 Hz
Metabolite t_R 16.1 min	250.9-s-361.2	491	6.20 (+0.01) $J_{H6\text{-}H8}$= 1.6 Hz	6.52 (+0.04) $J_{H8\text{-}H6}$= 1.6 Hz	7.25 (+0.30) $J_{H5'\text{-}H6'}$= 8.1 Hz	7.79 (+0.04) $J_{H2'\text{-}H6'}$= 1.6 Hz	7.76 (+0.07) $J_{H6'\text{-}H2'}$= 1.6 Hz $J_{H6'\text{-}H5'}$= 8.1 Hz

[1)] The ppm shift difference of the protons in the conjugates compared to the same protons in 3'-O-methylquercetin are put between brackets.
[2)] The symbol -s- in the UV-VIS signals indicates the presence of a shoulder; the symbol - - indicates the absence of a shoulder.
[3)] ^{1}H NMR identification of metabolites was hampered by instability, resulting in tentative assignment of the ^{1}H NMR signals.
[4)] ^{1}H NMR identification of metabolites was hampered by instability, preventing precise determination of coupling constants.
[5)] ^{1}H NMR identification of metabolites was strongly hampered by instability, preventing identification of the chemical shift values.

the sulfated group (resulting in chemical shift effects) while the H2' and H6' are *meta* to the sulfated group (resulting in almost no effect on the chemical shift values). The metabolite eluting at 17.2 min can therefore be tentatively identified as 3'-O-methylquercetin-4'-O-sulfate.

Identification of sulfated metabolites of 4'-O-methylquercetin

Figure 3B shows the HPLC chromatogram of the incubation of rat liver S9 homogenate with 4'-O-methylquercetin and with the cofactor PAPS and reveals the formation of two metabolites with retention times of 17.9 and 18.6 min. Table 3 summarizes the HPLC, UV-VIS and LC-MS data of the metabolites. LC-MS analysis of these metabolites attributes an m/z ratio of 395 to both peaks, confirming that the metabolites formed are mono-sulfated metabolites of 4'-O-methylquercetin.

The changes in ^{1}H NMR chemical shifts of the aromatic protons of the metabolite with a retention time of 17.9 min (Table 3) compared to 4'-O-methylquercetin identify this metabolite as 4'-O-methylquercetin-3'-O-sulfate. Following a similar argumentation, the metabolite with retention time 18.1 min can be identified as 4'-O-methylquercetin-7-O-sulfate.

Identification of glucuronidated metabolites of 3'-O-methylquercetin

Figure 4 A and B show the HPLC chromatograms of incubations of rat liver (A) and human liver (B) microsomes with 3'-O-methylquercetin and UDPGA, revealing the formation of three metabolites of 3'-O-methylquercetin, with retention times of 12.1, 13.5 and 16.1 min. The HPLC, UV-VIS and LC-MS data of the metabolites are listed in Table 2. Incubation of the samples with β-glucuronidase resulted in the complete elimination of the metabolite peaks and a corresponding increase in the peak of the parent compound 3'-O-methylquercetin (HPLC chromatograms not shown). Together with LC-MS analysis of the metabolites revealing an m/z ratio of 491 for these metabolites, this result confirms that the three metabolites are mono-glucuronidated derivatives of 3'-O-methylquercetin. Table 2 summarizes the ^{1}H NMR chemical shift values of the 3'-O-methylquercetin monoglucuronide derivatives, as well as of 3'-O-methylquercetin. Based on the changes in chemical shift values of the aromatic protons of the metabolites in comparison with 3'-O-methylquercetin, the ^{1}H NMR data of the metabolite with retention time 13.5 min identify this metabolite as 7-O-

Table 3 HPLC, UV-VIS, LC-MS and ^{1}H NMR data of the metabolites formed in the incubation mixture of microsomes or S9 homogenates with 4'-O-methylquercetin and PAPS or UDPGA[1].

Compound	UV-VIS[2] (nm)	m/z LC-MS (M-1)	^{1}H-NMR (ppm) H6	H8	H5'	H2'	H6'
4'-O-methylquercetin	254.5- -367.4	315	6.19 J_{H6-H8}= 1.2 Hz	6.42 J_{H8-H6}= 1.2 Hz	7.09 $J_{H5'-H6'}$= 8.7 Hz	7.66 $J_{H2'-H6'}$= 1.9 Hz	7.65 $J_{H6'-H2'}$= 1.9 Hz $J_{H6'-H5'}$= 8.7 Hz
Sulfation							
Metabolite[3] t_R 17.9 min	250.9-265.1-364.3	395	6.18 (-0.01) J_{H6-H8}= 1.9 Hz	6.39 (-0.03) n.d.	7.13 (+0.04) $J_{H5'-H6'}$= 8.7 Hz	8.23 (+0.57) n.d.	7.90 (+0.25) $J_{H6'-H2'}$= 1.9 Hz $J_{H6'-H5'}$= 8.7Hz
Metabolite[3] t_R 18.6 min	254.5- -367.4	395	6.51 (+0.32) J_{H6-H8}= 1.9 Hz	7.00 (+0.58) J_{H8-H6}=1.9 Hz	7.09 (+0.00) $J_{H5'-H6'}$= 9 Hz	8.15 (+0.49) n.d.	7.70 (+0.05) n.d.
Glucuronidation							
Metabolite[4] t_R 13.4 min	254.5- -364.3	491	-	-	-	-	-
Metabolite t_R 14.5 min	254.5- -367.5	491	6.43 (+0.24) J_{H6-H8}= 1.5 Hz	6.80 (+0.38) J_{H8-H6}= 1.5 Hz	7.12 (+0.03) $J_{H5'-H6'}$= 8.7 Hz	7.70 (+0.04) $J_{H2'-H6'}$= 2.1 Hz	7.69 (+0.04) $J_{H6'-H2'}$= 2.1 Hz $J_{H6'-H5'}$= 8.7 Hz
Metabolite t_R 17.4 min	250.9-s-364.3	491	6.20 (+0.01) n.d.	6.52 (+0.10) n.d.	7.18 (+0.09) $J_{H5'-H6'}$= 9.2 Hz	7.82 (+0.16) n.d.	7.95 (+0.30) n.d. $J_{H6'-H5'}$= 9.2 Hz

[1] The ppm shift difference of the protons in the conjugates compared to the same protons in 4'-O-methylquercetin are put between brackets.
[2] The symbol -s- in the UV-VIS signals indicates the presence of a shoulder; the symbol - - indicates the absence of a shoulder.
[3] ^{1}H NMR identification of metabolites was hampered by instability, preventing precise determination of some coupling constants.
[4] ^{1}H NMR identification of metabolites was strongly hampered by instability, preventing identification of the chemical shift values.

glucuronosyl 3'-O-methylquercetin. Similarly, the metabolite with a retention time of 16.1 min can be identified as 4'-O-glucuronosyl 3'-O-methylquercetin.
Due to the instability of the metabolite with retention time 12.1 min, no ^{1}H NMR data could be obtained (*25*). Taking into consideration that the metabolites eluting at 13.5 and 16.1 min have been identified as 7-O- and 4'-O-glucuronosyl 3'-O-methylquercetin respectively, and the fact that the metabolite with retention time 12.1 min has also been shown by LC-MS to be a monoglucuronide of 3'-O-methylquercetin with m/z ratio 491, this metabolite can either be 3-O-glucuronosyl 3'-O-methylquercetin or 5-O-glucuronosyl 3'-O-methylquercetin. The C_5-OH group, however, is unlikely to react with UDPGA, because the reactivity of the C_5-OH group is very low due to strong intramolecular hydrogen bonding with the keto-group at C_4 (*26*). Therefore the metabolite eluting at 12.1 min is most likely to be glucuronidated at the C_3-OH. Moreover, UV-VIS data (Table 2) of this metabolite show a hypsochromic shift of band II, which is typical for conjugation of the C_3-OH group (*22*). Therefore, this metabolite can be identified as 3-O-glucuronosyl 3'-O-methylquercetin.

Identification of glucuronidated metabolites of 4'-O-methylquercetin

Figure 4 C and D show the HPLC chromatograms of incubations of rat liver (C) and human liver (D) microsomes with 4'-O-methylquercetin and UDPGA, revealing the formation of three metabolites of 4'-O-methylquercetin with retention times of 13.4, 14.5 and 17.4 min. Table 3 presents the HPLC, UV-VIS and LC-MS data of these metabolites. Incubation of the samples with β-glucuronidase resulted in the complete elimination of the metabolite peaks and a corresponding increase in the peak of the parent compound 4'-O-methylquercetin (HPLC chromatograms not shown), confirming that the metabolites were glucuronidated derivatives of 4'-O-methylquercetin. LC-MS analysis of the metabolites attributed an m/z ratio of 491 to these metabolite peaks, confirming that the metabolites were monoglucuronidated derivatives of 4'-O-methylquercetin.
Table 3 summarizes the ^{1}H NMR chemical shift values of the 4'-O-methylquercetin metabolites, as well as of 4'-O-methylquercetin. Identification of the various ^{1}H NMR resonances has been achieved on the basis of chemical shift values and their splitting patterns. ^{1}H NMR data of the metabolite eluting at 14.5 min show changes in chemical shift values of the aromatic protons characteristic for conjugation of the 7-hydroxylgroup. Therefore this metabolite can be identified 7-O-glucuronosyl 4'-O-methylquercetin. Similarly, the ^{1}H NMR data of the metabolite eluting at 17.4 minutes identify this metabolite as 3'-O-glucuronosyl 4'-

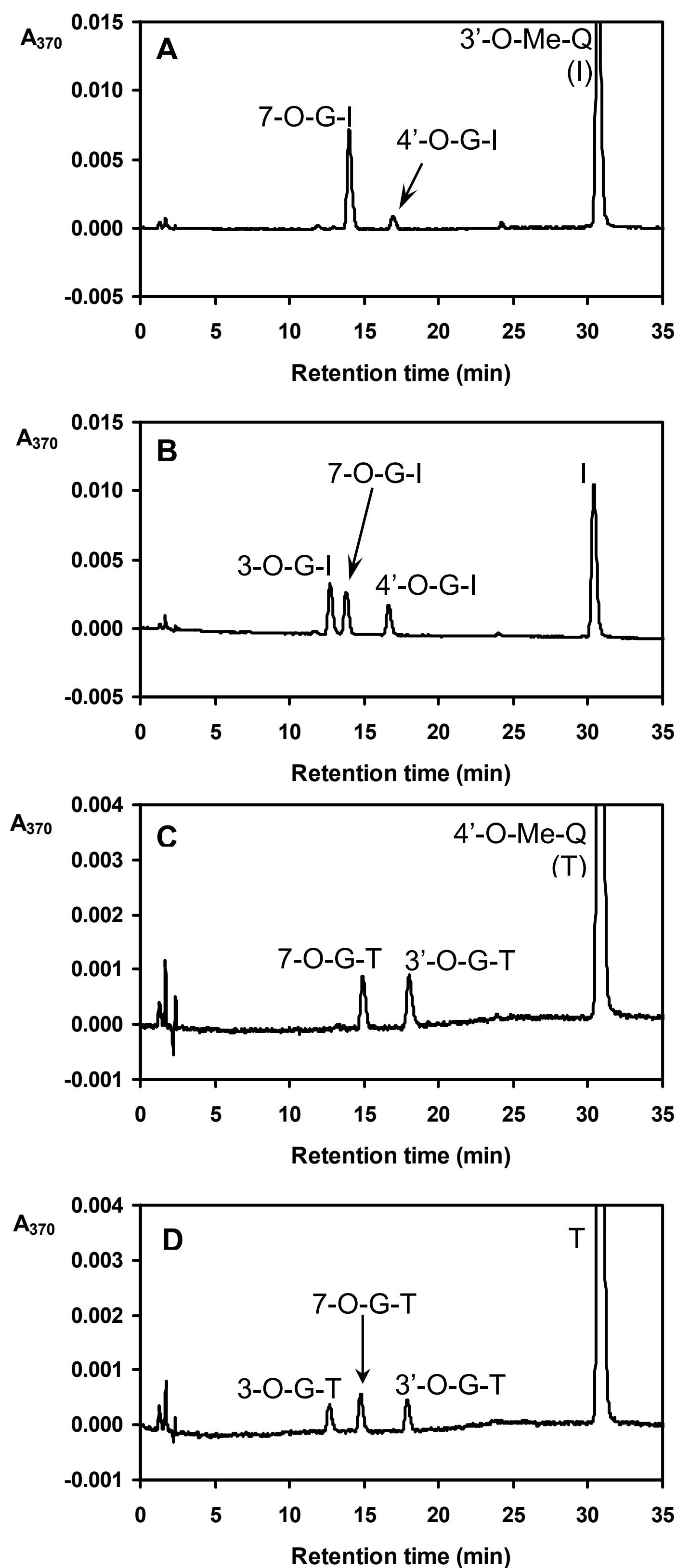

Figure 4 HPLC elution patterns of the incubation mixtures of 3'-O-methylquercetin with (A) rat liver or (B) human liver microsomes and of 4’-O-methylquercetin with (C) rat liver or (D) human liver microsomes with UDPGA, after 30-minute incubation at 37 °C. Q = quercetin, Me = methyl, I = 3’-O-methylquercetin (isorhamnetin), T = 4’-O-methylquercetin (tamarixetin), G = glucuronide.

O-methylquercetin. The metabolite with a retention time of 13.4 min was unstable, preventing ^{1}H NMR measurements. However, for reasons similar to those described for the identification of 3-O-glucuronosyl 3'-O-methylquercetin, the metabolite with a retention time of 13.4 min can be identified as 3-O-glucuronosyl-4'-O-methylquercetin.

Major inter-organ and inter-species differences in phase II metabolism of quercetin

Using the information on all newly identified quercetin metabolites, a series of incubations aiming at identifying some major inter-organ and inter-species differences in quercetin phase II metabolism were performed. These studies were carried out to compare the phase II metabolism of quercetin by rat versus man and liver versus small intestine.

Table 4 shows the ratios of formation of the mono-conjugates of quercetin by rat and human liver and small intestine *in vitro* cellular fractions (S9 or microsomes). Metabolite formation was linear in time (data not shown). All relevant model systems appeared to catalyze the O-methylation of quercetin, with human small intestine being somewhat less active. Furthermore, the regioselectivity for methylation was similar for all samples, resulting in the formation of 3'-O-methyl and 4'-O-methylquercetin at about 2:1 ratio. Incubations with a protein concentration of 1 mg/mL instead of 2.5 mg/mL did not alter the regioselectivity of the methylation of quercetin.

Incubations of quercetin with S9 homogenates in the presence of the cofactor PAPS revealed remarkable inter-species and inter-organ differences in the rate of sulfation of quercetin. Rat small intestine and human liver S9 homogenate did not or hardly show any sulfating activity of quercetin, whereas rat liver and human small intestine S9 homogenates metabolized quercetin to quercetin-7-O-sulfate and quercetin-3'-O-sulfate at similar ratios of about 1:9. The percentages of quercetin conversion indicate that rat liver S9 metabolized quercetin at a faster rate than human small intestine, but human small intestine was far more active than human liver. Incubations with a protein concentration of 1 mg/mL instead of 2.5 mg/mL did not alter the regioselectivity of the sulfation of quercetin.

The regioselectivity of the formation of monoglucuronidated derivatives of quercetin has been discussed previously (*21*). For comparison, the relative quantities of mono-glucuronidated metabolites of quercetin as well as the percentages of quercetin conversion are included in Table 4.

Table 4 Inter-organ and inter-species differences in the formation of phase II mono-conjugates of quercetin after 30 min incubation. Percentages of metabolites are expressed relative to the total amount of quercetin metabolites formed in the incubation. Values given represent the mean of two experiments. Variation between experiments was approximately 15% of the value given.

	Rat liver	Rat small intestine	Human liver	Human small intestine
Methylation				
Quercetin conversion (%)	6	4	5	1
3'-O-methylation (%)	65	67	66	62
4'-O-methylation (%)	35	33	34	38
Sulfation				
Quercetin conversion (%)	14	0	0	5
7-O-sulfation (%)	12	-	-	12
3'-O-sulfation (%)	88	-	-	88
Glucuronidation (21)				
Quercetin conversion (%)	18	25	22	61
3-O-glucuronidation (%)	0	9	13	20
7-O- glucuronidation (%)	40	41	65	4
3'-O- glucuronidation (%)	42	46	19	49
4'-O- glucuronidation (%)	18	4	3	27

Overall phase II metabolism of quercetin

To study the overall phase II metabolism of quercetin in terms of inter-species and inter-organ differences, incubations of quercetin with the four S9 homogenates in the simultaneous presence of the cofactors for methylation, glucuronidation and sulfation were performed and the resulting metabolite pattern was identified. For comparison, a series of commonly used other *in vitro* systems were also exposed to quercetin for determination of the phase II metabolite pattern. These included rat and human cryopreserved hepatocytes, the hepatic cell lines H4IIE from rat and HepG2 from human, the rat small intestine cell line IEC-6 and the colon cell lines DHD/K12/Trb from rat and HT29 from human. The results are listed in Table 5. All *in vitro* models tested metabolized quercetin, although the type and regioselectivity of the phase II metabolism of quercetin strongly varied with the origin of the *in vitro* model. All models glucuronidated quercetin with varying regioselectivity, whereas the methylating and sulfating capacities of the models showed the highest variation. Important to note is that *in vitro* models from a similar origin in terms of organ and species gave very different phase II metabolite patterns of quercetin.

Table 5 Phase II metabolite pattern of quercetin formed by various *in vitro* systems of rat and human origin. Q = quercetin, M = methyl, S = sulfate, G = glucuronide. Percentages of metabolites are expressed relative to the total amount of metabolites present in the incubation. Values given represent the mean of two experiments. Variation between experiments was approximately 15% of the value given. The metabolites Q-3'-M-4'-S, Q-4'-M-3'-S and 4'-M-7-S were not detected. Total percentage of metabolites per *in vitro* system may be less than 100%, due to the formation of metabolites of which the quantity was too small to allow identification.

Species	**Rat**						**Human**				
Organ	Liver	Liver	Liver	Small Intestine	Small Intestine	Colon	Liver	Liver	Liver	Small Intestine	Colon
***In vitro* system**	S9	H4IIE	Hepatocytes	S9	IEC-6	DHD/K12/Trb	S9	HepG2	Hepatocytes	S9	HT29
Phase II metabolites (%)											
Q-3'-M	3	0	2	8	37	46	3	50	1	0	0
Q-4'-M	1	0	1	4	19	18	0	19	0	0	0
Q-7-S	13	1	0	0	1	0	0	1	0	9	0
Q-3'-S	52	0	20	0	21	0	0	20	0	71	0
Q-3-G	0	0	0	10	0	0	18	3	5	4	29
Q-7-G	14	76	0	44	24	24	38	0	44	0	0
Q-3'-G	9	9	17	21	0	0	37	7	27	10	7
Q-4'-G	0	0	0	0	0	0	0	0	0	6	40

Table 5 (continued) Phase II metabolite pattern of quercetin formed by various *in vitro* systems of rat and human origin. Q = quercetin, M = methyl, S = sulfate, G = glucuronide. Percentages of metabolites are expressed relative to the total amount of metabolites present in the incubation. Values given represent the mean of two experiments. Variation between experiments was approximately 15% of the value given. The metabolites Q-3'-M-4'-S, Q-4'-M-3'-S and 4'-M-7-S were not detected. Total percentage of metabolites per *in vitro* system may be less than 100%, due to the formation of metabolites of which the quantity was too small to allow identification.

Species	**Rat**						**Human**				
Organ	Liver	Liver	Liver	Small Intestine	Small Intestine	Colon	Liver	Liver	Liver	Small Intestine	Colon
***In vitro* system**	S9	H4IIE	Hepatocytes	S9	IEC-6	DHD/K12/Trb	S9	HepG2	Hepatocytes	S9	HT29
Phase II metabolites (%)											
Q-3'-M-7-S	0	0	0	0	0	6	0	0	0	0	0
Q-3'-M-3-G	0	1	0	0	0	0	3	1	1	0	7
Q-3'-M-7-G	0	0	37	3	0	6	0	0	16	0	0
Q-3'-M-4'-G	0	0	12	0	0	0	0	0	2	0	15
Q-4'-M-3-G	0	8	0	0	0	0	0	0	0	0	0
Q-4'-M-7-G	5	1	8	0	0	0	0	0	5	0	0
Q-4'-M-3'-G	0	0	0	5	0	0	0	1	1	0	4

Inter-individual differences in phase II metabolism of quercetin

To study the inter-individual differences in phase II metabolism of quercetin, incubations of quercetin with hepatic S9 homogenates of 13 human donors in the presence of the cofactors for glucuronidation, sulfation and methylation were performed. Table 6 shows the rate, type and regioselectivity of the phase II conversion of quercetin by liver homogenates of 7 male and 6 female human donors. Conversion percentages of quercetin varied from 3 to 14%. Glucuronidation of quercetin to especially 7-O-glucuronosyl and to a lesser extent also 3'-O-glucuronosylquercetin as major metabolites were the main phase II metabolic pathways for all individuals tested. A third important metabolite for the majority of the samples was 3-O-glucuronosyl quercetin. All individuals, except SD107, methylated quercetin at at least one of the catechol hydroxyl groups, although the contribution of methylation was considerably lower than that of glucuronidation. The results also show that there are clear differences in sulfating capacity of quercetin between individuals, varying from 0 to 16% of total phase II metabolized quercetin. The 7- and the 3'-hydroxyl groups of quercetin are extensively conjugated by all human liver samples, mainly by glucuronidation and to a lesser extent by sulfation. The fact that glucuronidated or sulfated metabolites of 3'-O-methyl or 4'-O-methylquercetin were detected only in small amounts is not surprising in view of the low contribution of methylation to phase II metabolism of quercetin in these samples (from 2 to 21%).

Table 6 Inter-individual differences in the formation of phase II metabolites of quercetin by hepatic S9 homogenate of 7 male (M) and 6 female (F) human donors after 30 min incubation in the presence of the cofactors PAPS, SAM and UDPGA. Q = quercetin, M = methyl, S = sulfate, G = glucuronide. Percentages of metabolites are expressed relative to the total amount of metabolites present in the incubation. Values given represent the mean of two experiments. Variation between experiments was approximately 15% of the value given. Classification of donors according to the supplying companies Cellzdirect and InVitroTechnologies (see Materials and Methods section): M1 = SD004, M2 = SD102, M3 = SD103, M4 = SD107, M5 = SD122, M6 = 1016, M7 = 1020, F1 = SD002, F2 = SD120, F3 = SD127, F4 = SD134, F5 = 1007, F6 = 1008). The metabolites Q-3'-M-7-S, Q-4'-M-3'-S, Q-4'-M-7-S, Q-3'-M-4'-G and Q-4'-M-3'-G were not detected.

	Donor code												
	M1	**M2**	**M3**	**M4**	**M5**	**M6**	**M7**	**F1**	**F2**	**F3**	**F4**	**F5**	**F6**
Age	**56**	**6**	**5**	**16**	**57**	**42**	**37**	**52**	**53**	**1.5**	**74**	**53**	**61**
Quercetin conversion (%)	***10***	***5***	***8***	***5***	***10***	***12***	***6***	**14**	**6**	**3**	**7**	**5**	**13**
Phase II metabolites (%)													
Q-3'-M	3	2	3	0	2	4	9	2	3	8	6	3	1
Q-4'-M	1	0	0	0	0	1	5	1	0	3	2	0	0
Q-7-S	2	5	0	2	2	2	0	1	0	0	0	0	0
Q-3'-S	6	11	6	4	5	0	0	3	4	0	8	3	0
Q-3-G	11	14	15	11	7	12	9	7	7	0	14	22	17
Q-7-G	44	26	35	35	54	49	53	59	42	45	39	41	40
Q-3'-G	22	36	29	40	22	21	17	22	36	35	21	25	17
Q-4'-G	0	7	0	0	0	0	0	0	0	8	0	0	0
Q-3'-M-4'-S	0	0	0	0	0	0	0	0	0	0	0	0	4
Q-3'-M -3-G	4	0	4	0	1	3	0	1	2	2	3	0	3
Q-3'-M-7-G	6	0	0	0	5	7	7	4	7	0	8	6	7
Q-4'-M-3-G	0	0	7	8	0	0	0	0	0	0	0	0	0
Q-4'-M-7-G	1	0	0	0	0	1	0	1	0	0	0	0	0

Discussion

In this study, the structures of 14 relevant phase II metabolites of quercetin were unequivocally identified using HPLC, UV-VIS and ^{1}H NMR. Previously, a method for determination of the regioselectivity of glucuronidation of quercetin has been published, based on changes in the UV-spectra of the conjugates after the addition of shift reagents (*22*). Together, the data presented in this report provide the possibility to characterize the nature of phase II metabolites of quercetin in a variety of models. This is of use since quercetin is known to be extensively metabolized to methylated, glucuronidated and/or sulfated derivatives (*17-19, 27*), whereas little is known about the regioselectivity of these phase II conjugations. This regioselectivity is of importance because the biological activity of flavonoids is known to be dependent on the number and position of especially unconjugated free hydroxyl groups (*13, 28-32*).

Glucuronidation studies with quercetin showed that the 7-, 3-, 3'- and 4'-hydroxyl groups were all target for conjugation; the studies on sulfation of quercetin, however, showed a more restricted regioselectivity: besides the already reported quercetin-3'-O-sulfate metabolite (*17, 33*), the present study reveals that sulfation of the 7-hydroxyl group appears to be an important phase II metabolite pathway of quercetin.

Methylation of quercetin yielded two metabolites, 3'-O-methylquercetin and 4'-O-methylquercetin, in a 2:1 ratio. The preferential formation of 3'-O-methyl quercetin over 4'-O-methyl quercetin is in line with the regioselectivity of *in vivo* methylation of quercetin (*34, 35*), and might be the consequence of the fact that methylation of the 3'-hydroxylgroup is energetically more favorable than methylation of the 4'-hydroxylgroup (*20, 36*). The phenomenon is also observed for other substrates, such as catecholamines. Computer modeling showed that the energetically most favorable binding orientation of a catecholamine in the active site of catechol-O-methyltransferase (COMT) led to methylation of the hydroxyl group in the *meta*-position (*37*) and in the case of quercetin, the 3'-hydroxyl group is situated in *meta*-position with respect to the main part of the molecule.

Using the newly developed tool for phase II quercetin metabolite identification, the phase II metabolite patterns formed by a variety of *in vitro* model systems could be quantified. The data show that quercetin is extensively metabolized by all phase II biotransformation systems, although the type and regioselectivity of the conjugation of quercetin are dependent on the species and organ under study. Furthermore, the variation between the different *in vitro* models from corresponding origin is considerable. It could be established that the intestine,

known to express certain cytosolic sulfotransferase (ST) isoforms (*38, 39*), contributes significantly to phase II metabolism of quercetin, especially to its sulfation. Thus, the detection of quercetin sulfate metabolites in human plasma after consumption of a quercetin-containing meal (*17*) may be the result of sulfation reactions by small intestine enterocytes. The results also reveal that organ-dependent phase II metabolism in rat and man differ significantly. Remarkably, the phase II metabolite pattern formed by human small intestine S9 closely resembles that of rat liver S9, whereas that of rat small intestine S9 resembles that of human liver S9.

In Table 7, the phase II metabolism of quercetin by the *in vitro* systems studied is compared with *in vivo* plasma data (*17, 19*). Altogether, the identification of the various mono- and mixed quercetin phase II metabolites in the different *in vitro* models leads to the conclusion that none of the models produces the *in vivo* plasma metabolite pattern of quercetin (*17, 19*). From this, it is concluded that the plasma phase II metabolite pattern is likely to be the result of interplay of different organs with metabolizing capacity, especially the liver and the small intestine. Comparison of plasma phase II metabolism with the phase II metabolite patterns of the S9 homogenates studied (Table 7) suggests that in rat, methylation of quercetin takes place mainly in the intestine, sulfation in the liver and that glucuronidation is performed by both liver and small intestine. The human data suggest that in man, both liver and intestine perform glucuronidation of quercetin. However in contrast with the rat, methylation takes place mainly in the liver, whereas sulfation of quercetin is mainly performed by the intestine.

A closer study on the phase II metabolism of quercetin by hepatic S9 homogenates from individual human donors revealed that the liver showed little variability between individuals in the major phase II metabolism of quercetin. However, although contributing to a limited extent to the phase II metabolism of quercetin, clear differences in sulfating capacity between the individuals were found (Table 6).

Finally, data on the exact regioselectivity of phase II metabolism are of importance, because conjugation of the hydroxyl groups of quercetin generally attenuates the biological activity, especially when the catechol group (*13, 28, 29, 32, 40-42*) or the 3-hydroxyl group (*30*) are the target of conjugation. Table 8 summarizes the overall percentage of phase II conjugation at the various hydroxyl groups of quercetin in the different model systems and reveals that the preferential sites of conjugation by most of the *in vitro* models studied are especially the 3’- and 7- hydroxyl group, whereas 4’ and 3-hydroxyl moiety are a less common target for phase II conjugation. Altogether, the unequivocal identification of many phase II metabolites of

Table 7 Comparison of phase II metabolite patterns of quercetin formed by different *in vitro* models with *in vivo* plasma data of rat (*19*) and of human (*17*). Percentages given represent the relative contribution of the conjugate relative to the total amount of quercetin phase II metabolites. Since literature only identified the type but not the exact identity (i.e. regioselectivity) of the phase II metabolites of quercetin, the sum of all methylated, sulfated, and/or glucuronidated quercetin metabolites is presented (Hepa = hepatocytes; DHD = DHD/K12/Trb).

	Rat							**Human**					
	Liver			**Intestine**				**Liver**			**Intestine**		
	S9	H4IIE	Hepa	S9	IEC-6	DHD	**Plasma**	S9	HepG2	Hepa	S9	HT29	**Plasma**
Conjugated form of quercetin	%												
methylated	4	0	3	12	56	65	**0**	3	69	1	0	0	**0**
sulfated	65	1	20	0	22	0	**13**	0	21	0	80	0	**35**
methylated + sulfated	0	0	0	0	0	6	**79**	0	0	0	0	0	**0**
glucuronidated	23	85	17	75	24	24	**5**	93	10	76	20	76	**46**
methylated + glucuronidated	5	10	57	8	0	6	**4**	3	2	25	0	26	**19**

Table 8 Regioselectivity of the overall phase II metabolism of quercetin by different *in vitro systems* (Hepa = hepatocytes; DHD = DHD/K12/Trb). Percentages given represent the relative contribution of the conjugation of a certain hydroxyl group relative to the total amount of quercetin phase II metabolites. Total percentages of metabolites per *in vitro* system may exceed 100% because of the formation of mixed conjugates.

	Rat						**Human**				
	Liver			**Intestine**			**Liver**			**Intestine**	
	S9	H4IIE	Hepa	S9	IEC-6	DHD	S9	HepG2	Hepa	S9	HT29
Position of conjugation	%										
3'-OH	64	10	88	37	58	58	43	79	48	81	33
4'-OH	6	9	21	9	19	18	0	20	8	6	59
3-OH	0	9	0	10	0	0	21	4	6	4	36
7-OH	32	78	45	47	25	36	38	1	65	9	0

quercetin as presented in this study allows the identification of the exact quercetin phase II patterns, and opens the way for a better-funded assessment of the biological activity of quercetin in a variety of biological systems.

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3

The effect of catechol O-methylation on radical scavenging characteristics of quercetin and luteolin – A mechanistic insight

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Abstract

The biological effect of flavonoids can be modulated *in vivo* due to metabolism. The *O*-methylation of the catechol group in the molecule by catechol *O*-methyl transferase is one of the important metabolic pathways of flavonoids. In the present study, the consequences of catechol *O*-methylation for the pH-dependent radical scavenging antioxidant properties of quercetin and luteolin were characterized both experimentally and theoretically. Comparison of the pKa values to the pH-dependent TEAC antioxidant profiles reveals that *O*-methylation not only affects the TEAC as such but also modulates the effect of changing pH on this antioxidant activity due to an effect on the pKa for deprotonation. The pH-dependent TEAC curves and computer calculated electronic parameters: bond dissociation energy (BDE) and ionisation potential (IP) even indicate that *O*-methylation of the luteolin catechol group affects the antioxidant potential only because it shifts the pKa for deprotonation. *O*-Methylation of the quercetin catechol moiety affects antioxidant activity by both an effect on the pKa, and also by an effect on the electron and hydrogen atom donating properties of the neutral and the anionic form of the molecule. Moreover, *O*-methylation of a catechol OH-group in quercetin and luteolin has a similar effect on their TEAC profiles and on calculated parameters as replacement of the OH-group by a hydrogen atom. Altogether, the results presented provide new mechanistic insight in the effect of catechol *O*-methylation on the antioxidant characteristics of quercetin and luteolin.

Introduction

Natural polyphenols, especially flavonoids and their corresponding glycosides are important constituents of fruits, vegetables, nuts, tea, olive oil and red wine (*1, 2*). Quercetin (Figure 1), the main flavonol in our diet, is particularly abundant in onions and apples (*3*). Luteolin (Figure 1) was identified in red sweet pepper and celery (*4*). The daily consumption of polyphenols is variable according to food habits and may range from 25 mg/d to 1 g/d (*5, 6*).

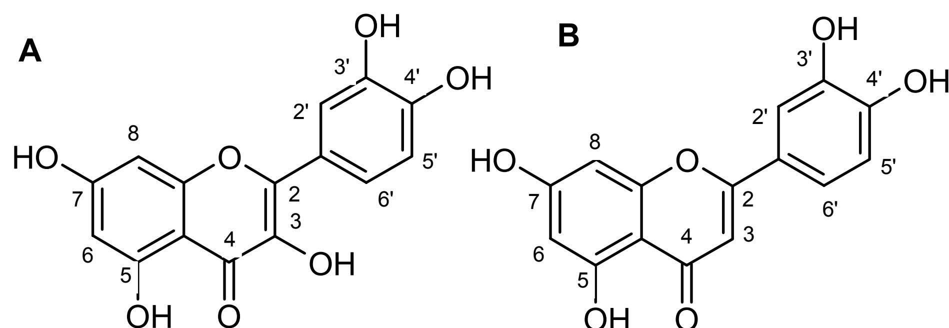

Figure 1 Structural formulas and numbering of the model flavonoids (A) quercetin and (B) luteolin.

The antioxidant properties of these compounds are often claimed to be, at least in part, responsible for the protective effects of these food components against cardiovascular diseases, certain forms of cancer and photosensitivity diseases (*7, 8*). The antioxidant activity of flavonoids is known to be highly dependent on their structure, particularly the availability of free hydroxyl groups (*8, 9*). Therefore, conjugation of the free hydroxyl moieties by phase II metabolism may influence the biological activity, including the antioxidant potential of the flavonoids. Both quercetin and luteolin are known to be extensively metabolised by the phase II biotransformation system, resulting in glucuronidated, sulfated and methylated derivatives (*2, 10*). The present study focuses on the consequences of catechol *O*-methylation for the antioxidant capacity of luteolin and quercetin, as the model flavonoids to be investigated. For long, it has been known that catechol-*O*-methyl transferases (COMT) are capable of the methylation of the catechol groups of flavonoids, especially quercetin (*11-13*). There are two different COMT isoforms: soluble COMT (s-COMT) in the cytoplasm, mainly present in periferal tissues (*14*), and membrane-bound COMT (mb-COMT) (*15*), mainly present in the brain area (*14*). Several reports showed the important contribution of methylation to the phase II metabolism of quercetin *in vivo*. In humans, 21 % of quercetin circulating in plasma after consumption of a quercetin-rich meal was present as 3’-*O*-methylquercetin (isorhamnetin),

partly conjugated as glucuronide- and/or sulfate conjugate (*16*). In rats, the contribution of *O*-methylation is even higher, since up to 86% of the total amount of circulating quercetin has been reported to be methylated at the 3'-hydroxyl group (*1, 11, 17*). Contrary to 3'-*O*-methylquercetin, 4'-*O*-methylquercetin is hardly recovered from plasma (*1, 11, 17-19*). This has been ascribed to a very efficient renal clearance, because 4'-*O*-methylquercetin can be detected in considerable amounts in bile and urine of rats fed quercetin (*11*).

Given the importance of catechol-*O*-methylation in the phase II biotransformation of flavonoids, the objective of the present study is to characterize the consequences of catechol-*O*-methylation for the antioxidant capacity of quercetin and luteolin, model flavonoids known to be present in significant amounts in our daily diets (*2, 5*).

By investigating the consequences of catechol-*O*-methylation of luteolin and quercetin both experimentally, taking into account the effect of pKa and pH on the antioxidant activity (*20, 21*), and by theoretical quantum mechanical calculations, new mechanistic insight in the effect of catechol-*O*-methylation on the antioxidant characteristics of these two important model flavonoids was obtained.

Materials and Methods

Materials

Isorhamnetin (3'-*O*-methyl-quercetin; 3'-*O*Me-Q), tamarixetin (4'-*O*-methyl-quercetin; 4-*O*Me-Q), chrysoeriol (3'-*O*-methyl-luteolin; 3'-*O*Me-L), diosmetin (4'-*O*-methyl-luteolin; 4'-*O*Me-L) were purchased from Indofine Chemical (Somerville, NJ, USA). Quercetin, luteolin, kaempferol and apigenin were obtained from Fluka (Buchs, Switzerland). 3',5,7-Trihydroxyflavone was synthesised according to the method of Gaydou and Bianchini (*22*). Microperoxidase-8 (MP-8) was obtained from Sigma (Steinheim, Germany). 2,2'-Azinobis(3-ethylbenzothiazoline-6-sulphonic acid) diammonium salt (ABTS), 6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid (Trolox) and ascorbic acid were purchased from Aldrich (Steinheim, Germany). Hydrogen peroxide (30%), methanol (HPLC grade) and acetonitrile (HPLC grade) were purchased form Merck (Darmstadt, Germany).

Determination of pKa

The pKa values of quercetin, 3'-*O*-methyl-quercetin, 4'-*O*-methyl-quercetin, luteolin, 3'-*O*-methyl-luteolin, 4'-*O*-methyl-luteolin and 3',5,7-hydroxyflavone was determined from absorption spectra as a function of pH as described by Sauerwald *et al*. (*23*).

TEAC assay

The Trolox® Equivalent Antioxidant Capacity (TEAC) was measured by the method of Miller *et al*. (*24*) with some modifications (*21*).
The TEAC value is based on the ability of the antioxidant to scavenge the blue-green colour $ABTS^{•+}$ radical cation relative to the $ABTS^{•+}$ scavenging ability of the water-soluble vitamin E analogue – Trolox®. The major advantage of the modified TEAC assay is that it permits studies of radical scavenging antioxidant activity over a wide pH range (i.e. pH 4.5 to 9.5).
Solubility of the 3'-*O*-methyl-luteolin and 4'-*O*-methyl-luteolin was checked at pH 4.5 and 7.5 and a linear relationship between absorbance and concentration was obtained up to 10 μM. Therefore, pH dependent TEAC profiles were measured at concentrations not exceeding 10 μM.

Quantum mechanical calculations

All geometries of molecules studied were optimised with the B3LYP hybrid density functional theory (DFT) by using a 6-31G(d) basis set as implemented in the Gaussian 98 computational package (Gaussian Inc., Pittsburgh, PA, USA). Single-point energies were then evaluated by using a higher 6-311 G(d,p) basis set. The calculated deprotonation energies (DE), ionisation potentials (IP) and bond dissociation energies (BDE) were not corrected for zero–point–energy assuming a negligible error and thus saving computer-time.
The DE was calculated as the electronic energy of the deprotonated anion minus the electronic energy of the parent molecule. The BDE for homolytic O-H bond cleavage in the neutral flavonoid (BDE(N)) was calculated as the energy of the radical resulting from the hydrogen atom abstraction minus the energy of the neutral molecule. The IP for the neutral flavonoid (IP(N)) was calculated as the energy of the radical cation resulting from the electron abstraction minus the energy of the neutral parent molecule.
Similarly the BDE for homolytic O-H bond cleavage in the deprotonated anionic flavonoid (BDE(A)) was calculated as the energy of the radical of the most stable hydroxyflavone anion minus the energy of this most stable anion. The IP of the most stable anion (IP(A)) was calculated as the energy of phenoxyl radical formed by electron abstraction from the most stable monoanion minus the energy of this most stable monoanion.

Results

pKa values of quercetin and luteolin O-methyl derivatives

Table 1 presents the experimental pKa values of the various model compounds, as determined in the present study or obtained from the literature. Table 1 lists also the calculated relative deprotonation energies (DE) of various hydroxyl groups of querectin and luteolin as well as of their catechol *O*-methyl metabolites. These calculated DE values reflect the ease of deprotonation. In addition, Table 1 presents the pKa values of the various model compounds predicted using the calculated DE and the quantitative structure activity relationship (QSAR) obtained in our previous study (*20*) for the relationship between the calculated DE and the pKa of a series of hydroxyflavones. Comparison of the experimental pKa values to those predicted using the previously defined QSAR illustrates that this recently developed QSAR model predicts the actual pKa values of the *O*-methyl metabolites relatively well. The results also illustrate that *O*-methylation of the catechol moiety significantly affects the ease of deprotonation, reflected by a significant increase in the calculated DE for deprotonation and a marked increase in the experimental as well as the predicted pKa values. Comparison of the pKa values of the *O*-methylated metabolites and the corresponding aglycones indicates deprotonation to be dependent on the position of *O*-methylation, 4'-*O*-methylation increasing the pKa to a somewhat larger extent than 3'-*O*-methylation. Calculations of the DE values for deprotonation of the $C_{4'}$-*O*- or $C_{3'}$-*O*-methylated metabolites theoretically corroborates that the effect on the ease of deprotonation is more pronounced upon $C_{4'}$-*O*- than upon $C_{3'}$-*O*-methylation. The pKa values obtained also indicate that, as for many hydroxyflavones, also for the *O*-methylated derivatives deprotonation equilibria are expected to occur within the physiological pH range.

Finally, the DE values presented in Table 1 for deprotonation of the various moieties also indicate that the *O*-methylation of the catechol moiety at both $C_{4'}$-OH and $C_{3'}$-OH changes the preferential site of OH deprotonation from $C_{4'}$-OH to C_7-OH. This holds for the 4'-*O*-methyl derivatives of both quercetin and luteolin as well as for the 3'-*O*-methyl derivative of quercetin. For luteolin, however, C3'-OH-methylation does not affect the order of preference for deprotonation and C4'-OH remains the site that can be more easily deprotonated than C_7-OH, although the relative difference between the DE for 4'-OH and 7-OH deprotonation reduces in the 3'-O-methyl-luteolin as compared to luteolin itself.

Table 1 Experimental pKa values, calculated deprotonation energies (DE) and theoretically predicted pKa values of the hydroxyl moiety of quercetin and luteolin as well as of their *O*-methylated metabolites.

Compound	pKa observed	DE [kcal/mol]	pKa predicted [1)]
Quercetin (3',4',3,5,7-pentahydroxyflavone)	7.65 7.03 [3)]	331.6 (4') [2)] 338.1 (7)	7.00
3'-*O*Me-Q (Isorhamnetin)	7.80	338.7 (7) 342.0 (4')	8.06
4'-*O*Me-Q (Tamarixetin)	8.17	339.7 (7) 350.9 (3')	8.21
Luteolin (3',4',5,7-tetrahydroxyflavone)	7.06	327.9 (4') 341.7 (7)	6.37
3'-*O*Me-L (Chryoseriol)	7.70	338.1 (4') 342.3 (7)	7.96
4'-*O*Me-L (Diosmetin)	7.90	343.7 (7) 346.9(3')	8.82

[1)] Predictions of pKa values was done using the calculated DE and the QSAR obtained in previous studies (*20*): pKa=0.1525 DE-43.596; r=0.9808 (DFT).
[2)] the number between brackets refers to the position of OH moiety.
[3)] pKa value taken from (*23*).

pH Dependent TEAC antioxidant activity of luteolin and quercetin and their catechol O-methyl metabolites

Previous studies revealed a significant influence of pH on antioxidant behaviour of polyphenols, showing an increase in TEAC value around the pKa of the compounds (*20, 21*). The results presented in Figure 2, which show the pH dependent TEAC profiles of quercetin and luteolin aglycones as well as of their *O*-methylated metabolites, reveal that the quercetin and luteolin *O*-methyl metabolites behave in a similar way. The antioxidant action of Trolox C (standard) was previously shown to be unaffected over the whole pH range tested (*21*). Thus, the TEAC values for all compounds increase with increasing pH. Comparison of the pKa values (Table 1) to the pH-dependent TEAC profiles leads to the conclusion that this increase in TEAC value occurs around the pKa , which suggests that it is related to deprotonation of the flavonoid under study. Upon deprotonation quercetin and luteolin, but also their *O*-methylated derivatives become better antioxidants reflected in a significant pH-dependent increase in the TEAC values around their pKa.

Figure 2B shows that 3'-*O*-methyl-luteolin and 4'-*O*-methyl-luteolin are not active as

$ABTS^{\bullet+}$ radical cation scavengers in the pH range between 4.5 - 6.5. pH-Dependent UV-spectroscopy was performed and showed that changes in TEAC observed are not due to solubility effects since a linear relation between absorbance and concentration of the compounds was obtained (data not shown).

O-Methylation of the $C_{4'}$-OH and $C_{3'}$-OH position in the quercetin and luteolin molecules results in a decrease of their TEAC antioxidant activity compared to their aglycone form over the whole pH range tested (Figure 2). This effect is more pronounced for quercetin than for luteolin. For luteolin the effect can even be overcome at high pH, since at pH 9.5 the TEAC values of luteolin and its *O*-methylated analogues is similar.

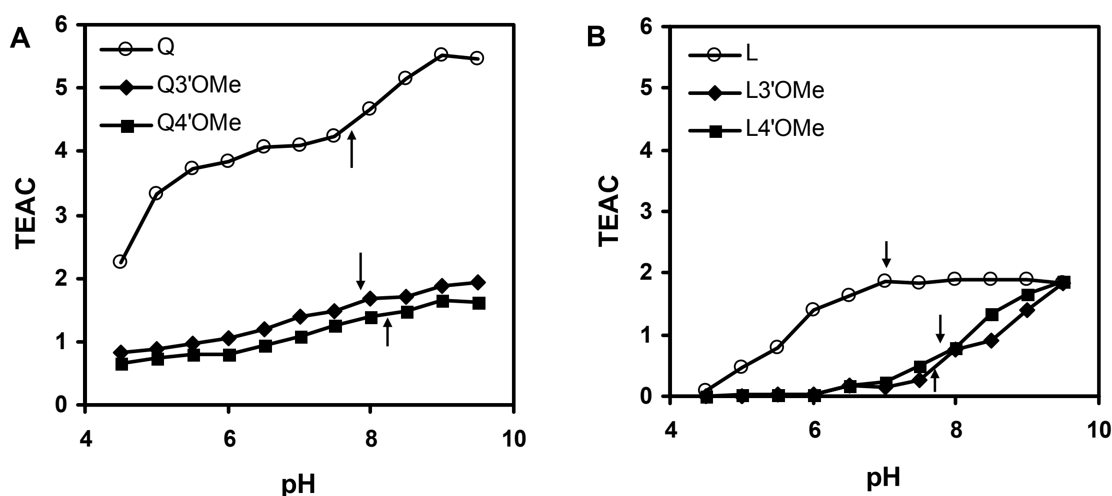

Figure 2 Effect of pH on the TEAC value of (A) quercetin and *O*-methylated metabolites of quercetin and (B) luteolin and *O*-methylated metabolites of luteolin. Arrows indicate the experimental pKa value of preferably deprotonated OH-group in molecule**.**

pH Dependent TEAC profiles of kaempferol, apigenin and 3',5,7-trihydroxyflavone

Figure 3 presents the pH-dependent TEAC profiles of kaempferol (4',3,5,7-*tetra*-hydroxyflavone) (Figure 3D), apigenin (4',5,7-*tri*-hydroxyflavone) (Figure 3E), and 3',5,7-*tri*-hydroxyflavone (Figure 3F). For comparison, Figure 3a-c present the curves for 3'-*O*-methyl-quercetin (Figure 3A), 3'-*O*-methyl-luteolin (Figure 3B), and 4'-*O*-methyl-luteolin (Figure 3C). Comparison of Figure 3A to 3D, 3B to 3E and 3C to 3F, respectively, reveals that the pH dependent TEAC profiles of 3'-*O*-methyl-quercetin, 3'-*O*-methyl-luteolin and 4'-

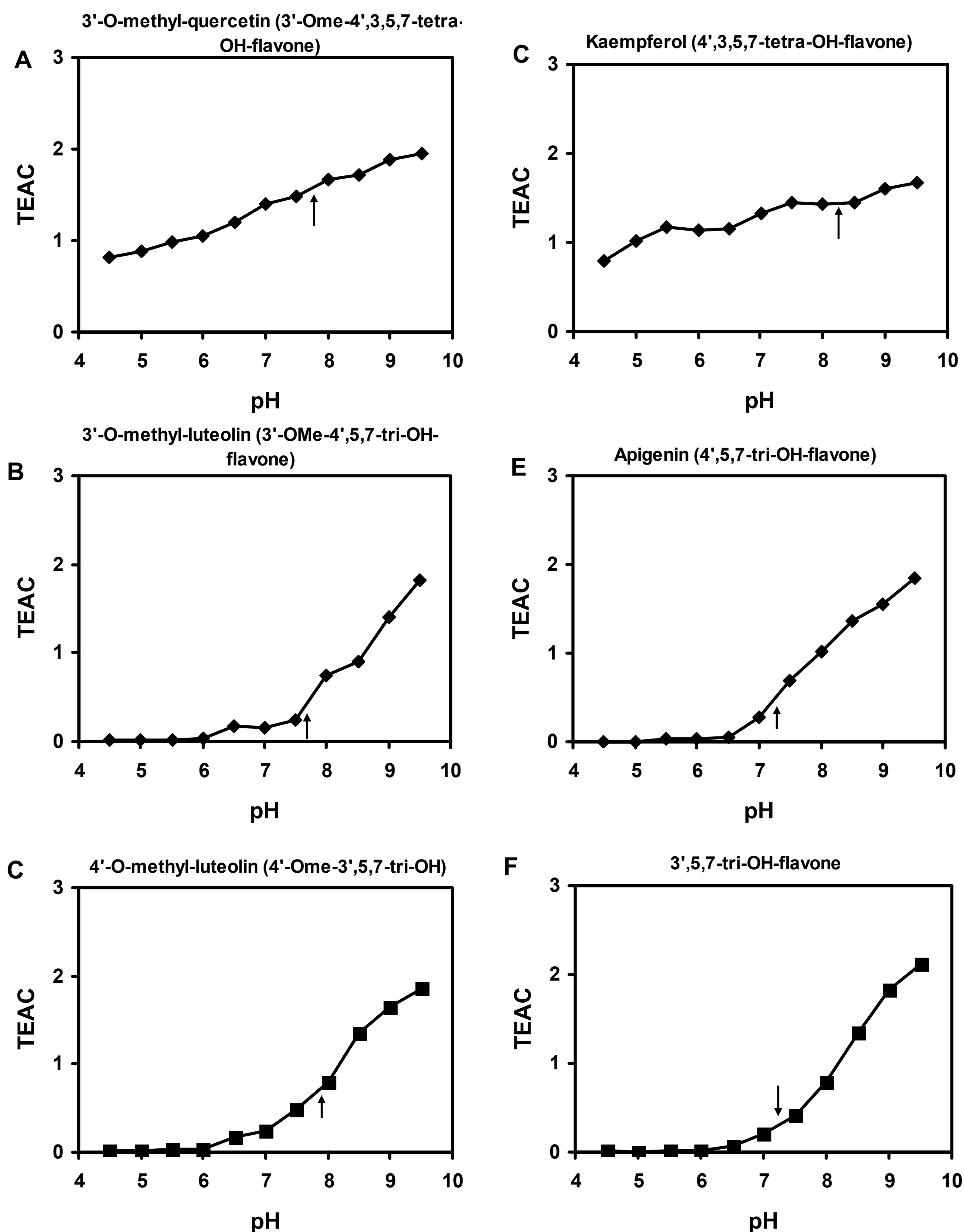

Figure 3 pH-Dependent TEAC profiles of selected quercetin and luteolin *O*-methylated metabolites and hydroxyflavone analogues in which OH group is replaced by a hydrogen atom: (a) 3'-*O*-methyl-quercetin, (b) 3'- *O*-methyl luteolin, (c) 4'- *O*-methyl-luteolin, (d) 4',3,5,7-tetrahydroxyflavone; (e) 4',5,7-trihydroxyflavone (f) 3',5,7-trihydroxyflavone. Arrows indicate the experimental pKa value of preferably deprotonated OH group in molecule.

O-methyl-luteolin are very similar to the pH-dependent TEAC profiles of 4',3,5,7-hydroxyflavone, 4',5,7-hydroxyflavone and 3',5,7-hydroxyflavone, respectively. This

illustrates that *O*-methylation of a catechol OH group effectively results in pH dependent TEAC profiles similar to the pH dependent TEAC profile of the flavonoid analogue in which this OH group is replaced by a hydrogen atom.

Calculated parameters for the ease of electron and hydrogen atom donation by the metabolites of quercetin and luteolin

To obtain more insight in the effect of *O*-methylation on the radical scavenging antioxidant activity of quercetin and luteolin and in the effect of protonation states on the TEAC activity of quercetin, luteolin, and their *O*-methylated metabolites, the TEAC values, derived in the present study, were compared to the theoretically calculated electronic parameters. Table 2 presents these electronic descriptors for the neutral (N) and anionic (A) form of the compounds under investigation, including OH bond dissociation energy (BDE), representing the ease of hydrogen atom donation and ionisation potential (IP) representing the ease of electron donation. The results show that *O*-methylation of the OH group at the C4' and C3' positions both in quercetin and luteolin increases the BDE(N) in comparison to the BDE calculated for the parent aglycones. This would provide an explanation why 4'-*O*-methyl-quercetin, 3'-*O*-methyl-quercetin, 4'-*O*-methyl-luteolin and 3'-*O*-methyl-luteolin in their neutral form can be less effective antioxidants (reflected in relatively lower TEAC values) than quercetin and luteolin respectively. Upon *O*-methylation the BDE(N) increases because the phenoxyl radical, resulting from hydrogen atom donation by the neutral forms of *O*-methylated derivatives, can not be as effectively stabilized by hydrogen bonding within the catechol moiety, making the radical relatively less stable.

Additionally, Table 2 presents the calculated electronic parameters of the deprotonated forms of the $C_{4'}$-*O*-methylated derivatives with a deprotonated 7-OH moiety and $C_{3'}$-*O*-methylated derivatives with deprotonated 4'-OH or 7-OH moiety, providing BDE(A) and IP(A) values for the monoanionic forms of investigated compounds. These data provide more insight in the mechanism underlying the increase in TEAC value with increasing pH, i.e. upon deprotonation, of the $C_{4'}$-and $C_{3'}$-*O*-methylated derivatives of quercetin and luteolin. The actual mechanism for the antioxidant action of these deprotonated forms can still be either hydrogen atom or electron donation or both in different rates. Therefore, Table 2 lists, not only the BDE values for hydrogen atom donation from the weakest remaining OH moieties in the anion (BDE(A)), but also the ionisation potential of the deprotonated anionic molecules (IP(A)). Comparison of the BDE values of the deprotonated forms (BDE(A)) to the BDE

values for the neutral forms (BDE(N)) (Table 2) reveals that there is a decrease in the BDE values upon hydroxyflavone deprotonation, but the decrease is not significant. This implies that on the basis of BDE values the observed increase in radical scavenging capacity of the antioxidants upon deprotonation cannot be explained. In contrast, the parameter reflecting the ease of electron donation, i.e., ionisation potential (IP) is greatly influenced by the deprotonation step; the ionisation potentials drop significantly. This result is in an agreement with previous findings for other hydroxyflavones (*20*)and supports the conclusion that upon deprotonation the TEAC value of hydroxyflavones and their metabolites increases (radical scavenging capacity increases) because electron donation becomes the major mechanism for antioxidant action.

Table 2 Calculated bond dissociation energies (BDE) as well as ionisation potentials (IP) for the neutral (N) and monoanionic (A) form of quercetin, luteolin and their *O*-methylated metabolites.

Compound	BDE(N) [kcal/mol]	IP(N) [eV]	BDE(A) [kcal/mol]	IP(A) [eV]
Quercetin (3',4',3,5,7pentahydroxyflavone)	78.6 (4') [1)]	7.03	73.7 (7) [1)]	2.69 (4') [1)]
3'-*O*Me-Q (Isorhamnetin)	86.9 (3)	6.93	77.1 (4')	3.04 (7)
4'-*O*Me-Q (Tamarixetin)	86.8 (3)	6.92	80.2 (3')	2.99 (7)
Luteolin (3',4',5,7-tetrahydroxyflavone)	80.4 (4')	7.42 (7.32)[2)]	80.7 (7)	2.93 (4')
3'-*O*Me-L (Chrysoeriol)	88.0 (4')	7.35 (7.26)	81.1 (7)	2.83 (4')
4'-*O*Me-L (Diosmetin)	89.0 (3')	7.36 (7.25)	82.0 (3')	2.86 (7)

[1)] the number between brackets refers to the position of OH-moiety.
[2)] the value between brackets indicates the IP calculated as the energy of the radical cation resulting from electron abstraction and stabilised through proton transfer from C_5-OH to C_4=O minus the energy of the neutral parent molecule.

Moreover, the results show that *O*-methylation of the OH group at the $C_{4'}$- or $C_{3'}$-position in quercetin enhances the IP(A) in comparison to the parent aglycone. This would qualitatively explain why $C_{4'}$- and $C_{3'}$-*O*-methylated derivatives of quercetin derivatives, even upon deprotonation, can be less effective antioxidant, reflected in lower TEAC values than the deprotonated form of quercetin itself (Figure 2A).

In contrast to quercetin, *O*-methylation of the OH-group at the $C_{4'}$- or $C_{3'}$-position in luteolin does not enhance the IP(A) (Table 2). This would explain why 4'-*O*-methyl-luteolin and $C_{3'}$-*O*-methyl-luteolin at high pH values, at which the monoanionic form of the compounds prevails, show nearly the same antioxidant activity as luteolin (Figure 2B). This also implies that *O*-methylation has no effect on the TEAC antioxidant activity of luteolin in its deprotonated form. Thus the pH dependent TEAC curves presented in Figure 2a and 2b and the results of the calculations presented in Table 2 indicate that for luteolin, *O*-methylation of the catechol group affects the antioxidant potential only because it shifts the pKa for deprotonation.

For quercetin however, *O*-methylation of the catechol moiety affects antioxidant activity by both an effect on the pKa for deprotonation, but also by a differential effect on the electron and hydrogen donating properties of the neutral and the anionic form of the molecule.

Finally, to explain the observation that *O*-methylation of a catechol group results in antioxidant behaviour similar to the analogue in which the OH group is replaced by a hydrogen atom, the calculated molecular parameters BDE and IP of 3'-*O*-methyl-quercetin, 3'-*O*-methyl-luteolin and 4'-*O*-methyl-luteolin were compared with BDE and IP of their corresponding analogues in which the $C_{3'}$-OH or $C_{4'}$-OH group is substituted by a hydrogen atom (Table 2 and 3).

Table 3 Experimental and theoretically predicted pKa values of analogues of quercetin and luteolin *O*-methylated metabolites, in which the 3'- or 4'-OH group is replaced by a hydrogen atom as well as calculated deprotonation energies (DE), bond dissociation energies (BDE) and ionisation potentials (IP) for the neutral (N) and the anionic (A) forms of these flavonoids.

Compound	pKa observed	DE [kcal/mol]	pKa predicted	BDE(N) [kcal/mol]	IP(N) [eV]	BDE(A) [kcal/mol]	IP(A) [eV]
4',3,5,7-OH	8.29 [1)]	338.7(7) [2)] 339.1(4')	8.10	86.8(3) [2)]	7.08	74.1(4') [2)]	2.73 (7) [2)]
4',5,7-OH	7.37 [3)]	335.5(4') 342.7(7)	7.56	89.5(4')	7.52 (7.33) [4)]	81.1(7)	3.00 (7)
3',5,7-OH	7.25	340.6(7) 346.6(3')	8.45	91.6(3')	7.67 (7.49) [4)]	85.5(3')	3.01 (7)

[1)] pKa value taken from (*25*).
[2)] the number between brackets refers to the position of OH moiety.
[3)] pKa value taken from (*26*).
[4)] the value between brackets indicates the IP calculated as the energy of the radical cation resulting from electron abstraction and stabilised through proton transfer from C_5-OH to C_4=O minus the energy of the neutral parent molecule.

The results show that *O*-methylation of a catechol OH group has a similar effect on the BDE and IP of quercetin and luteolin as OH group removal, that is, replacement of the OH moiety by a hydrogen atom.

Discussion

In the present study the effect of catechol-*O*-methylation on the antioxidant characteristics of two model flavonoids, quercetin and luteolin, was investigated using experimental as well as theoretical methods.

Several *in vivo* studies pointed at catechol-*O*-methylation as a very important phase II metabolism pathway for both quercetin (*11-13*) and luteolin (*4, 10, 13*). In addition, a few studies investigated the possible consequences of catechol *O*-methylation for antioxidant activity of flavonoids, generally reporting a reduction in antioxidant efficacy upon methylation (*8, 27-30*), although others reported the opposite (*31*). Because the 4'-OH group is generally suggested to be the hydroxyl moiety primarily involved in both deprotonation, and in hydrogen donation upon radical scavenging action of quercetin and luteolin (*20, 32*), it was expected that especially *O*-methylation of the 4'-OH moiety may affect deprotonation as well as radical scavenging activities of the flavonoids studied. Results obtained, however, reveal that methylation of the 3'-OH affects both characteristics to almost the same extent as 4’-OH methylation. This supports the view, frequently expressed (*8, 9*) that for optimal antioxidant activity a functional catechol moiety with both 3’-OH and 4'-OH in their unconjugated form, is of importance.

Clearly the reduction in radical scavenging capacity of both luteolin as well as quercetin upon methylation of one of their catechol OH group is in line with other reports indicating that *O*-methylation of the catechol moiety reduces the radical scavenging activity of flavonoids (*8, 28, 30*). The pH dependent TEAC measurements and the theoretical computer calculations of the present study provide new insight into the mechanism behind this effect of *O*-methylation. First of all *O*-methylation was shown to affect the pKa for deprotonation of the flavonoid molecule. Theoretical calculations corroborated that *O*-methylation of the catechol group results in a significant change in deprotonation characteristics, increasing the DE for deprotonation and shifting the preferential site for deprotonation more in favour of 7-OH deprotonation, the latter because 4'-OH deprotonation either becomes impossible due to methylation of the site, or because 4'-OH deprotonation becomes energetically less favourable

upon 3'-*O*-methylation. As a result, the pKa of the flavonoid increases by about 0.2-0.8 pH units upon methylation of its catechol moiety.

These results indicate that the reduction in TEAC activity of the flavonoids upon their *O*-methylation can be, at least in part, ascribed to an increase in their pKa values, resulting in a lower extent of deprotonation at physiological pH. The deprotonated forms of the flavonoids are most active in radical scavenging reactions such as in the TEAC assay (*20*). Therefore, this shift in the deprotonation equilibrium upon *O*-methylation, resulting in a reduced fraction of deprotonated flavonoid molecules at physiological pH for the *O*-methylated metabolites as compared to the parent flavonoids, results in lower TEAC values at physiological pH. Measurement of the pH dependent TEAC profiles as performed in the present study corroborates this conclusion and even suggests that in the case of luteolin the shift in pKa could almost fully explain the effects of *O*-methylation on TEAC activity. This, because at higher pH values the TEAC values of luteolin, 3'-*O*-methyl-luteolin and 4-'*O*-methyl-luteolin become similar again. Thus, the pH dependent TEAC profiles of luteolin and its *O*-methylated analogues reveal a shift in pKa of about 0.6-0.8 pH units as the major cause for the differences in TEAC activity of luteolin and its *O*-methyl derivatives. Results from DFT calculations supported the conclusion that upon full deprotonation luteolin and its *O*-methylated metabolites have almost equal radical scavenging capacities reflected by almost similar IP(A) and BDE(A) values for the different compounds.

For quercetin, the situation appeared different. For quercetin, as for luteolin, *O*-methylation of its catechol moiety increases the pKa for its deprotonation, again in line with computer DFT calculations. However, upon full deprotonation the difference in TEAC value between quercetin and its *O*-methylated analogues still exists. Thus for quercetin, the increase in the pKa for deprotonation contributes to the reduction in TEAC value at physiological pH upon *O*-methylation, but it cannot be the only or not even the dominant mechanism causing the reduction in TEAC at physiological pH upon catechol *O*-methylation. For quercetin, in contrast to luteolin, the radical scavenging characteristics of the deprotonated form of the flavonoid are significantly influenced by *O*-methylation of the catechol moiety. *O*-Methylation of both the 3'-OH and the 4'-OH group of quercetin increases the IP(A) for electron donation and also the BDE(A) for hydrogen donation by the flavonoid anion. This explains why for quercetin, also above the pKa, there is a difference in the TEAC value between the aglycon and its *O*-methylated metabolites resulting in decreased radical scavenging capacity upon *O*-methylation.

Additional results of the present study reveal that *O*-methylation of a catechol OH moiety in luteolin or quercetin, effectively results in removal of the OH moiety as far as antioxidant behaviour is concerned. This conclusion follows from the observation that the pH dependent TEAC profile of 3'-*O*-methyl-quercetin is similar to the pH dependent TEAC profile and TEAC activities of 4', 3, 5, 7-tetrahydroxyflavone, that of 3'-*O*-methyl-luteolin similar to that of 4',5,7-trihydroxyflavone, and, finally, that of 4'-*O*-methyl-luteolin similar to that of 3',5,7-trihydroxyflavone. DFT calculations also corroborate this conclusion showing that catechol *O*-methylation has the same electronic effect on the molecule and its DE, IP and BDE characteristics as replacement of the OH-group by a hydrogen atom, i.e. removal of the hydroxyl group from the molecule. The mechanistic explanations for these similar effects of *O*-methylation or hydroxyl elimination from the flavonoid molecule on its antioxidant and deprotonation characteristics were elucidated in the present study. Clearly, there appear to be two mechanisms underlying the reduction in radical scavenging capacities of flavonoids upon *O*-methylation of their catechol moiety, and/or effective elimination of a catechol hydroxyl group. First, *O*-methylation (OH-elimination) increases the pKa of the flavonoid resulting in lower levels of deprotonation of the flavonoids at physiological pH and, thus, reduced antioxidant properties. Second, *O*-methylation (OH-elimination) may affect the electronic characteristics of especially the deprotonated form of the flavonoid, reducing its capacity for electron and hydrogen atom donation. Especially of interest is the fact that the results of the present study reveal that the extent to which these two mechanisms are relevant depends on the substituent pattern of the flavonoid. Especially the absence of the 3-OH substituent reduces the consequences of *O*-methylation for the antioxidant properties of the deprotonated form. This may relate to the fact that the presence of the 3-OH is crucial in generating possibilities for conjugating interactions between the B and the C rings. When absent, modifications in the B ring, including *O*-methylation or OH removal, apparently affect the electron donating characteristics of the molecule to a lesser extent than when the 3-OH is present.

Finally, it is of interest to stress that in theory *O*-methylation may also hamper the radical scavenging capacity of the flavonoids by hampering a possible second electron oxidation. Such a second electron oxidation by the flavonoid would give rise to a flavonoid quinone-type reaction (*33, 34*). However, formation of a quinone, upon a second electron oxidation, is expected to be hampered by methylation at the 4'-OH but not by methylation at the 3'-OH moiety of quercetin or luteolin. This because methylation of the 3'-OH leaves the possibility

for formation of a quinone methide metabolite from quercetin and luteolin. This would imply that if *O*-methylation would slow down the radical scavenging capacity by preventing quinone formation upon a second elecgtron donation, methylation at the 4'-OH should have a larger effect than methylation of the 3'-OH. The experimental data obtained in the present study reveal that this is not the case, sinced methylation of the 3'-OH and 4'-OH was shown to affect the radical scavenging ability to exactly the same extent. Altogether the results of the present study provide new mechanistic insight in the effect of *O*-methylation of flavonoids, a physiologically relevant phase II modification, on their pH dependent antioxidant characteristics.

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4

Formation of transient covalent protein and DNA adducts by quercetin in cells with and without oxidative enzyme activity

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Abstract

This study investigates the role of cellular tyrosinase and/or peroxidase-like oxidative enzyme activity in the covalent binding of quercetin to glutathione, protein and DNA as well as the stability of quercetin DNA adducts in time. This was done by studying the formation of glutathionyl quercetin adducts in various *in vitro* models, and the covalent binding of radio-labeled quercetin to protein and DNA in cells with elevated peroxidase or tyrosinase levels, and in cells devoid of nucleotide excision repair (NER).

Cells with elevated tyrosinase or peroxidase levels contained approximately two times higher levels of covalent quercetin adducts than cells without detectable levels of these oxidative enzymes. However, this difference was smaller than expected based on the differences in tyrosinase and/or peroxidase levels, indicating that these types of oxidative enzyme activities do not play a major role in the cellular pro-oxidant activity of quercetin. Furthermore, quercetin DNA adducts were of transient nature, independent of the presence of NER, suggesting chemical instability of the adducts. Whether this transient nature reflects real reversibility, or formation of genotoxic depurinated sites remains to be investigated at the molecular level.

Together, these data indicate that formation of covalent quercetin adducts can be expected in all cells, independent of their oxidative enzyme levels, whereas the transient nature of the DNA adducts formed may limit or cause their ultimate biological impact. If the transient nature represents chemical reversibility of the adduct formation, it would provide a possible explanation for the apparent lack of *in vivo* carcinogenicity of this *in vitro* mutagen. Therefore, *in vitro* mutagenicity studies should focus more on the transient nature of DNA adducts responsible for the mutagenicity *in vitro*, since this transient nature of DNA adducts may play an essential role in whether the genotoxicity observed *in vitro* will have any impact *in vivo*.

Introduction

Flavonoids are important constituents of various fruits, vegetables, seeds and nuts (*1, 2*). The Western-European diet contains on average approximately 3 to 58 mg of flavonoids per day, to which the flavonol quercetin (Figure 1) contributes approximately 40 to 100% (*3*). For decades, quercetin has been widely investigated, mainly because of its putative health-promoting effects, reflected in for example potential protection against coronary heart disease (*3, 4*). These health claims are merely supported by *in vitro* evidence related to the strong antioxidant activity (*5*) and ability of quercetin to modulate the activity of numerous enzymes involved in signal transduction, cell growth (*2, 6*) and biotransformation (*7*). Nevertheless, the supposed health claims associated with quercetin have elicited interest in and provided possibilities for its use as food supplement and in functional food applications. A consequence of this trend is an increased human exposure to quercetin, up to 40-fold the present average daily intake, based on the recommended dosing of food supplements.

However, at the same time, other studies pointed at possible adverse health effects caused by quercetin. Quercetin appears to be genotoxic in various *in vitro* systems (*8-10*), even without metabolic activation (*8*). The so-called pro-oxidant activity of quercetin is thought to play a role in the mutagenic activity of quercetin (*2*). The mechanism suggested to underly the carcinogenic activity of various compounds, including estrogens (*11, 12*), involves metabolic activation leading to the formation of catechol metabolites and subsequent oxidation to *o*-quinone and in some cases *p*-quinone methide metabolites. Quercetin already possesses a catechol group in the B-ring (Figure 1), which, due to its oxidant properties, provides a basis for the pro-oxidative toxic effects of this flavonoid (*13*). Figure 1 depicts the reaction mechanism possibly involved in the pro-oxidant activity of quercetin leading to the formation of quinone-type metabolites and covalent adducts to cellular (macro-) molecules, as hypothesized previously (*14, 15*). Evidence for this mechanism was generated by structure activity studies on mutagenicity of quercetin and quercetin derivatives (*8*) and further supported by the glutathione trapping method, as well as quantum mechanical calculations (*14*).

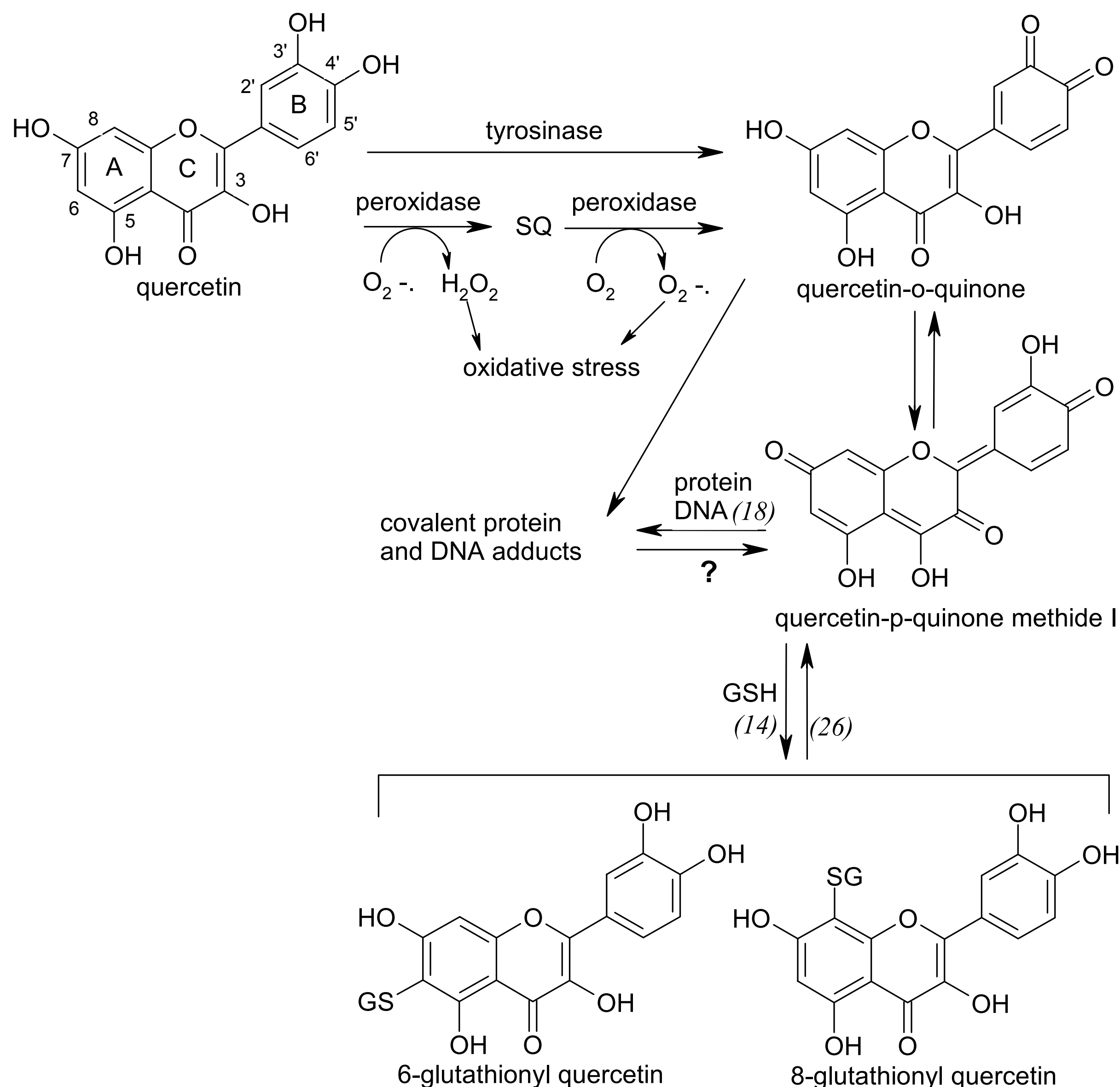

Figure 1 Schematic presentation of the pro-oxidant activity of quercetin, as hypothesized previously (*14, 15*), leading to the formation of 6- and 8-glutathionyl quercetin.

The oxidation of quercetin to quercetin-*o*-semiquinone and quercetin-*o*-quinone may occur chemically in the presence of molecular oxygen and/or superoxide anion, especially when metal ions, including copper and iron, are present (*13*). In addition, previous work showed a central role for oxidative enzyme activity in the formation of pro-oxidant quinone type metabolites of quercetin, both in a test tube using the glutathione trapping method and in a tyrosinase-rich cellular *in vitro* model B16-F10 (*14-16*). Besides being prone to redox cycling, quinone-type metabolites, especially quinone methides are potent electrophiles (*17*). Recently, evidence for covalent binding of quercetin to DNA and protein was obtained in several *in vitro* systems (*18*).

In spite of the genotoxic effects of quercetin in various *in vitro* systems (*8-10*), quercetin appears not to be carcinogenic *in vivo* (*19-21*). One reason for the difference in the effects of quercetin between the *in vitro* and *in vivo* situation may be the extensive phase II metabolism of quercetin *in vivo* (*22, 23*). Because conjugation of the hydroxyl groups of quercetin generally attenuates its biological activity (*24, 25*), phase II metabolism of quercetin may eliminate the genotoxic effects found *in vitro*. A second explanation for the discrepancy between *in vitro* and *in vivo* behavior of quercetin may be that DNA adduct formation by quercetin may be reversible in time. Evidence for the transient nature of covalent quercetin adducts was obtained previously for covalent glutathionyl quercetin adducts (*18, 26*) but the possible reversible nature of quercetin DNA adducts has not been investigated before.

To obtain more insight into the consequences of the intracellular pro-oxidant activity of quercetin, the objective of the present study was two-fold: 1) to study the role of tyrosinase- and peroxidase-type oxidative enzyme activity in the formation of covalent glutathione, protein and DNA adducts, and 2) to study the stability of quercetin DNA adducts in the course of time. This was done by studying the formation of glutathionyl quercetin adducts in various *in vitro* model systems, as well as the covalent binding of radio-labeled quercetin to protein and DNA of cells with elevated peroxidase or tyrosinase levels, or in cells with or without the nucleotide excision repair (NER) system. The NER system is the DNA repair machinery typically involved in the removal of stable bulky adducts (*27*). Because quercetin adducts represent bulky adducts, one can foresee that the NER system might be recruited for the possible removal of quercetin DNA adducts.

Materials and Methods

Materials

Dimethylsulfoxide (DMSO), glutathione, quercetin dihydrate and trichloroacetic acid were obtained from Acros Organics (New-Jersey, USA). Phenol:chloroform premixed with isoamylalcolhol (25:24:1) was obtained from Amresco (Solon, Ohio, USA). Sodium dodecylsulfate (SDS) was purchased from BDH Biochemical (Poole, UK). [4-^{14}C]Quercetin dihydrate (specific activity 53.1 mCi/mmol) was a generous gift from dr. J.M.M. van Amelsvoort from Unilever Research Vlaardingen (the Netherlands) and was purchased from Chemsyn Science Laboratories (Lenexa, Kansas, USA). Tris was purchased from Invitrogen (Paisley, UK). Ethylene-bis-(oxyethylenenitrolo)-tetra-acetic acid (EGTA) was purchased from Janssen Chimica (Geel, Belgium). Trifluoracetic acid was purchased from J.T. Baker

(Philipsburg, NJ). Acetonitril was from LabScan Ltd. (Dublin, Ireland). Ascorbic acid, ethylene-diamine-tetra-acetic acid (EDTA), methanol, di-potassium hydrogen phosphate, ethanol, ether, hydrochloric acid, magnesium chloride, potassium chloride, potassium dihydrogen phosphate, sodium chloride and sodium hydroxide were from Merck (Darmstadt, Germany). Pellet paint Co-precipitant® was obtained from Novagen (Madison, WI, USA). Bovine serum albumin, cetyltrimethylammonium bromide, dopamine (β-(3,4-dihydroxyphenyl)-ethylamine hydrochloride), glucose, guaiacol (2-methoxyphenol), HEPES, horseradish peroxidase (HRP; EC 1.11.1.7), hydrogen peroxide (H_2O_2), lactoperoxidase (LPO; from bovine milk, EC 1.11.1.7), myeloperoxidase (MPO; from human leukocytes, EC 1.11.1.7), phenylmethylsulfonyl fluoride, proteinase K, ribonuclease A (RNAse; from bovine pancreas, EC 3.1.27.5), spermidine, spermine, tyrosinase (from mushroom, EC 1.14.18.1) and Triton X-100 were obtained from Sigma (Saint Louis, MO, USA).

Dulbecco's Modified Eagle's Medium (DMEM), RPMI-1640, non-essential amino acids, gentamicin, L-glutamine, sodium pyruvate, bovine insulin, fetal calf serum (FCS), phosphate buffer solution (PBS), penicillin-streptomycin, Hank's Balanced Salt solution (HBSS) were obtained from Gibco (Paisley, UK). William's Medium E (WME) and Eagle's modified Minimum Essential Medium (EMEM) were obtained from Sigma (Saint Louis, MO, USA). Antibiotic/antimycotic (consisting of penicillin, streptomycin and amphotericin) was purchased from Cellgro (Amsterdam, The Netherlands).

Cell lines

The human promyelotic leukemia cell line HL60, the mouse melanoma cell line B16-F10, the human hepatocarcinoma cell line HepG2 and the human colon carcinoma cell line Caco-2 were purchased from the American Type Culture Collection (Manassas, VA, USA). Both HepG2 and Caco-2 cells proved to be excellent cellular models to study the covalent binding of quercetin to DNA and protein (*18*). In addition, HL60 and B16-F10 cells are known to express elevated levels of peroxidase and tyrosinase activity, respectively (*16, 28*) and were therefore used to study the effect of the intracellular presence of these types of oxidative activity on covalent adduct formation of quercetin with glutathione, DNA and protein. The mouse embryonic fibroblasts MEF-XPA+/+ and MEF-XPA-/- were obtained as previously described (*29*). Cell lines were grown at 37°C in a humidified atmosphere at 5% CO_2 in media supplemented with 10% fetal bovine serum, and if applicable, other additions, as indicated in Table 1.

Table 1 Composition of culture media for the cell lines used in this study.

Cell line	Culture medium	Antibiotic	Other additions
B16F10	DMEM	50 μg/mL gentamycin	25 mM HEPES
Caco-2	EMEM	100 IU/mL penicillin 100 IU/mL streptomycin	Non-essential amino acids
HepG2	WME	100 IU/mL penicillin 100 IU/mL streptomycin 0.25 μg/mL amphotericin	2 mM L-glutamine
HL-60	RPMI-1640	50 μg/mL gentamycin	2 mM L-glutamine
MEF	DMEM	100 IU/mL penicillin 100 IU/mL streptomycin	Non-essential amino acids

Oxidative enzyme incubations

Standard reaction mixtures in a final volume of 1 mL consisted of (final concentrations, added in this order) 25 mM KP_i pH 7.0, 5 mM reduced glutathione (GSH), enzyme (100 U/mL tyrosinase, 1 U/mL MPO, LPO or HRP) and 100 μM quercetin from a 10 mM stock solution in DMSO. The reaction was started by the addition of 0.3 mM H_2O_2 from a 5.2 mM stock solution in nanopure. Incubations were performed in a 37°C stirring water bath for 8 minutes. Then, samples were frozen in liquid nitrogen and stored at -80°C until HPLC analysis.

Tyrosinase and peroxidase activity in cell lines

Preparation of cell homogenate

Preparation of cell homogenate was based on Kagan *et al.* (2001) (*28*), with some minor modifications. To measure the activity of peroxidase and tyrosinase in cell lines, confluent cells were scraped in 6 mL assay buffer. The assay buffer consisted of 0.1 M KP_i pH 7.0, supplemented with 0.1% Triton X-100, 0.1 mM phenylmethyl-sulfonyl fluoride and 0.02% cetyltrimethylammonium bromide. The cells were then sonicated for 15 min on ice. After centrifugation at 4000 rpm (1310 g) for 5 min, the supernatant was used in the peroxidase and tyrosinase assays. Protein was quantified according to Lowry *et al.* (*30*) using bovine serum albumin as the standard.

Peroxidase assay

The peroxidase assay was based on a method previously described (*31*) with some minor modifications. A typical incubation mixture in assay buffer (final volume 1 mL) consisted of cell homogenate and 15 mM guaiacol from a 3 M stock solution in DMSO. The reaction was started by the addition of 0.26 mM H_2O_2 from a 5.2 mM stock solution in reaction buffer. After rapid homogenization, the absorbance at 470 nm was followed over time at room temperature using a spectrophotometer coupled to a recorder. Peroxidase catalyzes the oxidation of guaiacol to tetraguaiacol. From the change in A_{470} over time, the enzyme activity can be calculated for the reaction: 4 guaiacol + 2 H_2O_2 $\rightarrow$ tetraguaiacol + 4 H_2O

with $\varepsilon(\text{tetraguaiacol})_{470} = 26600\ M^{-1}cm^{-1}$.

Peroxidase activity was expressed in nmol tetraguaiacol/minute/mg protein, after correction for the activity of the control incubation in which H_2O_2 was absent. The conditions used were such that the enzyme activities were linear in time and proportional to the amount of protein present in the incubations (data not shown).

Tyrosinase assay

A typical incubation mixture (final volume 1 mL) in assay buffer consisted of cell homogenate and 12.8 mM dopamine from a 1280 mM stock solution in 0.1 M KP_i pH 7.0. After quick homogenization, the absorbance at 475 nm was followed in time at room temperature, using a spectrophotometer coupled to a recorder. Tyrosinase catalyses the oxidation of dopamine to dopamine-*o*-quinone. Dopamine-*o*-quinone immediately disproportionates into aminochrome. From the change in A_{475} over time, the enzyme activity can be calculated for the reaction: dopamine $\rightarrow$ dopamine-*o*-quinone $\rightarrow$ aminochrome

with $\varepsilon(\text{aminochrome})_{475} = 3058\ M^{-1}cm^{-1}$ (*32*).

Activities were expressed in nmol aminochrome/minute/mg protein after correction for the activity of the control incubation in which dopamine was absent. The conditions used were such that the enzyme activities were linear in time and proportional to the amount of protein present in the incubations (data not shown).

Determination of quercetin glutathione conjugates

Incubations with Caco-2 and HepG2 cells, growing in monolayers, were performed as described before (*16*). HL-60 cells, growing in suspension, were harvested, centrifuged at 1500 rpm (200 g; Eppendorf Centrifuge 5415C, Hamburg, Germany) for 8 min at 4°C and

diluted to a concentration of $2x10^6$ cells/mL in HBSS without phenol red, supplemented with 5 mM glutathione for quercetin stabilization. Cells were exposed in 48-wells plates (0.5 mL/well) to 75 μM quercetin from a 15 mM stock solution in DMSO. In control incubations, either quercetin or HL-60 cells were absent. The cells were incubated in a humidified atmosphere with 5% CO_2 at 37°C. After 30, 60, 90 and 120 min, samples were frozen in liquid nitrogen and stored at -80°C until HPLC analysis of the supernatant. Glutathionyl conjugates of quercetin were quantified on the basis of their peak area in the HPLC chromatogram at 290 nm, using reference curves of 6- and 8-glutathionylquercetin.

Covalent protein adduct formation

The determination of the amount of [4-^{14}C]-quercetin covalently bound to protein was based on a method previously described (*18*). Cells were grown in monolayers in 6-wells plates until confluency. Then, cells were exposed in triplicate (2 wells per sample) to 5 μM [4-^{14}C]-quercetin in the presence of 1 mM ascorbic acid for quercetin stabilization (*33*) at 37°C in a humidified atmosphere. After 0 min, 10 min and 2 hours, cells were harvested according to the following procedure. The cells were scraped in 2 mL ice cold 0.9% sodium chloride.

The HL-60 cells, growing in suspension, underwent a different treatment. Exposure took place in 2 mL Eppendorf tubes containing 1 mL of a $6x10^6$ cells/mL cell suspension. The cells were exposed in triplicate to 5 μM [4-^{14}C]-quercetin at 37°C in a humidified atmosphere. Because ascorbic acid is a known inhibitor of peroxidase activity (*31*), 5 mM reduced glutathione was used for quercetin stabilization also preventing the auto-oxidation of quercetin (data not shown). After 0 min, 10 min and 2 hours, cells were harvested according to the following procedure. At the end of the incubation, 0.8 mL icecold 0.9% saline was added to the cell suspension. All centrifugation steps in the protocol for the determination of covalent binding to protein were performed using an Eppendorf Centrifuge, type 5415C (Hamburg Germany), unless stated otherwise.

After quick homogenization, the cells were pelleted by centrifugation at 14000 rpm (16000 g) for 20 sec. Because of this treatment, the minimum time until the end of the exposure was approximately 1 min. Therefore the first sample that could be collected represents a t = 1 min sample. Supernatant was discarded and the cells were washed three times with ice cold 0.9% saline. After the third washing step, cells were resuspended in 2 mL ice-cold 0.9% sodium chloride. The suspensions obtained from both cells growing in monolayer and cells growing in suspension were sonicated using a small Polytron for 2x 10 seconds on ice. The protein

was precipitated with 1 mL 25% (w/v) trichloroacetic acid in de-ionized water. After centrifugation at 3000 rpm (2000 g, Hermle Labortechnik C Centrifuge, type Z400K, Wehingen, Germany) for 5 min, the pellet was resuspended in 1 mL 5% (w/v) trichloroacetic acid in de-ionized water. The suspension was centrifuged at 11000 rpm (10000 g) for 5 min, after which the pellet was subsequently washed twice with 1 mL 80% (v/v) methanol in de-ionized water, twice with 1 mL hot 80% (v/v) methanol in de-ionized water (heated to approximately 55°C), twice with 1 mL methanol:ether (50:50) and finally twice with 1 mL 80% (v/v) methanol in de-ionized water. The pellet was digested overnight at room temperature in 1 mL 0.5 M sodium hydroxide. Protein was quantified according to Lowry *et al.* (*30*) using bovine serum albumin as a standard and the radioactivity in the protein fraction was measured using a scintillation counter.

Covalent DNA adduct formation

For determination of covalent binding of [4-^{14}C]-quercetin to DNA, cells were grown and exposed in a similar way as described above for covalent protein adduct formation (*18*). After exposure times of 0 hour, 10 min and 2 hours, the exposure medium was removed and the cells were washed three times with a cold 0.9% saline solution. Then, in the case of cells growing in monolayers, 0.7 mL lift buffer was added to the wells and the plates were incubated for 5 min at 37°C in a humidified atmosphere, to allow detachment of the cells. The lift buffer consisted of 10 mM Tris-HCl pH 8.0 containing 0.14 M NaCl and 1 mM EDTA. The cell suspension was centrifuged at 2000 rpm (325 g) for 5 min to obtain a cell pellet. All centrifugation steps in the protocol for the determination of covalent binding to DNA were performed using an Eppendorf Centrifuge, type 5415C (Hamburg Germany).

Cell pellets obtained from the procedures described for cells growing in monolayers and cells growing in suspension were gently resuspended in 0.7 mL cold swell buffer containing 7 µL 10% Triton-X100 and left on ice for 10 min. The swell buffer consisted of 100 mM HEPES pH 8.0, containing 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine 0.1 mM EDTA and 0.1 mM EGTA. The nuclei were precipitated by centrifugation at 14000 rpm (16000 g) for 2 min and resuspended in 0.6 mL swell buffer. To further purify the nuclei, this suspension was centrifuged in 30% sucrose in swell buffer for 10 min at 4000 rpm (1310 g). The supernatant was discarded. The pellet was subsequently resuspended in 0.5 mL extraction buffer to which 1 µL 10 mg/mL RNAse in extraction buffer, 3 µL 20 mg/mL proteinase K in demineralised water and 1.5 µL 20% sodium dodecylsulfate in demineralised water were added. The

extraction buffer consisted of 10 mM Tris-HCl pH 8.0, containing 40 mM EDTA. The mixture was incubated overnight at room temperature.

Then, the DNA was extracted by the addition of 900 μL phenol:chloroform:isoamylalcohol (25:24:1). The mixture was vortexed for 30 sec and transferred to Phase Lock Gel vials (Eppendorf, Hamburg, Germany) and centrifuged at 14000 rpm (16000 g) for 4 min. The aqueous phase was decanted to a clean eppendorf vial. Then, 30 μL ice cold 3 M sodium acetate pH 5.2, 2 μL Pellet Paint Co-Precipitant and 1 mL ice cold 100% ethanol were added to the aqueous phase to precipitate the DNA. After vortexing, the DNA was pelleted by centrifugation for 5 min at 14000 rpm (16000 g). The DNA pellet was washed twice with 1 mL ice cold 100% ethanol, after which the pellet was dissolved in 0.5 mL de-ionized water.

The DNA was quantified by measurement of A_{260} and purity was checked by using the A_{260}/A_{280} ratio (1.6< ratio <1.8). Then, the radioactivity present in the DNA was measured using a scintillation counter.

Extended time course of DNA adduct formation

To study the time course of DNA adduct formation in HepG2 cells, a similar experimental set up was used as described before in the section 'Covalent DNA adduct formation'. HepG2 cells were exposed for 2 hours to 5 μM [4-^{14}C]-quercetin in the presence of 1 mM ascorbic acid, after which the medium was replaced by quercetin-free medium containing 1 mM ascorbic acid. Upon replacement of the exposure medium by quercetin-free medium, cell samples in triplicate were taken at the indicated time points up to 24 hours. Calculation of the half time of decomposition of covalent quercetin DNA adducts was performed with the equation 1:

$$T_{1/2} = \frac{\ln 2}{\lambda} \qquad \textit{equation 1}$$

in which $T_{1/2}$ = half time (h), and λ = first order dissociation rate constant (h^{-1}).

The first order dissociation rate constant λ was derived from equation 2:

$$N(t) = N(0) \times e^{-\lambda t} \qquad \textit{equation 2}$$

in which N(t) = DNA adduct level at time t (pmol/mg)

N(0) = DNA adduct level at 0 hours (pmol/mg)

Covalent DNA adduct formation in NER-deficient mouse embryo fibroblasts (MEF-XPA-/-)

MEF-XPA-/- cells and the corresponding wild type MEF-XPA+/+ cells (Passage 4-6) were seeded in 150 cm^2 tissue culture flasks and grown to 70-80% confluency. Then, the medium was removed and 8 mL exposure medium was added to the cells. Exposure medium consisted of DMEM supplemented with non-essential amino acids, 1 mM ascorbic acid and 5 μM [4-^{14}C]-quercetin from a 10 mM stock solution in DMSO. Flasks were incubated in duplicate for the indicated periods at 37°C in a humidified atmosphere with 5% CO_2. At the end of the incubation time, exposure medium was removed and the cell layer was washed three times with 5 mL ice cold 0.9% saline. Then, cells were detached from the bottom of the flask by incubation with 8 mL lift buffer for 20 minutes in the cell incubator. The cell suspension was subsequently centrifuged at 2000 rpm (325 g, Eppendorf Centrifuge, type 5415C, Hamburg Germany) for 5 minutes, after which the cell pellet was treated as described above in the section 'Covalent DNA adduct formation' to isolate DNA and determine the amount of covalently bound [4-^{14}C]-quercetin.

HPLC analysis

HPLC analysis was performed with a Waters M600 liquid chromatography sytem (Millipore Corporation, Bedford MA), using an Alltima C18 column (4.6 x 150 mm; Altech, Breda, The Netherlands). The column was eluted with water containing 0.1% (v/v) trifluoracetic acid, using a linear gradient with 5-30% acetonitrile in 18 min, followed by 2 min of isocratic elution with 30% acetonitrile, followed by 30-40% acetonitrile from 20 to 25 min, 40-60% acetonitrile from 25 to 28 min, 60-100% acetonitrile from 28 to 30 min and 100% acetonitrile from 30 to 35 min. A flow rate of 0.7 mL/min and an injection loop of 5 μL were used. Detection was performed with a Waters 996 photodiode array detector and performed between 200 and 450 nm. Chromatograms presented are based on detection at 290 nm.

Statistical analysis

The statistical significance of differences was evaluated using Student's two-tailed unpaired *t*-test for equal or unequal variations. The F-test was used to distinguish between equal or unqual variations. Differences were significant if $P < 0.05$.

Results

Oxidation of quercetin by different oxidative enzymes in the presence of glutathione

To determine which enzymes might catalyze the formation of quercetin quinone metabolites, several enzymes with peroxidase or tyrosinase activity were incubated with quercetin using the glutathione trapping method (*14*). Figure 2 shows the chromatograms of the incubation of quercetin with tyrosinase (A), horseradish peroxidase (B), myeloperoxidase (C), and lactoperoxidase (D) in the presence of glutathione. The chromatograms all reveal the formation of two peaks with retention times of 9.8 and 11.7 min. Based on the retention times and the UV-spectra, which were similar to those previously reported for glutathionyl quercetin adducts identified by LC-MS, UV and NMR (*14*), these metabolites were identified as 8- and 6-glutathionyl quercetin, respectively (for structure, see Figure 1). In all samples, the metabolites were formed at a ratio of approximately 0.49:0.51 respectively, as reflected by the areas of the peaks in the HPLC chromatograms, which was in line with previous results (*14*). The results indicate that all of these four enzymes are able to catalyze the conversion of quercetin to quinone-type metabolites.

Determination of peroxidase and/or tyrosinase enzyme activity in various cell lines

To characterize cell lines that might be appropriate *in vitro* models to study the importance of cellular tyrosinase- and peroxidase-type antioxidative enzyme activities in the formation of covalent quercetin protein and DNA adducts, the homogenates of Caco-2, HepG2, B16-F10 and HL-60 cells were screened for peroxidase and tyrosinase activity. In the human promyelotic leukemia cell line HL-60, the peroxidase activity was 175 ± 5 nmol tetraguaiacol/min/mg protein, identified according to literature as myeloperoxidase (*28*). In the mouse melanoma cell line B16-F10, a tyrosinase activity of 981 ± 153 nmol aminochrome/min/mg protein was determined. Caco-2 and HepG2 cells showed no detectable enzyme activity in either the peroxidase or the tyrosinase assay. Based on the results of this experiment, B16-F10 and HL-60 cells proved to be appropriate cell models to study the covalent protein and DNA binding of quercetin in cells containing tyrosinase or peroxidase activity respectively, whereas Caco-2 and HepG2 appear to represent cell lines without these activities.

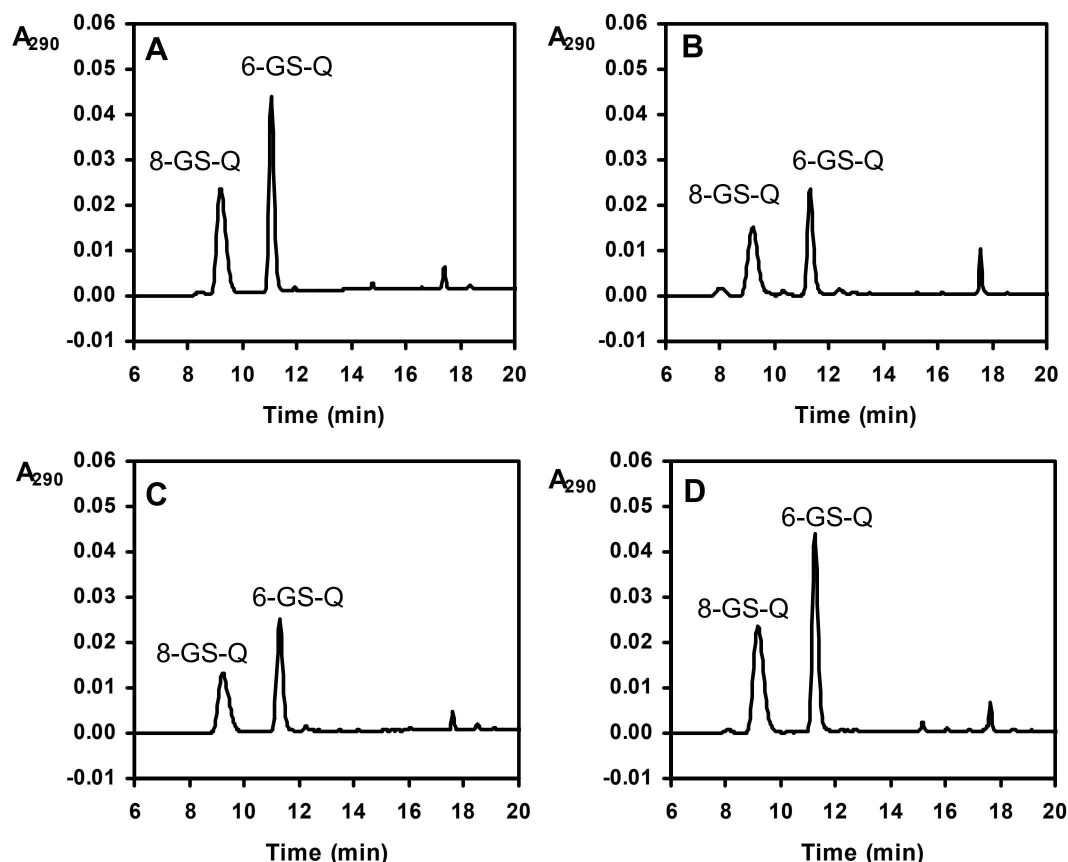

Figure 2 HPLC chromatograms of the incubation of tyrosinase (A), horseradish peroxidase (B), myeloperoxidase (C) or lactoperoxidase (D) with quercetin (retention time 22.4 min) in the presence of glutathione. 6-GS-Q = 6-glutathionyl quercetin, 8-GS-Q = 8-glutathionyl quercetin, identified previously (*14*).

Formation of quercetin glutathione conjugates in cells with or without tyrosinase- or peroxidase-type enzyme activities

Given the absence of tyrosinase or peroxidase-like enzyme activity in Caco-2 and HepG2 cells, these cell lines were used for comparison to investigate the influence of elevated tyrosinase and/or peroxidase-like enzyme activities found in B16-F10 and HL-60 on the formation of covalent glutathione adducts by quercetin. The pro-oxidant activity of quercetin has previously been shown to be relevant in tyrosinase-rich B16-F10 mouse melanoma cells exposed to quercetin, reflected by the formation and excretion of glutathionyl quercetin adducts by these cells when exposed to quercetin (*16*). To investigate whether similar to tyrosinase-rich B16-F10 cells, peroxidase-rich HL-60 cells also excrete glutathionyl conjugates of quercetin in the medium, HL-60 cells were incubated with quercetin in the presence of glutathione for stabilization, also preventing the auto-oxidation of quercetin (data not shown). Table 2 shows the amounts of 6- and 8-glutathionyl quercetin formed in the medium of HL-60 cells and for comparison also B16-F10 cells (*16*) after a 1-hour incubation

with quercetin. No glutathionyl quercetin adducts were detected in the medium of quercetin-exposed HepG2 and Caco-2 cells, in which no tyrosinase or peroxidase-type oxidative enzyme activity was detected and in which the quercetin was also stabilized by addition of glutathione to the incubation medium. The absence of glutathionyl conjugates of quercetin in the medium of cells without any oxidative activity provides the evidence that the quercetin glutathione conjugates detected in the medium of cell lines with tyrosinase- or peroxidase-type enzyme activities were formed intracellular and not by auto-oxidation in the medium. Altogether, the results indicate that especially cells rich in tyrosinase- or peroxidase-type enzyme activity, including B16-F10 (*16*) and HL-60 cells respectively, excrete significant amounts of quercetin glutathione conjugates in the medium during exposure to quercetin.

Table 2 Amounts of 6-glutathionyl quercetin (6-GSQ) and 8-glutathionyl quercetin (8-GSQ) formed in the medium of cell lines after 1 hour of incubation with 75 μM quercetin. The detection limit for 6- and 8-GSQ was approximately 0.4 μM. *n.d.* = not detectable.

Cell line	8-GSQ (μM)	6-GSQ (μM)	Ratio 8-GSQ: 6-GSQ
B16F10 (*16*)	2.93	3.07	0.49 : 0.51
Caco-2	*n.d.*	*n.d.*	-
HepG2	*n.d.*	*n.d.*	-
HL-60	2.98	3.05	0.49 : 0.51

Formation of covalent protein adducts in cells exposed to [4-^{14}C]-quercetin

Figure 3 shows the quantification of the formation of covalent protein adducts in Caco-2 (A), HepG2 (B), B16-F10 (C) and HL-60 (D) cells exposed to quercetin up to 2 hours in the presence of ascorbic acid. In Caco-2 cells (panel A), the amount of protein adducts significantly increased up to 2 hours, to a maximum of 95 pmol quercetin/mg protein. In HepG2, B16-F10 and HL-60 cells (panels B, C and D), maximum protein binding was detected after 10 minutes, and amounted to 322, 144 and 120 pmol quercetin/mg protein, respectively. The amount of covalent protein binding in HepG2 cells is remarkably higher than in the other cell lines tested, which might be related to the preferential binding of quercetin to human serum albumin (*34*), a protein expressed in large quantities by this cell line (*35*). In three of the four cell lines, the amount of covalent protein adducts was lower

after 2 hours as compared to the amount present after 10 minutes. The decrease was significant for both HepG2 and B16-F10 cells. These results provide an indication that the covalent binding of quercetin to cellular macromolecules is reversible.

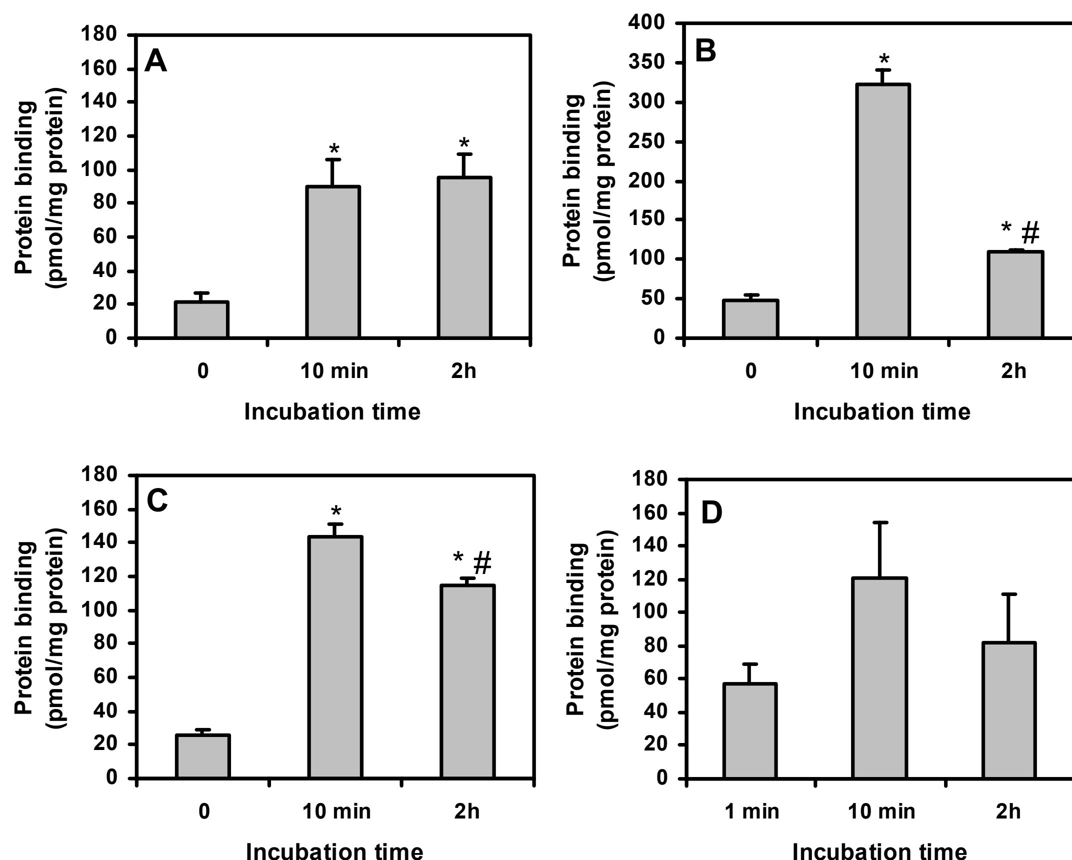

Figure 3 Covalent binding of [4-^{14}C]-quercetin to protein of Caco-2 (A), HepG2 (B), B16F10 (C) and HL-60 (D) cells, exposed to 5 μM [4-^{14}C]-quercetin in the presence of 1 mM ascorbic acid for the indicated time periods. Results are presented as mean +/- SEM. * Significantly different from t = 0 ($P < 0.05$). # Significantly different from t = 10 min ($P < 0.05$).

Formation of covalent DNA adducts in cells exposed to [4-^{14}C]-quercetin

Figure 4 shows the results from the quantification of the formation of covalent DNA adducts in Caco-2 (A), HepG2 (B), B16-F10 (C) and HL-60 (D) cells exposed to quercetin up to 2 hours in the presence of ascorbic acid. Due to the absence of tyrosinase- and peroxidase-type oxidative enzyme activity in Caco-2 and HepG2 cells, these cell lines were used for comparison to investigate the influence of elevated tyrosinase and peroxidase activities found in respectively B16-F10 and HL-60 cells on covalent DNA adduct formation by quercetin. In all cell lines, except for HL-60 (panel D), the amount of DNA adducts increased significantly up to 2 hours of incubation. In HL-60 cells, the amount of DNA adducts after 2 hours of

incubation was significantly lower (approximately 50%) than the amount present after 10 minutes, providing an indication that the formation of covalent DNA adducts might be a reversible process. The maximum amounts of DNA adducts formed in B16-F10 and HL-60 cells (panels C and D) were comparable, amounting to 33 and 37 pmol quercetin/mg DNA respectively. This is approximately two times higher than the maximum amount of DNA adducts detected in the incubations with Caco-2 or HepG2 cells (panels A and B). The results show that in the cell lines with detectable peroxidase or tyrosinase activity (HL-60 and B16-F10) significantly higher levels of covalent DNA adducts are formed ($P<0.05$) as compared to the cell lines without detectable peroxidase and/or tyrosinase activity (HepG2 and Caco-2), although the difference between the cell lines does not match the much larger difference in peroxidase and tyrosinase activities.

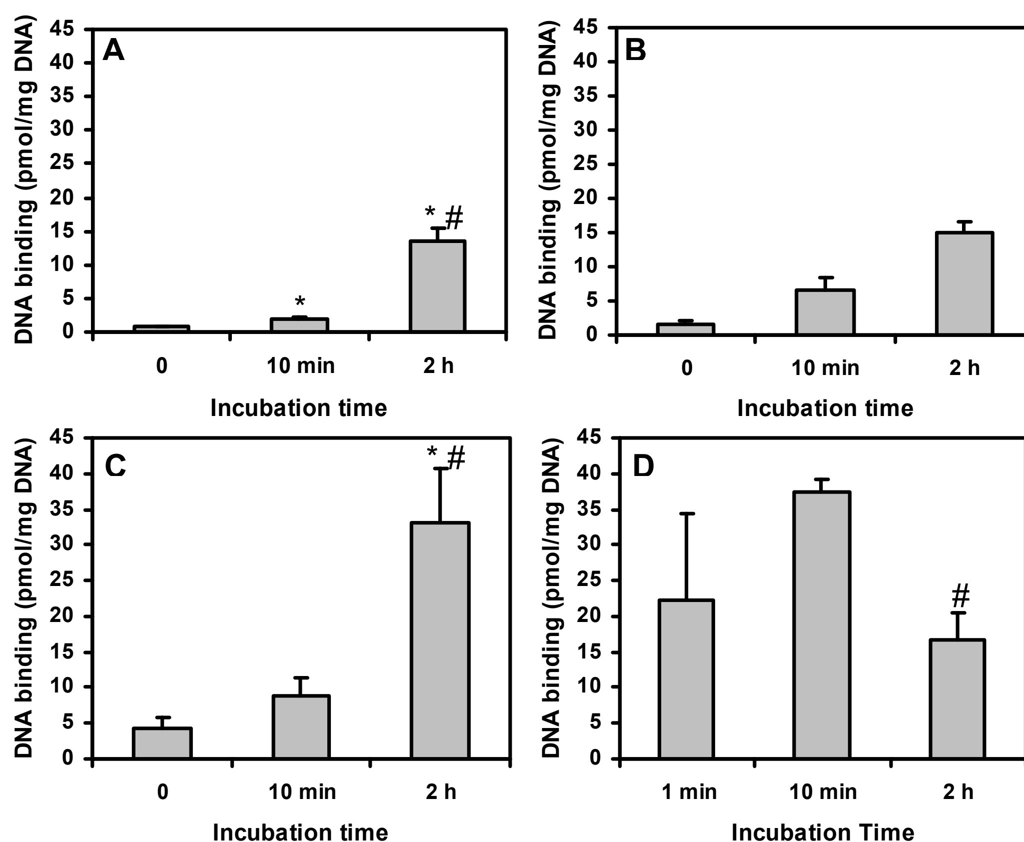

Figure 4 Covalent binding of [4-^{14}C]-quercetin to DNA of Caco-2 (A), HepG2 (B), B16F10 (C) and HL-60 (D) cells, exposed to 5 μM [4-^{14}C]-quercetin in the presence of 1 mM ascorbic acid for the indicated time periods. Results are presented as mean +/- SEM. * Significantly different from t = 0 ($P < 0.05$). # Significantly different from t = 10 min ($P < 0.05$).

Time course of covalent DNA adduct formation in HepG2 cells exposed to [4-^{14}C]-quercetin

Because the quantification of covalent protein and DNA adduct formation during exposure to quercetin gave indications for the reversibility of this process (Figures 3B, C and D and Figure 4D), the stability of DNA adducts was investigated in HepG2 cells exposed to quercetin for 2 hours, after which the medium was replaced by medium containing no quercetin.

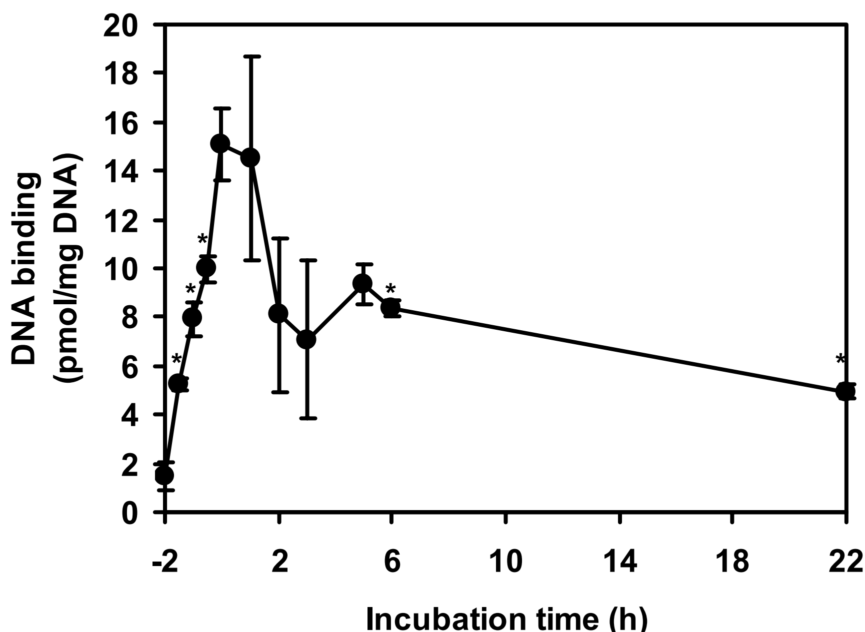

Figure 5 Time course of covalent binding of [4-^{14}C]-quercetin to DNA of HepG2 cells exposed to 5 μM [4-^{14}C]-quercetin for 2 hours in the presence of 1 mM ascorbic acid. At t = 0, the exposure medium was replaced by quercetin-free medium containing 1 mM ascorbic acid. Results are presented as mean +/- SEM.* Significantly different from t = 2 h ($P < 0.05$).

Figure 5 shows the change in the level of cellular quercetin DNA adducts in time in HepG2 cells. The amount of quercetin covalently bound to DNA increased during the 2-hour exposure to 13 pmol quercetin/mg DNA (from t = -2h to t = 0 in the figure). After removal of quercetin from the medium (t = 0), the amount of covalent DNA adducts in the HepG2 cells significantly decreased over time. After 22 hours, the covalent DNA adduct level was significantly lower than the amount present at the end of the exposure (t = 0), amounting to approximately 30% of the level present at t = 0. In view of the fact that the doubling time of the batch of HepG2 cells used was approximately 6 to 7 days, the corresponding 22-hour decrease in covalent quercetin binding per mg DNA cannot be ascribed to the cell division-related increase in DNA. Therefore, the present results provide evidence that the covalent

binding of quercetin to DNA is partly reversible in time, with a half-life of approximately 13.6 hours, as calculated from the data obtained after 22 hours.

Formation of covalent DNA adducts in nucleotide excision repair (NER) deficient mouse embryonic fibroblasts exposed to [4-^{14}C]-quercetin

To investigate whether NER is involved in the reversibility of covalent binding of quercetin to DNA, the amount of quercetin DNA adducts and the time-dependent decrease in these adducts was determined in wild-type (MEF-XPA+/+) as well as in NER-deficient (MEF-XPA-/-) cells. The MEF-XPA-/- cells are mouse embryonic fibroblasts, originating from NER-deficient mice, knocked out for the XPA gene, encoding a protein responsible for the recognition of DNA damage in the process of NER (*29*). The MEF-XPA+/+ cells are mouse embryonic fibroblasts originating from wild type mice.

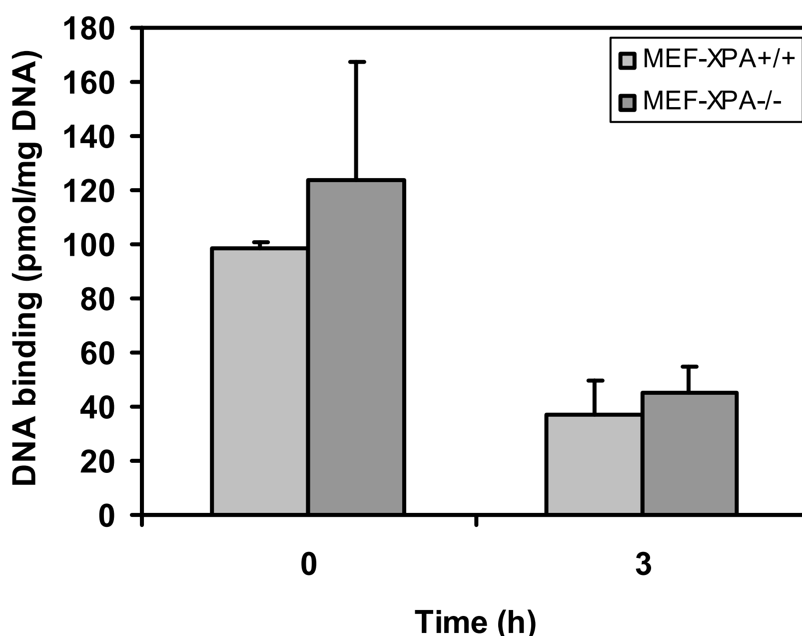

Figure 6 Covalent binding of [4-^{14}C]-quercetin to DNA of two types of mouse embryo fibroblast cells (MEF), exposed to 5 μM [4-^{14}C]-quercetin in the presence of 1 mM ascorbic acid for 4 hours. At t = 0, the medium was replaced by quercetin-free medium containing 1 mM ascorbic acid. XPA+/+: wild type; XPA-/-: knock out for the XPA-gene, involved in NER (*29*). Results are presented as mean +/- SEM.

Figure 6 shows the extent of covalent DNA binding in these cell lines exposed to quercetin for 4 hours (t=0 in the figure), after which the medium was replaced by quercetin-free medium. After 3 hours (t=3), the quercetin DNA adduct level had decreased to approximately 36% of the level present at t=0, indicating that DNA adduct formation was reversible in time

for both cell lines. The amount of DNA adducts detected in DNA repair deficient MEF-XPA-/- cells did not differ significantly from the amount of DNA adducts detected in the wild type cells (MEF-XPA+/+), indicating that nucleotide excision repair does not play a major role in the reversibility of covalent quercetin DNA adduct formation.

Discussion

This study shows for the first time that the pro-oxidant quinone/quinone methide chemistry of quercetin, reflected in the formation of covalent quercetin glutathione, protein and DNA adducts and thought to play a role in the genotoxicity of quercetin (*8, 14*) is relevant in all cells, independent of the presence of tyrosinase or peroxidase-type oxidative enzyme activity. Furthermore, covalent quercetin DNA adducts appeared to be of a transient nature, which might be a reason for the lack of *in vivo* carcinogenic effects of this *in vitro* mutagen.

It is known that oxidative enzymes, including peroxidase and tyrosinase, may be involved in the mechanism underlying the adverse effects of various compounds, including antipsychotic drugs (*36*) and pro-carcinogens (*37*). Moreover, oxidative enzymes, including uterine peroxidase, horseradish peroxidase, lactoperoxidase and tyrosinase are known to catalyze the formation of covalent DNA and/or protein adducts by various compounds, including 4-hydroxytamoxifen, catechol estrogens and dopamine (*38-40*). The present study shows that these enzymes, including horseradish peroxidase, myeloperoxidase, lactoperoxidase and tyrosinase, are able to catalyze the formation of quinone-type metabolites of quercetin, reflected by the formation of 6- and 8-glutathionyl quercetin using the glutathione trapping method. An important question raised by these results is whether the pro-oxidant activity of quercetin is also relevant in whole cells, tissues or even organisms exposed to this flavonoid.

Quercetin has already been reported to bind covalently to cellular protein (*18, 41*) and DNA (*18*), presumably following its oxidation to quinone/quinone methides. To obtain better insight into the consequences of the intracellular pro-oxidant activity of quercetin and to investigate whether tissues expressing peroxidase and/or tyrosinase activity, including breast, uterus and skin tissue as well as leukocytes (*42, 43*), are more susceptible to covalent binding of quercetin to protein and/or DNA, the formation of covalent quercetin protein and DNA adducts was studied. This was done in the cell lines HL-60 and B16-F10 with elevated levels of peroxidase and tyrosinase respectively, and compared to covalent binding in HepG2 and Caco-2 cells, shown in this study not to contain any detectable activity of tyrosinase or peroxidase-type oxidative enzymes. The extent of covalent DNA and/or protein adduct

formation appeared to be increased by the presence of detectable peroxidase and/or tyrosinase activity. However, the difference in the levels of covalent DNA and/or protein adduct formation between the cell lines with and without detectable peroxidase and tyrosinase activity did not match the much larger difference in their levels of these oxidative enzyme activities. Therefore, the present study suggests that tyrosinase and/or peroxidase-type oxidative enzyme activities do not play a major role in the intracellular formation of pro-oxidant metabolites of quercetin. This might be explained by the intracellular compartmentalization of oxidative enzymes, such as tyrosinase and peroxidases, known to be sequestered in compartments surrounded by membranes (*44-46*). As a result, oxidative metabolites of quercetin, formed in the compartments containing these types of oxidative enzyme activities, may have limited access to DNA or protein. This may explain why only a limited increase in DNA and protein adducts is found in cell types containing elevated levels of tyrosinase or peroxidase activities. A second possible reason for the relative low contribution of these types of oxidative enzyme activities to the cellular pro-oxidant activity of quercetin could be that pro-oxidant quinone-type metabolites may be very efficiently scavenged by glutathione and thus detoxified (*41, 47*). Temporarily elevated intracellular quercetin quinone metabolite levels may be conjugated to glutathione quickly after their formation, even before covalent binding to protein or DNA can occur. In support of this, the present study shows that glutathionyl quercetin adducts could only be detected in the medium of cell lines with elevated tyrosinase or peroxidase activities. However, scavenging of quinone-type metabolites by glutathione has been suggested to result in glutathione depletion, considered to be an adverse effect (*48*). The detection of approximately 6 μM glutathionyl adducts in the medium of cells exposed to quercetin (Table 2) would result from the consumption of 3 nmol glutathione from the cells present in the incubation ($1x10^6$). Taking into account an average glutathione concentration of 5 mM (*49*) and a cell volume of $2.5x10^{-8}$ cm^3 (*50*), this corresponds to only 2.5% of the total amount of glutathione present in the cells. Therefore, the pro-oxidant activity of quercetin in these cell types is not expected to result in significant glutathione depletion.

In view of all these considerations, chemical oxidation of quercetin, resulting from the antioxidant activity of this flavonoid, is probably a more important determinant than tyrosinase and/or peroxidase activity for the consequences of the pro-oxidant activity of quercetin. From these results, it can be concluded that the formation of covalent protein and/or DNA adducts is probably not restricted to tissues with elevated tyrosinase- and/or

peroxidase-type oxidative enzyme activity, but rather, given the general nature of oxidant reactions, relevant in all cell types, irrespective of their levels of these types of oxidative enzymes.

Covalent addition to DNA may lead to the formation of permanent mutations (*51*). In spite of unambiguous reports on the *in vitro* genotoxicity of quercetin (*8, 10*), various studies aiming at the assessment of carcinogenic activity of quercetin have generated controversial results (*19-21*). The present study provides evidence that, similar to the binding of oxidized quercetin to glutathione (*26*), the formation of covalent adducts between quercetin and DNA is transient in time (Figure 5), although the loss of quercetin DNA adducts was not complete within 22 hours. The transient character of quercetin DNA adducts may be the result of either an enzymatic or a chemical mechanism. The results with the NER-deficient cells (Figure 6) suggest that the loss of quercetin DNA adducts is probably a chemical process rather than a process in which the cellular NER machinery is involved. In theory, the loss of covalently bound quercetin may be due to either full reversibility of the adduct formation, as seen for quercetin adducts with glutathione and other thiol reagents (*26*), or to depurination of the DNA base involved (*11, 51*). Considering the experimental set up used in this study, one cannot discriminate between these two possibilities. A chemical process leading to depurination of DNA adducts might be analogous to the mechanism suggested for the genotoxicity of quinones of natural and synthetic estrogens (*11, 51*). In general, apurinic sites formed in the DNA due to the loss of such depurinating adducts can lead to mutations by error-prone base excision repair (BER) or misreplication, which substantially increases the risk for cancer and other diseases (*52, 53*). Whether the transient nature of quercetin DNA adducts reflects full reversibility of the adduct formation and thus detoxification, or formation of genotoxic depurinated sites requires additional investigation of the transient nature of quercetin DNA adducts at the molecular level.

The fact that the relative amounts of covalent quercetin protein adducts on a milligram basis were approximately 6 to 9 times higher than the amounts of covalent quercetin DNA adducts may be explained by the fact that quinone-type metabolites react faster with thiol groups from protein or glutathione than with nucleophilic groups present in the DNA (*54*).

The transient nature of quercetin DNA adducts, when proceeding by a fully reversible mechanism, may be an important mechanism underlying the apparent lack of *in vivo* carcinogenic effects of quercetin (*19-21*), in spite of its genotoxicity (*8-10*). A possible reason for the observation of mutagenic effects of quercetin *in vitro* in spite of the transient nature of

quercetin DNA adducts could be that the dissociation rate of quercetin DNA adducts is low (Figure 5) compared with the relatively high cell division rate of the *in vitro* systems as well as short exposure times usually used in genotoxicity studies (*8*). This may lead to an increased incidence of permanent DNA lesions due to quercetin DNA adduct formation, leading to mutagenic effects of quercetin in short-term assays using these *in vitro* systems, but not in a more chronic *in vivo* situation. Alternatively, when the transient nature of the quercetin DNA adducts would be due to depurination of the DNA adducts, the lack of carcinogenicity could be ascribed to the fact that quercetin is a weak carcinogen, for which adequate *in vivo* models are hard to define.

In addition, it is important to note that, besides the possible reversible nature of covalent quercetin DNA adduct formation, the extensive phase II metabolism of quercetin (*22, 23*) may also be a determining factor in the lack of carcinogenic effects of this flavonoid, because conjugated quercetin derivatives are known to have an attenuated biological activity as compared to the aglycone (*24, 25*). Especially conjugation of the catechol moiety could attenuate the pro-oxidative quinone chemistry of quercetin. Together, the data obtained in this study indicate that formation of quercetin DNA adducts can be expected in all cells, independent of their level of tyrosinase and/or peroxidase-type oxidative enzyme activities, whereas the transient nature of the DNA adducts may to some extent limit or cause the ultimate biological impact of the adducts formed. The present study illustrates that mutagenicity studies should put more emphasis on the transient nature of the DNA adducts responsible for the mutagenicity *in vitro*, since this transient nature of the formed DNA adducts may play an essential role in whether the genotoxicity observed *in vitro* will have any impact *in vivo*.

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5

Consequences of quercetin methylation for its covalent glutathione and DNA adduct formation

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Abstract

This study investigates the pro-oxidant activity of 3'- and 4'-O-methylquercetin, two relevant phase II metabolites of quercetin without a functional catechol moiety, which is generally thought to be important for the pro-oxidant activity of quercetin. Oxidation of 3'- and 4'-O-methylquercetin with horseradish peroxidase in the presence of glutathione yielded two major metabolites for each compound, identified as the 6- and 8-glutathionyl conjugates of 3'- and 4'-O-methylquercetin. Thus, catechol-O-methylation of quercetin does not eliminate its pro-oxidant chemistry. Furthermore, the formation of these A-ring glutathione conjugates of 3'- and 4'-O-methylquercetin indicates that quercetin *o*-quinone may not be an intermediate in the formation of covalent quercetin adducts with glutathione, protein and/or DNA. In additional studies, it was demonstrated that covalent DNA adduct formation by a mixture of [4-^{14}C]-3'- and 4'-O-methylquercetin in HepG2 cells amounted to only 42% of the level of covalent adducts formed by a similar amount of [4-^{14}C]-quercetin. Altogether, these results reveal the effect of methylation of the catechol moiety of quercetin on its pro-oxidant behavior. Methylation of quercetin does not eliminate but considerably attenuates the cellular implications of the pro-oxidant activity of quercetin, which might add to the mechanisms underlying the apparent lack of *in vivo* carcinogenicity of this genotoxic compound. The paper also presents a new mechanism for the pro-oxidant chemistry of quercetin, eliminating the requirement for formation of an *o*-quinone, and explaining why methylation of the catechol moiety does not fully abolish formation of reactive DNA binding metabolites.

Introduction

Quercetin (Figure 1) is one of the most studied flavonoids. It is present, mainly as glycoside, in many fruits, vegetables, nuts and seeds and, as such, a component of the daily human diet (*1*). The average intake is approximately 16 mg/day in the Netherlands (*2*). For long, quercetin has been a compound of interest, mainly due to its presumed health promoting effects, reflected in for example protection against cardiovascular diseases (*3, 4*). Although the scientific evidence for the health claims is limited and mainly based on *in vitro* data on its antioxidant activity (*5*), its inhibiting effect on the proliferation of some cancer cells *in vitro* (*6, 7*) and/or on its capacity to modulate the activity of enzymes involved in cell division and biotransformation (*8-10*), the compound is widely available on the Internet as a constituent of many food supplements. Consumption of these supplements according to the advised dosing increases the daily intake to 10- to even 1000-fold the dose obtained from a supplement-free Western diet (*2*).

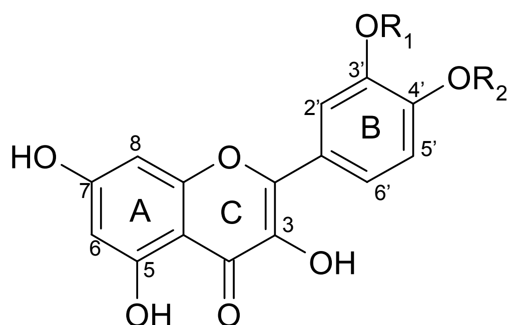

Figure 1 Structural formula of quercetin (R_1 = R_2 = H), 3'-O-methylquercetin (isorhamnetin; R_1 = CH_3, R_2 = H) and 4'-O-methylquercetin (tamarixetin; R_1 = H, R_2 = CH_3), and numbering of their carbon atoms and aromatic rings.

In addition to the beneficial health claims, various studies have generated indications for possible adverse health effects of this compound. Quercetin appears to stimulate the proliferation of estrogen receptor-positive cells *in vitro* at concentrations physiologically relevant *in vivo* (*11, 12*). Moreover, quercetin is genotoxic in various *in vitro* systems (*13-15*). Furthermore, evidence for covalent binding of quercetin to cellular protein and DNA (*16*) has been reported. The pro-oxidant activity of quercetin, leading to the formation of reactive electrophilic quinone-type metabolites, is thought to be involved in these genotoxic effects (*13, 17*). However, studies aiming at the assessment of *in vivo* carcinogenic effects of quercetin have generated controversial results (*18-20*).

A possible explanation suggested for the apparent lack of carcinogenic activity of quercetin *in vivo* in spite of its *in vitro* genotoxicity, may be that *in vitro* tests on genotoxicity are

usually performed with the aglycon (*13-15*). *In vivo*, quercetin is extensively metabolized to methylated, sulfated and glucuronidated phase II conjugates. In human plasma, 35% of circulating quercetin consists of sulfate conjugates, 46% of glucuronide conjugates and 19% of glucurono-methylated conjugates (*21*), while in rat, circulating quercetin consists of 13% sulfated-, 79% sulfo-methylated-, 5% glucuronidated- and 4% glucurono-methylated conjugates (*22*). The 3'- and 4'-hydroxylgroups are important targets for phase II metabolism of quercetin (*23*) and conjugation of these hydroxyl groups of the catechol moiety of quercetin generally attenuates the biological activity of quercetin (*24-27*), a phenomenon also observed in genotoxicity tests (*13, 14*).

Given the fact that the catechol moiety of quercetin is generally considered to play an essential role in its pro-oxidant chemistry, and the fact that especially the 3'- and 4'-hydroxyl moieties of this catechol group are a target for phase II metabolism, the question rises whether phase II metabolism of quercetin has consequences for the pro-oxidant activity of quercetin. Therefore, the objective of the present study was to investigate the possible effect of phase II modification of the quercetin catechol moiety on its pro-oxidant chemistry and behaviour. This was done by studying the formation of covalent glutathione and DNA adducts by two relevant phase II metabolites of quercetin, i.e. 3'-O-methylquercetin (isorhamnetin) and 4'-O-methylquercetin (tamarixetin), presented in Figure 1, in two different *in vitro* model systems. These compounds can be considered model compounds for the quercetin metabolites conjugated at either of the catechol hydroxyl groups. Furthermore, various studies have shown that many cell types, including hepatocytes, blood cells, kidney cells and intestinal cells contain enzyme activities capable of the deconjugation of the glucuronidated and/or sulfated conjugates of quercetin or methylquercetin, thereby liberating quercetin or the methylated derivatives of quercetin in the cell (*28, 29*). Moreover, neutrophils are known to excrete β-glucuronidase activity into the plasma upon stimulation of the cell by an antigen (*30*). Therefore, the occurrence of unconjugated methylated conjugates of quercetin *in vivo* may be expected, not only intracellular but also in plasma and interstitial fluid at sites of inflammation. First, the quinone chemistry of 3'- and 4'-O-methylquercetin during oxidation by horseradish peroxidase/hydrogen peroxide was studied using the glutathione trapping method (*17*). To extend the results to a physiologically more relevant *in vitro* system, the extent of covalent DNA binding caused by exposure to methylated phase II metabolites of quercetin was studied in the hepatocarcinoma cell line HepG2, a cell line previously shown to be a good model for the study of covalent quercetin DNA adduct formation (*16*). These *vitro*

studies reported in the present study can be considered explorative and preparative for possible future studies aiming at the investigation of the pro-oxidant activity of quercetin *in vivo*.

Materials and Methods

Materials

Deuterium oxide was purchased from Apollo Scientific (Stockport, UK). Dimethyl sulfoxide (DMSO), glutathione and quercetin dihydrate were obtained from Acros Organics (New-Jersey, USA). Phenol:chloroform premixed with isoamylalcolhol (25:24:1) was obtained from Amresco (Solon, Ohio, USA). Sodium dodecylsulfate (SDS) was purchased from BDH Biochemical (Poole, UK). [4-^{14}C]-Quercetin dihydrate was a generous gift from dr. J.M.M. van Amelsvoort from Unilever Research Vlaardingen (The Netherlands) and was purchased from Chemsyn Science Laboratories (Lenexa, Kansas, USA). Tris was purchased from Invitrogen (Paisley, UK). Ethylene-glycol-bis-(β-aminoethylether)-NNN’N’-tetra-acetic acid (EGTA) was purchased from Janssen Chimica (Geel, Belgium). Trifluoro-acetic acid was purchased from J.T. Baker (Philipsburg, NJ). Acetonitril was from LabScan Ltd. (Dublin, Ireland). 3’-O-Methylquercetin (isorhamnetin) and 4’-O-methylquercetin (tamarixetin) were obtained from Extrasynthèse (Genay, France). Ascorbic acid, ethylene-diamine-tetra-acetic acid (EDTA), dipotassium hydrogen phosphate, ethanol, hydrochloric acid (HCl), magnesium chloride, phosphoric acid, potassium chloride, potassium dihydrogen phosphate and sodium chloride were from Merck (Darmstadt, Germany). Pellet paint Co-precipitant® was obtained from Novagen (Madison, WI). S-adenosylmethionine, horseradish peroxidase (HRP; EC 1.11.1.7), hydrogen peroxide (H_2O_2), proteinase K, ribonuclease A (RNAse; from bovine pancreas, EC 3.1.27.5), spermidine, spermine and Triton X-100 were obtained from Sigma (Saint Louis, MO). L-glutamine and fetal calf serum (FCS) were obtained from Gibco (Paisley, UK). William’s Medium E was obtained from Sigma (Saint Louis, MO). Antibiotic/antimycotic was purchased from Cellgro (Amsterdam, The Netherlands).

The human hepatocarcinoma cell line HepG2 was purchased from the American Type Culture Collection (Manassas, VA, USA). Rat liver S9 homogenate was obtained from BioPredic (Rennes, France).

Oxidation of methylated conjugates of quercetin by horseradish peroxidase in the presence of glutathione

Typical incubations in 50 mM potassium phosphate pH 7.0 contained 1 mM glutathione, 0.1 μM horseradish peroxidase and 150 μM substrate. The substrates used were quercetin, 3'-O-methylquercetin (isorhamnetin) or 4'-O-methylquercetin (tamarixetin), all added from a 10 mM stock solution in DMSO. The reaction was started by the addition of 200 μM hydrogen peroxide. The reaction mixtures were incubated in a water bath at 25°C for 5 minutes, after which the samples were stored at -80°C until HPLC analysis.

To obtain higher amounts of glutathione adducts appropriate for identification by ^{1}H NMR, incubations of the following composition were made. The initial mixture in 50 mM potassium phosphate pH 7.0 contained 1 mM glutathione, 0.1 μM horseradish peroxidase and 150 μM substrate. The reaction was started by the addition of 200 μM hydrogen peroxide. The following additions were made to the incubation in the course of time for the generation of sufficient amounts of GSH conjugates of 3'- and 4'-O-methylquercetin for ^{1}H NMR experiments. Substrate (150 μM) was added every 5 min during the 30-minute incubation period, 0.1 μM HRP was added at 0, 5, 15, and 20 min of incubation, and glutathione (1 mM) was added at 0 and 15 min of incubation. Thirty minutes after the start of the incubation, the samples were stored at -80°C until further analysis. The samples thus obtained appeared to contain high concentrations of well-dissolved glutathione adducts, in spite of the low water-solubility of the substrates, added to the different samples in a final concentration of 900 μM. The samples were analyzed using HPLC (injection volume 50 μL). Product peaks of five successive runs were collected, pooled, freeze-dried and dissolved in 25 mM potassium phosphate pH 7.0, made with deuterated water (HDO), for ^{1}H NMR analysis. The yield of each glutathionyl conjugate is estimated to be approximately 5 nmoles.

Methylation of quercetin by rat liver S9 protein

Determination of conditions for the methylation of quercetin

The methylation of quercetin was performed essentially as described before (*23*). Typical reaction mixtures in 0.1 M potassium phosphate pH 7.5 contained 1 mM ascorbic acid for flavonoid stabilization (*12*), 10 mg/mL rat liver S9 protein and 50 μM quercetin added from a 10 mM stock solution in DMSO. The reaction was started by the addition of 10 mM S-adenosylmethionine from a 50 mM stock solution in buffer. After thorough homogenization,

the mixture was incubated at 37°C in a water bath. The samples were gently homogenized every 60 minutes. After 3 hours, samples were stored at -80°C until HPLC-analysis for the quantification of the concentrations of free quercetin and its methylated metabolites.

Methylation of [4-^{14}C]-quercetin

To obtain methylated [4-^{14}C]-quercetin conjugates, similar incubations were performed as described above for the synthesis of methylated quercetin with the following modification. Cold quercetin was replaced by [4-^{14}C]-quercetin with a specific activity of 53.3 mCi/mmol. After 3 hours of incubation, the reaction mixture, in which [4-^{14}C]-quercetin was completely converted to methylated [4-^{14}C]-quercetin as determined by HPLC-analysis (see Results section), was used to make the exposure medium for HepG2 cells.

Dissociation of quercetin and its methylated conjugates from protein

Quercetin is known to extensively bind to protein (*31*). To dissociate quercetin and its metabolites from protein for the quantification of total quercetin conversion, the samples were treated essentially as described by Sesink *et al.* (*32*): 100 μL acetonitril was added to 50 μL incubation mixture and the mixture was thoroughly vortexed. Then, 50 μL 20% phosphoric acid in nanopure containing 1g/L ascorbic acid was added. After vortexing, the mixture was centrifuged for 10 minutes at 13800g (13000 rpm using an Eppendorf 5415C Centrifuge, (Hamburg Germany)). The supernatant was analyzed using HPLC. The methylated conjugates of quercetin were quantified on the basis of their peak area in the HPLC chromatogram at 370 nm, using reference curves of 3'-O-methylquercetin and 4'-O-methylquercetin.

HPLC analysis

HPLC was performed on a Waters M600 liquid chromatography system, using an Alltima C18 5U column (4.6 x 150 mm; Alltech, Breda, The Netherlands), coupled to a Waters 996 photodiode array detector. Before injection, incubation mixtures were centrifuged for 4 minutes at 16000 g (14000 rpm, using an Eppendorf 5415C Centrifuge (Hamburg, Germany)).

Glutathione adducts of methylated conjugates of quercetin were detected at a wavelength of 295 nm. In a typical run, aliquots of 50 μL of the 14000 rpm-supernatant were injected. Samples were eluted at a flow of 1 mL/min starting at 5% acetonitril in nanopure water containing 0.1% trifluoroacetic acid, going to 70% acetonitril in 20 minutes, followed by an

isocratic elution at 70% acetonitril for 2 minutes after which the percentage acetonitril was decreased to 5% in 1 minute. Then, the column was re-equilibrated at the initial conditions. Methylated conjugates of quercetin were detected at a wavelength of 370 nm. In a typical run, aliquots of 10 μL of the 14000 rpm-supernatant were injected. Samples were eluted at a flow of 1 mL/min starting by an isocratic elution at 35% acetonitril in nanopure water containing 0.1% trifluoroacetic acid for 15 minutes, going to 80% acetonitril in 2 minutes, followed by an isocratic elution at 80% acetonitril for 1 minute after which the percentage acetonitril was decreased to 0% in 1 minute, followed by an isocratic elution at 0% acetonitril for 5 minutes. Then, the column was re-equilibrated at the initial conditions.

LC-MS analysis

To identify the glutathione conjugates formed in incubations with 3'-O- or 4'-O-methylquercetin with horseradish peroxidase/H_2O_2 in the presence of GSH, incubation samples were also analyzed by LC-MS. Mass spectrometric analysis was performed using a Thermo Electron LCQ equipment (Bremen, Germany) in the positive electrospray mode using a spray voltage of 5 kV and a capillary temperature of 275°C with nitrogen as sheath and auxillary gas. For LC, an Alltima C18 5U column (2.1 x 150 mm; Alltech, Breda, The Netherlands) was used. Samples were eluted at a flow of 0.2 mL/min, using a gradient starting at 0.1% acetic acid in nanopure containing 5% acetonitril, increasing to 15% acetonitril in 10 min. Then acetonitril was increased to 30% in 5 min and subsequently to 60% in 5 min. This percentage was kept for 2 min, after which the column was re-equilibrated at the initial conditions.

^{1}H NMR characterization of glutathione adducts of 3'- and 4'-O-methylquercetin

^{1}H NMR measurements were performed on a Bruker DPX 400 spectrometer. Compounds were dissolved in DMSO-d_6. Temperature was 298 K. A 1.5-s presaturation delay, a 70° angle, and a 2.2-s acquisition time (7575-Hz sweep width, 32 K data points) were used. The data were processed using an exponential multiplication of 0.5 or 1.0 Hz and zero filling to 64 K data points. Resonances are reported relative to water at 4.75 ppm.

Quantification of covalent DNA adduct formation in HepG2 cells exposed to methylated [4-^{14}C]- quercetin

HepG2 cells were cultured in William's medium E, supplemented with 10% FCS, 2 mM L-glutamine and antibiotic/antimycotic (consisting of 100 IU/mL penicillin, 100 IU/mL streptomycin and 0.25 μg/mL amphotericin). For experiments, cells were plated in 6-wells plates and grown to approximately 80% confluency.

The quantification of covalent DNA adduct formation in HepG2 cells was performed essentially as described before (*16*). Cells were grown in monolayers in 6-wells plates until confluency. Then, cells were exposed in triplicate (2 wells per sample) at 37°C in a humidified atmosphere. The exposure medium for the cells consisted of William's Medium E, without FCS or antibiotics, supplemented with 1 mM ascorbic acid for flavonoid stabilization (*12*) and 5 μM methylated [4-^{14}C]-quercetin. Methylated [4-^{14}C]-quercetin was obtained using rat liver S9 incubations as described above in the section "Methylation of quercetin by rat liver S9 protein". These rat liver S9 incubations, containing methylated [4-^{14}C]-quercetin, were diluted in incubation medium to a final concentration of 5 μM methylated quercetin metabolites. For comparison, control cells were exposed to exposure medium of similar composition and treatment as the medium used for exposure to methylated [4-^{14}C]-quercetin, only omitting the cofactor for methylation, S-adenosylmethionine.

After exposure times of 0 min, 10 min and 2 hours, the exposure medium was removed and the cells were washed three times with a cold 0.9% saline solution. Then, 0.7 mL lift buffer was added to the wells and the plates were incubated for 5 min at 37°C in a humidified atmosphere, to allow detachment of the cells. The lift buffer consisted of 10 mM Tris-HCl pH 8.0 containing 0.14 M NaCl and 1 mM EDTA. The cell suspension was centrifuged at 2000 rpm (325 g) for 5 min to obtain a cell pellet. All centrifugation steps in the protocol for the determination of covalent binding to DNA were performed using an Eppendorf Centrifuge, type 5415C (Hamburg Germany).

Cell pellets were gently resuspended in 0.7 mL cold swell buffer containing 7 μL 10% Triton-X100 and left on ice for 10 min. The swell buffer consisted of 100 mM HEPES pH 8.0, containing 10 mM KCl, 0.75 mM spermidine, 0.15 mM spermine 0.1 mM EDTA and 0.1 mM EGTA. The nuclei were precipitated by centrifugation at 14000 rpm (16000 g) for 2 min and resuspended in 0.6 mL swell buffer. To further purify the nuclei, this suspension was centrifuged in 30% sucrose in swell buffer for 10 min at 4000 rpm (1310 g). The supernatant was discarded. The pellet was subsequently resuspended in 0.5 mL extraction buffer to which

1 μL 10 mg/mL RNAse in extraction buffer, 3 μL 20 mg/mL proteinase K in nanopure and 1.5 μL 20% sodium dodecylsulfate in nanopure were added. The extraction buffer consisted of 10 mM Tris-HCl pH 8.0, containing 40 mM EDTA. The mixture was incubated overnight at room temperature.

Then, the DNA was extracted by the addition of 900 μL phenol:chloroform:isoamylalcohol (25:24:1). The mixture was vortexed for 30 sec and transferred to Phase Lock Gel vials (Eppendorf, Hamburg, Germany) and centrifuged at 14000 rpm (16000 g) for 4 min. The aqueous phase was decanted to a clean eppendorf vial. Then, 30 μL ice cold 3 M sodium acetate pH 5.2, 2 μL Pellet Paint Co-Precipitant and 1 mL ice cold 100% ethanol were added to the aqueous phase to precipitate the DNA. After vortexing, the DNA was pelleted by centrifugation for 5 min at 14000 rpm (16000 g). The DNA pellet was washed twice with 1 mL ice cold 100% ethanol, after which the pellet was dissolved in 0.5 mL de-ionized water. The DNA was quantified by measurement of A_{260} and purity was checked by using the A_{260}/A_{280} ratio (1.6< ratio <1.8). Then, the radioactivity present in the DNA was measured using a scintillation counter.

Results

Identification of glutathione adducts of methylated conjugates of quercetin

Figure 2 shows the HPLC chromatograms of the incubations of quercetin (A), 3'-O- (B) and 4'-O-methylquercetin (C) with HRP/H_2O_2 in the presence of glutathione. The ^1H NMR data of the metabolites formed in the incubation with quercetin (panel A), previously identified as 6- and 8-glutathionyl quercetin (*17*), are given in Table 1. Table 2 shows the UV-VIS data of the metabolites formed in the incubations presented in Figure 2.

In the HPLC chromatogram of the incubation of 3'-O-methylquercetin with HRP/H_2O_2 in the presence of glutathione (Figure 2B), two major metabolites are formed with retention times of 9.7 and 10.3 minutes, both having a λ_{max} of 298 nm (Table 2). Using LC-MS, an *m/z* ratio of 622 was found for the $[M+H]^+$ ion of each of the two metabolites, indicating that the metabolites are mono-glutathione adducts of 3'-O-methylquercetin. Table 3 summarizes the ^1H NMR chemical shift values and coupling constants of the resonances of the aromatic protons of 3'-O-methylquercetin and its two glutathione adducts. Identification of the ^1H NMR resonances has been achieved on the basis of the ^1H NMR chemical shifts and their splitting patterns, as well as on the basis of comparison with literature data (*17, 33*).

Table 1 ^{1}H NMR data of the metabolites of the incubation of quercetin with HRP/H_2O_2 in the presence of glutathione (*17*).

	Chemical shift values (ppm)				
	H6	**H8**	**H5'**	**H2'**	**H6'**
Quercetin (Q)	6.18 J_{H6-H8} =2.1 Hz	6.40 J_{H8-H6} =2.1 Hz	6.87 $J_{H5'-H6'}$ =8.5 Hz	7.67 $J_{H2'-H6'}$ =2.1 Hz	7.53 $J_{H2'-H6'}$ =2.1 Hz $J_{H6'-H5'}$ =8.5 Hz
Peak 1 t_r=9.1min 8-GS-Q	5.88	-	6.85 $J_{H5'-H6'}$ =8.5 Hz	7.32 $J_{H2'-H6'}$ =2.1 Hz	7.25 $J_{H2'-H6'}$ =2.1 Hz $J_{H6'-H5'}$ =8.5 Hz
Peak 2 t_r=9.7min 6-GS-Q	-	6.02	6.80 $J_{H5'-H6'}$ =8.5 Hz	7.30 $J_{H2'-H6'}$ =2.1 Hz	7.23 $J_{H2'-H6'}$ =2.1 Hz $J_{H6'-H5'}$ =8.5 Hz

Table 2 UV-VIS data of major metabolites present in the incubation of quercetin as well as 3'-O- and 4'-O-methylquercetin with HRP/H_2O_2 in the presence of glutathione (see Figure 2). Of each peak, the corresponding retention time, as well as the λ_{max} values in the UV-VIS spectrum are given. Q = quercetin; 3'-O-MeQ = 3'-O-methylquercetin; 4'-O-MeQ = 4'-O-methylquercetin.

	UV-VIS data					
	Peak 1	**Peak 2**	**Peak 3**	**Peak 4**	**Peak 5**	**Peak 6**
Q	9.1 min 298 nm	9.7 min 298 nm	11.1 min 250-345 nm			
3'-O-MeQ	9.7 min 298 nm	10.3 min 298 nm				
4'-O-MeQ	9.0 min 231-283-312 nm	9.6 min 260-293 nm	10.4 min 298 nm	10.6 min 298 nm	13.4 min 293 nm	14.7 min 274-293 nm

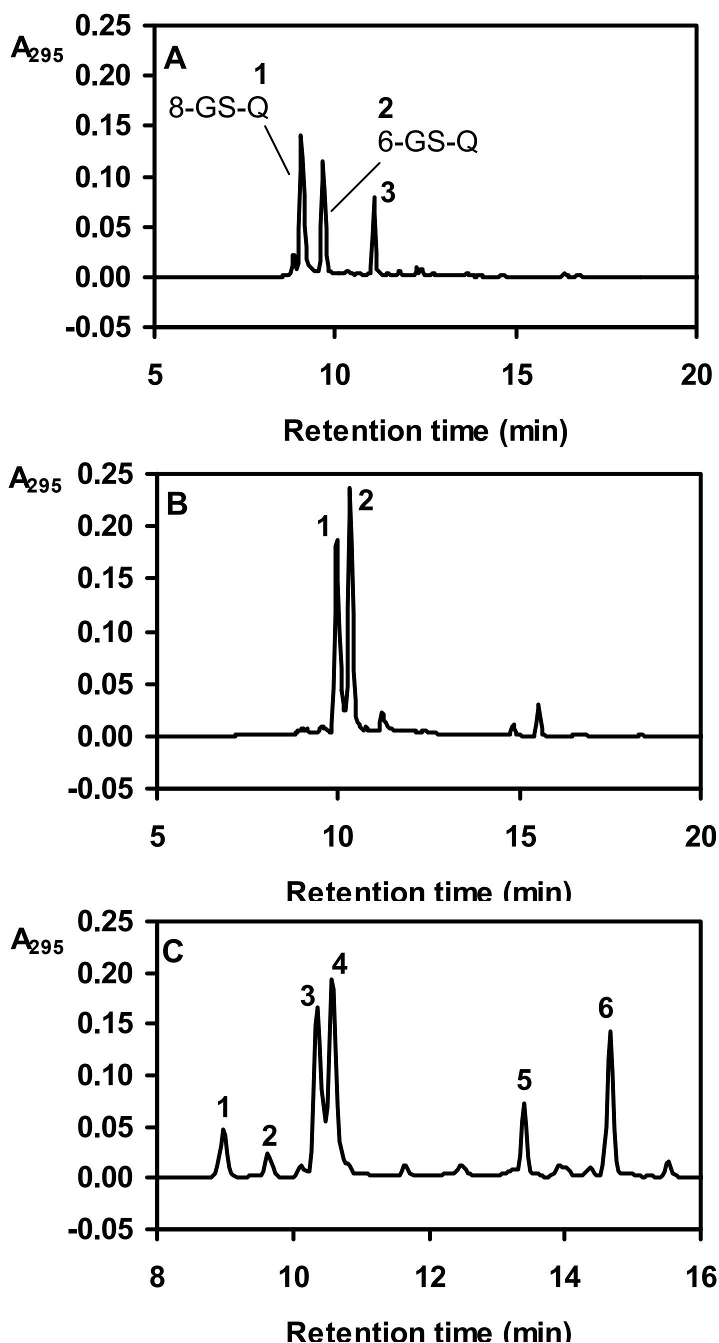

Figure 2 HPLC chromatogram of the incubation of (A) quercetin (Q), (B) 3'-O-methylquercetin (isorhamnetin) and (C) 4'-O-methylquercetin (tamarixetin) with horseradish peroxidase and H_2O_2 in the presence of glutathione. Retention times of quercetin, 3'-O-methylquercetin and 4'-O-methylquercetin under the HPLC conditions used were 16.3, 18.4 and 18.5 min, respectively.

Table 3 ^{1}H NMR data of the metabolites of the incubation of 3'-O-methylquercetin (isorhamnetin) or 4'-O-methylquercetin (tamarixetin) with HRP/H_2O_2 in the presence of glutathione.

	Chemical shift values (ppm)				
	H6	**H8**	**H5'**	**H2'**	**H6'**
3'-O-methylquercetin (isorhamnetin)	6.19 J_{H6-H8}=1.7 Hz	6.48 J_{H8-H6} =1.7 Hz	6.95 $J_{H5'-H6'}$ =8.4 Hz	7.75 $J_{H2'-H6'}$ =1.7 Hz	7.69 $J_{H2'-H6'}$ =1.7 Hz $J_{H6'-H5'}$ =8.4 Hz
Peak 1 t_r = 9.7 min	5.81	-	6.77 $J_{H5'-H6'}$ =8.4 Hz	7.29 $J_{H2'-H6'}$ =1.6 Hz	7.23 $J_{H2'-H6'}$ =1.6 Hz $J_{H6'-H5'}$ =8.4 Hz
Peak 2 t_r = 10.3 min	-	5.96	6.71 $J_{H5'-H6'}$ =8.5 Hz	7.27 $J_{H2'-H6'}$ =1.6 Hz	7.21 $J_{H2'-H6'}$ =1.6 Hz $J_{H6'-H5'}$ =8.5 Hz
4'-O-methylquercetin (tamarixetin)	6.19 J_{H6-H8} =1.2 Hz	6.42 J_{H8-H6}=1.2 Hz	7.09 $J_{H5'-H6'}$ =8.7 Hz	7.66 $J_{H2'-H6'}$ =1.9 Hz	7.65 $J_{H2'-H6'}$ =1.9 Hz $J_{H6'-H5'}$ =8.7 Hz
Peak 3 t_r = 10.4 min	5.81	No signal	6.87 $J_{H5'-H6'}$ =8.4 Hz	7.23 $J_{H2'-H6'}$ =1.8 Hz	7.26 $J_{H2'-H6'}$ =1.8 Hz $J_{H6'-H5'}$ =8.4 Hz
Peak 4 t_r = 10.6 min	No signal	5.96	6.82 $J_{H5'-H6'}$ =8.5Hz	7.19 $J_{H2'-H6'}$ =2.0 Hz	7.23 $J_{H2'-H6'}$ =2.0 Hz $J_{H6'-H5'}$ =8.5 Hz

An additional resonance was observed at 5.7 ppm, which disappeared in time, with no change in intensity or chemical shift value of the other resonances. This resonance at 5.7 ppm was tentatively assigned to the amide proton of the cysteine residue of glutathione, which can form a very tight intramolecular hydrogen bond towards the hydroxyl group at the 7-position (*34*). The proton resonances in the aliphatic region (data not shown) were in agreement with previous results (*17*), supporting that the metabolites are glutathionyl conjugates of 3'-O-methylquercetin. Comparison of the chemical shift values of the aromatic protons of the metabolite with a retention time of 9.7 min with the chemical shift values of 3'-O-methylquercetin (Table 3) clearly reveals the loss of the signal originating from proton C_8-H, as well as the J_{HH} coupling of 1.7 Hz between C_6-H and C_8-H. The data indicate that the metabolite still contains the $C_{5'}$-H and $C_{6'}$-H protons, reflected by a doublet and a double doublet, respectively, in the 6.7-7.3 ppm region. The signals originating from these protons

show a reciprocal J_{HH} coupling of 8.4 Hz. In addition, the $C_{6'}$-H proton shows a J_{HH} coupling of 1.6 Hz to the $C_{2'}$-H proton, of which the ^{1}H NMR signal is also still present, reflected by the doublet in the 7.2-7.3 ppm region with a J_{HH} coupling of 1.6 Hz. Altogether, these data point at the loss of proton C_8-H, indicating that this proton may be replaced by a glutathionyl moiety. Based on the LC-MS and NMR data the metabolite with a retention time of 9.7 min can therefore be identified as 8-glutathionyl 3'-O-methylquercetin. Based on a similar argumentation, the metabolite with a retention time of 10.3 min, of which the ^{1}H NMR data (Table 3) point at the loss of proton C_6-H, can be identified as 6-glutathionyl 3'-O-methylquercetin.

The HPLC chromatogram of the incubation of 4'-O-methylquercetin with HRP/H_2O_2 in the presence of glutathione (Figure 2C) reveals the formation of 6 metabolite peaks, of which the UV-VIS data are given in Table 2. In view of the fact that glutathione conjugation of quercetin shifts the UV-spectrum of the parent compound from λ_{max} values of approximately 250 and 370 nm to one λ_{max} value of approximately 295 nm (*17*), only peaks 3, 4 and 5, having retention times of 10.4, 10.6 and 13.4 min respectively, appeared to have UV-VIS spectra corresponding to possible glutathionyl adducts of the parent compound, with λ_{max} values of 298, 298 and 293 nm respectively (Table 2). Using LC-MS, an *m/z* ratio of 622 was found for the $[M+H]^+$ ion of both peak 3 and 4, indicating that these metabolites are glutathione adducts of 4'-O-methylquercetin. Because the UV-VIS data obtained for peaks 1, 2 and 6 were not in accordance with the UV-VIS spectrum that would be expected for glutathionyl conjugates of 4'-O-methylquercetin (*17*) and because LC-MS analysis of these peaks demonstrated that these three metabolites, and also the metabolite eluting as peak 5 in the HPLC chromatogram, were not mono-glutathionyl conjugates of 4'-O-methylquercetin, for which an $[M+H]^+$ ion at an *m/z* ratio of 622 would be expected, these metabolites were not considered for further identification.

Table 3 summarizes the ^{1}H NMR chemical shift values and coupling constants of the resonances of the aromatic protons of 4'-O-methylquercetin and the two major glutathione conjugates of 4'-O-methylquercetin having a retention time of 10.4 and 10.6 min. Based on an argumentation similar to the one described above for the identification of 8-glutathionyl 3'-O-methylquercetin, the metabolite with a retention time of 10.4 min (peak 3) can be identified as 8-glutathionyl 4'-O-methylquercetin and the metabolite with a retention time of 10.6 min (peak 4) can be identified as 6-glutathionyl 4'-O-methylquercetin.

Conditions for methylation of quercetin by rat liver S9 protein

HPLC analysis of the incubation of quercetin with rat liver S9 protein in the presence of S-adenosylmethionine and ascorbic acid after 3 hours of incubation at 37°C revealed that, in line with previous results (*23*), quercetin was completely converted to 3'-O-methylquercetin and 4'-O-methylquercetin (chromatograms not shown). The results indicate that the incubation conditions are appropriate for the complete conversion of 50 μM quercetin to methylated conjugates. To obtain a final concentration of 5 μM methylated [4-^{14}C]-quercetin for the exposure of HepG2 cells, similar incubations were carried out with [4-^{14}C]-quercetin. These incubations, were diluted in culture medium to reach a final concentration of 5 μM methylated [4-^{14}C]-quercetin, representing a mixture of [4-^{14}C]-3'-O- plus 4'-O-methylquercetin.

Covalent binding of methylated quercetin to the DNA of HepG2 cells

Figure 3 shows the results of the quantification of the covalent DNA binding in HepG2 cells exposed to 5 μM [4-^{14}C]-3'-O- plus 4'-O-methyl-quercetin or unmethylated [4-^{14}C]-quercetin, all in the presence of S9 protein, resulting from dilution of the S9 incubation with, respectively without S-adenosylmethionine. For both exposure conditions, the amount of covalent DNA binding increased in time up to 2 hours. In the cells exposed to unmethylated quercetin, the maximum binding after 2 hours of incubation amounted to 107 pmol quercetin/mg DNA. In the cells exposed to a similar amount of methylated quercetin, the maximum DNA binding after 2 hours of incubation was significantly lower, namely 47 pmol/mg DNA which represents only 42% of the amount formed in incubations with unmethylated [4-^{14}C]-quercetin. Although these results were obtained in a cellular *in vitro* system consisting of HepG2 cells grown under different conditions as compared to the *in vivo* situation, the results indicate that methylation of quercetin considerably decreases the potential of quercetin to form covalent adducts with cellular DNA.

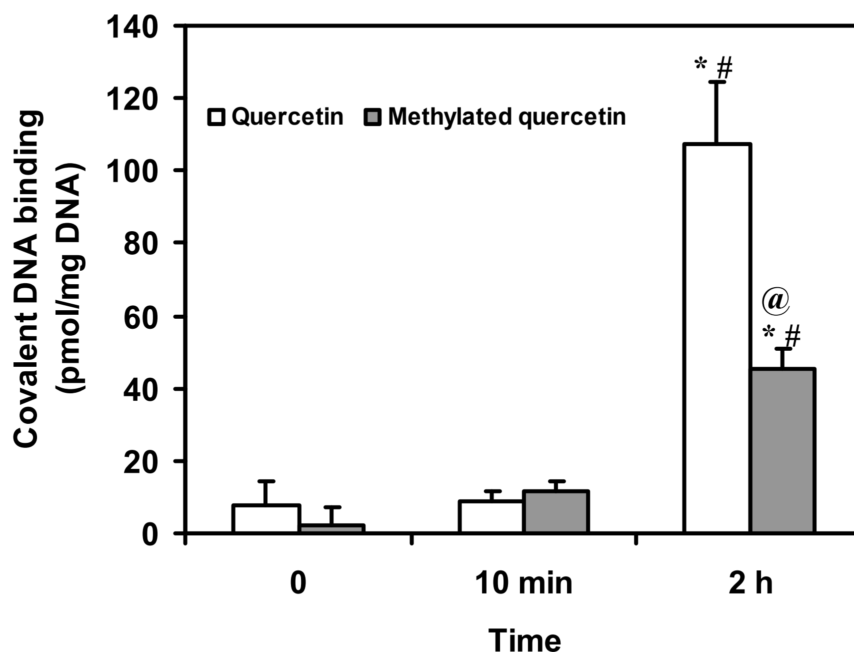

Figure 3 Quantification of covalent binding of methylated and unmethylated [4-^{14}C]-quercetin to the DNA of HepG2 cells exposed to 5 μM of the indicated compounds. 1 mg DNA is equivalent to 3.2×10^6 DNA bases. * Significantly different from corresponding t = 0 sample ($P < 0.05$); # Significantly different from corresponding t = 10 min sample ($P < 0.05$); @ Significantly different from t = 2 h sample of (unmethylated) quercetin series.

Discussion

The present study shows for the first time that catechol-O-methylated metabolites of quercetin form covalent adducts with glutathione as well as DNA. Previously, the formation of mutagenic quercetin metabolites and also of 6- and 8-glutathionyl quercetin conjugates after oxidation of quercetin was hypothetically explained by oxidation of quercetin to the *o*-quinone followed by swift isomerization to the *p*-quinone methides (*13, 17*). Because the methylation of a catechol hydroxyl group impedes the conversion of the catechol moiety to an *o*-quinone, which is a crucial event in this mechanism, this implies that this mechanism does not provide a satisfying explanation for the formation of 6- and 8-glutathionyl conjugates of the methylated derivatives of quercetin. Alternatively, direct oxidation of compounds containing a catechol group to quinone methides via disproportionation of semi-quinone radicals, instead of via *o*-quinone formation, has already been reported for various compounds, including diterpenone catechol and also quercetin (*35, 36*). The present study now provides additional evidence that the oxidation of the B-ring catechol group is not necessarily the first event in the oxidation of quercetin, although the present results do not fully exclude that the formation of GSH conjugates of quercetin and its methylated derivatives may occur following different mechanisms. Figure 4 proposes a mechanism that

includes two sequential one-electron oxidation steps of the aromatic π-system of quercetin or its methylated derivatives. This mechanism is widely applicable for various flavonoids, including both 3'-O- and 4'-O-methylquercetin, and may explain why conjugation of quercetin at the catechol hydroxyl groups does not eliminate the pro-oxidant chemistry of quercetin.

Altogether, these results support that oxidation of quercetin and/or of its methylated derivatives does not necessarily proceed by the intermediate formation of the *o*-quinone and therefore, the presence of an intact catechol group should not be considered a prerequisite for pro-oxidant chemistry of a metabolite of quercetin. Thus, conjugation of the catechol group of quercetin by phase II metabolism is not expected to completely eliminate the toxic pro-oxidant activity of this flavonoid. To validate these results using a physiologically more relevant *in vitro* model system, the extent of covalent DNA adduct formation by a mixture of 3'- and 4'-O-methylquercetin was compared with covalent adduct formation by quercetin. The reason that covalent DNA adduct formation by quercetin and its methylated derivatives was chosen as an end point to study the cellular events following the pro-oxidant chemistry of these flavonoids is that the formation of covalent DNA adducts of radioactively labeled quercetin proved to be a more sensitive method than the formation of covalent GSH conjugates. Covalent quercetin GSH conjugates could only be detected in the medium of cells with elevated levels of oxidative enzyme activity, such as B16F10 cells and HL60 cells, rich in tyrosinase- and peroxidase activity, respectively. In contrast, covalent adducts of quercetin with DNA were found in all cell types studied, including in addition to the B16F10 and HL60 cells also HepG2 and Caco-2, cells, which do not contain any detectable intracellular levels of oxidative enzyme activity (*37*). In line with previous results (*16*), exposure to quercetin led to the formation of covalent DNA adducts in HepG2 cells. The results also showed that methylation of quercetin to a mixture of 3'- and 4'-O-methylquercetin decreased the amount of DNA adducts in HepG2 cells by 58%, as compared with unmethylated quercetin. The decrease in covalent adduct formation due to catechol-O-methylation of quercetin provides an indication that in spite of retention of the capacity to form covalent glutathione and DNA adducts, methylation of quercetin probably affects the efficiency of the intracellular pro-oxidant chemistry of quercetin. Recently, indications were obtained that intracellular oxidative enzyme activity does not play a major role in the intracellular pro-oxidant chemistry of quercetin. This was concluded from experiments showing that the levels of covalent quercetin DNA adducts in cells with elevated levels of oxidative enzyme activity, such as the

B16F10 and the HL60 cell line, were only approximately two times higher than in cells without detectable levels of this type of enzyme activity, such as the HepG2 and the Caco-2 cell line. This difference in covalent quercetin DNA adduct levels was smaller than might be expected on the basis of the differences in levels of oxidative enzyme activity between the cells. These results therefore suggested that the intracellular pro-oxidant activity of quercetin was merely determined by its chemical oxidation resulting from its antioxidant activity, rather than by the intracellular levels of oxidative enzyme activity (*37*).

For both chemical oxidation as well as enzymatic oxidation, the efficiency of electron donation by the parent compound (Figure 4) may be considered an important factor (*38*). Therefore, the extent of pro-oxidant chemistry of quercetin-related compounds may also be dependent on their electron donating capacity. Methylation of the catechol group of quercetin has recently been demonstrated to affect the radical scavenging, and thus electron donating, capacity of quercetin by two mechanisms, namely by increasing the pKa for deprotonation and by decreasing the ease of electron and hydrogen donation by quercetin (*39*). This effect of methylation on the electron donating capacity of quercetin may provide an explanation for the formation of lower amounts of DNA adducts by 3'-O- and 4'-O-methylquercetin than by non-methylated quercetin. The results furthermore suggest that the antioxidant behavior as determined by the TEAC assay (*39*) may have predictive value for the intracellular pro-oxidant behavior of quercetin and/or its derivatives.

The redox potential of the metabolite, which is related to the number and position of free hydroxyl groups (*5, 40*), and thus the regioselectivity of phase II conjugation are expected to determine the ultimate biological effect of quercetin. Phase II metabolism of quercetin may thus greatly influence the biological activity in *vivo*. The extensive phase II metabolism of quercetin (*21, 22*) may therefore be an explanation for the apparent lack of *in vivo* carcinogenicity of quercetin (*18-20*), despite clear *in vitro* genotoxic effects (*13-15*). In support of this suggestion, genotoxicity assays with conjugated derivatives of quercetin generally show attenuated effects as compared with the aglycone (*13, 14*).

In conclusion, the present study shows for the first time that, similar to quercetin, catechol-O-methylated metabolites of quercetin, isorhamnetin and tamarixetin, can form covalent adducts with glutathione and DNA. However, exposure to a mixture of methylated conjugates of quercetin led to the formation of significantly lower amounts of DNA adducts in HepG2 cells as compared to the aglycone, indicating that the pro-oxidant chemistry of methylated derivatives of quercetin is less efficient than of the aglycone. Therefore, the present study

provides evidence that methylation of the catechol group of quercetin attenuates but does not eliminate the pro-oxidant properties of the flavonoid quercetin, and this may add to the apparent lack of *in vivo* carcinogenicity in spite of *in vitro* mutagenicity of this compound. The paper also presents a new mechanism for the pro-oxidant chemistry of quercetin, eliminating the requirement for formation of an *o*-quinone, and explaining why methylation of the catechol moiety does not fully abolish formation of reactive DNA binding metabolites.

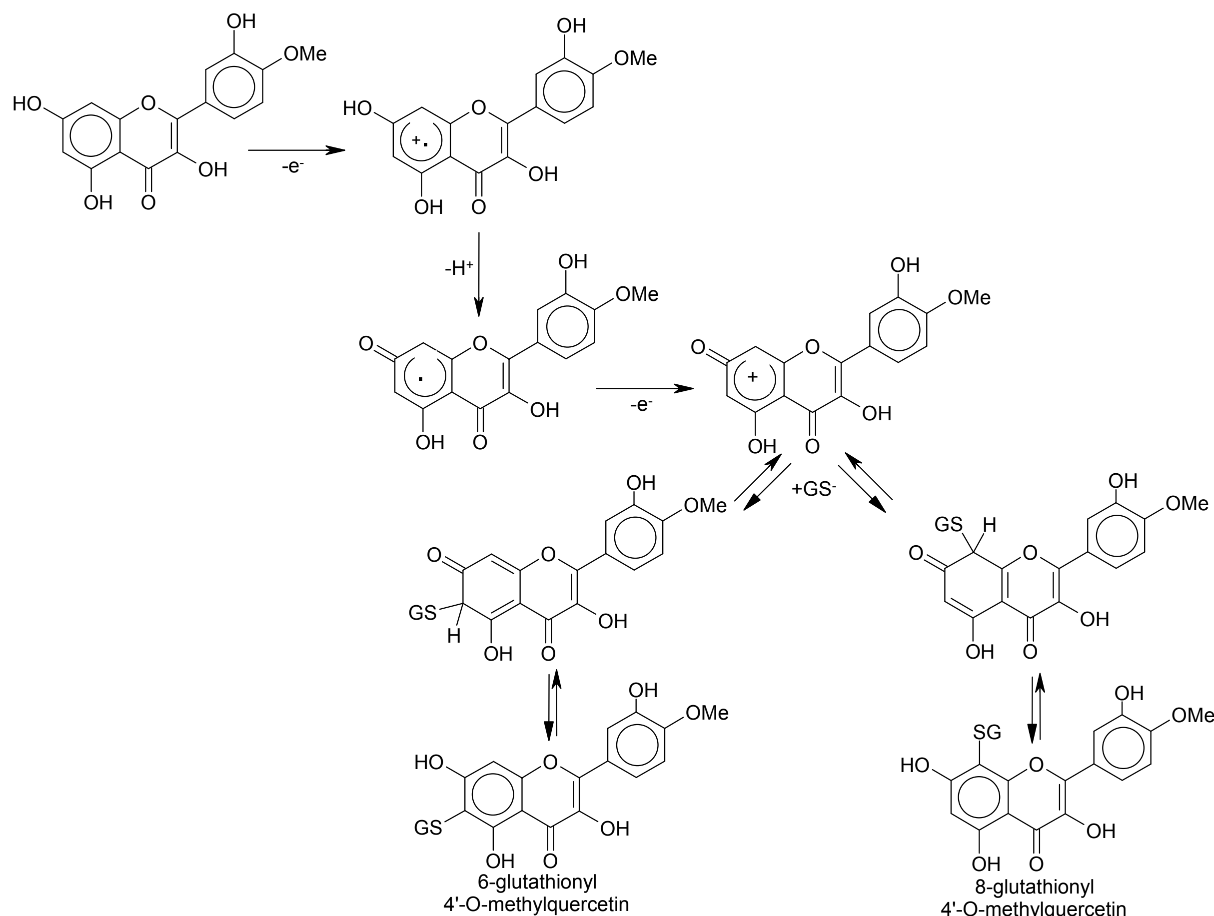

Figure 4 Possible mechanism underlying the formation of 6- and 8-glutathionyl conjugates of 4'-O-methylquercetin upon oxidation by HRP/H_2O_2 in the presence of glutathione.

Acknowledgements

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6

Biphasic modulation of cell proliferation by quercetin at concentrations physiologically relevant in humans

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Cancer Lett (2003) 200(1): 41-47

Abstract

Optimal in vitro conditions regarding quercetin solubility and stability were defined. Using these conditions, the effect of quercetin on proliferation of the colon carcinoma cell lines HCT-116 and HT29 and the mammary adenocarcinoma cell line MCF-7 was investigated. For the colon carcinoma cell lines, at relatively high concentrations, a significant decrease in cell proliferation was observed, providing a basis for claims on the anticarcinogenic activity of quercetin. However, at lower concentrations, a subtle but significant stimulation of cell proliferation was observed for all cell lines tested. These results point at a dualistic influence of quercetin on cell proliferation that may affect present views on its supposed beneficial antiproliferative effect.

Introduction

Quercetin is a flavonoid ubiquitously present in many fruits, vegetables, seeds, nuts, olive oil, tea and red wine (*1*). Average human daily quercetin intake in The Netherlands has been estimated at 23 mg (*2*). For several decades, quercetin has been a compound of interest, due to its potential health-promoting effects, including protection against certain forms of cancer and cardiovascular, photosensitivity and age-related diseases (*1*). The health claims associated with quercetin provide a basis for its increasing use as a functional food ingredient and/or food supplement, and for the large number of in vivo as well as in vitro studies aiming at defining the mechanisms and limits of its beneficial effects.

Evaluating currently available in vitro data on toxicity as well as on possible beneficial effects of quercetin, it appears that two important aspects linked to studies of quercetin in aqueous environment are generally not taken into account. The first aspect is its solubility in aqueous media, varying in the literature from practically insoluble (*3*) to concentrations in the millimolar range used for in vitro toxicity studies (*4, 5*). The second aspect is the sensitivity of quercetin towards autooxidation resulting in poor stability in aqueous aerobic environment (*6*). Conclusions on effects of quercetin on cell proliferation have often been drawn from exposure times up to several days, without any data on actual stability of the compound under these test conditions (*4, 5, 7-10*). Taking these aspects into account, reported data may be uncertain with respect to the concentration of quercetin actually causing the effects observed. Therefore, the objectives of the present study were twofold: first to provide data on solubility and stability of quercetin in media that are considered representative for the media currently in use for tissue culture purposes, and second to characterize the effects of quercetin on cell proliferation using the optimized in vitro conditions.

Materials and Methods

Materials

Dimethylsulphoxide (DMSO) and quercetin were from Acros Organics (New Jersey, USA). Ascorbic acid, dipotassium hydrogenphosphate, potassium dihydrogenphosphate and sodium hydroxide were from Merck (Darmstadt, Germany). Acetonitril was from Lab-Scan Ltd. (Dublin, Ireland). Trifluoracetic acid was from J.T. Baker (Philipsburg NJ). Gentamicin and McCoy’s 5A medium (modified: without serum, with L-glutamine) were from Gibco Invitrogen Corporation (Paisley, Scotland). The human colon carcinoma cell lines HCT-116 and HT29, and the mammary adenocarcinoma cell line MCF-7 were from the American Type

Culture Collection (Manassas VA). Cells were cultured in 75-cm^2 culture flasks in a humidified atmosphere at 37°C and 5% CO_2, in McCoy's 5A medium for HCT-116 and HT29, and in Dulbecco's Modified Eagle medium (DMEM) for MCF-7. Both media were supplemented with 10% fetal calf serum (FCS) and 50 μg/mL gentamicin.

Stability studies

Quercetin was dissolved in test medium to a final concentration of 50 μM from a 10 mM stock solution in DMSO. Test media were 0.1 M potassium phosphate pH 7.4 and McCoy's 5A cell culture medium. To assess the effect of ascorbic acid on quercetin stability, 1 mM ascorbic acid was dissolved in both test media, after which the pH was set at 7.4. Quercetin solutions were incubated at 37°C and 5% CO_2 in 24-wells plates either or not containing HCT-116 cells. The media were harvested at different time points up to 24 hours and stored at -80°C until analysis.

After thawing, the 14000-rpm supernatant was analyzed with HPLC, performed with a Waters M600 liquid chromatography system (Millipore Corporation, Bedford MA). Quercetin was quantified using an Alltima C18 5U column (4.6 x 150 mm; Alltech, Breda, The Netherlands), eluting at a flow of 1 mL/min. The injection volume was 10 μL. The gradient used for analysis of quercetin in the 0.1 M potassium phosphate pH 7.4 series was described previously (*11*). The McCoy's medium series were analyzed with a gradient starting at 0% acetonitril in nanopure water containing 0.1% trifluoracetic acid for 2 min, going from 0% to 70% acetonitril in 22 min, keeping this percentage for 2 min and returning to 0% acetonitril in 1 min. Detection was performed between 230 and 420 nm using a Waters 996 photodiode array detector. Quercetin peak areas at 370 nm were used to quantify the amount of quercetin; amounts are expressed as percentage of the peak area at t = 0 min.

Solubility studies

Quercetin solutions were made in test medium from 200 times concentrated stock solutions in DMSO in final concentrations up to 300 μM. Test media were 0.1 M potassium phosphate pH 7.4, and McCoy's 5A medium with or without 10% FCS, all containing 1 mM ascorbic acid. Quercetin solutions were incubated for 24 hours in a humidified atmosphere at 37°C and 5% CO_2 in 12-wells plates either or not containing HCT-116 cells.

Then, the contents of the wells were homogenized and analyzed with static light scattering (detection angle 90°, temperature 37°C) to determine the state of aggregation of quercetin in the media. The static light scattering equipment consisted of a Lexel-85 Argon Ion Laser (Palo Alto), wavelength 514.5 nm, complemented with an ALV/SP-125 goniometer and an ALV-800 transputerboard, coupled to a single photon detector and an ALV static and dynamic enhancer (ALV-GmbH, Langen, Germany). Data were processed using an ALV-5000/E tau digital correlator. Each sample was measured 10 times, each measurement lasting 10 seconds.

Cell viability and proliferation

Cell suspensions ($2x10^5$ cells/mL for the colon cell lines, $0.5x10^5$ cells/mL for MCF-7) were plated in culture medium in 96-wells plates (100 µL/well) and incubated to allow attachment. After the attachment period (24 hours for the colon cell lines, 72 hours for the MCF-7 cell line), the cells were exposed to quercetin. The exposure medium of the colon cell lines was based on 1 mM ascorbic acid in McCoy's 5A medium without FCS; for the MCF-7 cell line, only 200 µM ascorbic acid in DMEM was used, due to the toxicity of ascorbic acid for this cell line. Exposure media were supplemented with quercetin from 200 times concentrated stock solutions in DMSO. Ascorbic acid was dissolved in culture medium, and the pH was set at 7.4; after sterilization using Schleicher & Schuell FP30/0.2CA-S filters (Dassel, Germany), quercetin was added. Cells were exposed to 100 µL medium with final quercetin concentrations as indicated, in a humidified atmosphere at 37°C and 5% CO_2 for 24 hours. Due to the limited stability of quercetin in DMEM, the exposure medium of MCF-7 cells was refreshed after 6 and 12 hours.

Cell viability was measured using the LDH-leakage method (*12*) with some minor adaptations for 96-wells plates. The extent of proliferation was determined with the BrdU-labeling method using the Cell Proliferation ELISA, BrdU (colorimetric) kit from Roche Diagnostics GmbH (Mannheim, Germany). Results were expressed as percentage of the proliferation of control cells exposed to 0.5% DMSO in culture medium containing 1 mM ascorbic acid.

Statistical analysis of data

Statistical analysis of data was carried out using the Dunnett T3 test (ANOVA). Differences were significant if at least $P<0.05$.

Results

Quercetin stability

Figure 1 presents the results of studies on the stability of a 50 µM solution of quercetin incubated under various conditions. In 0.1 M potassium phosphate pH 7.4 quercetin proved to be unstable, with a half-life of 10 hours. In McCoy's 5A culture medium of similar pH, degradation of quercetin was even faster, showing a half-life of 2 hours. Addition of 1 mM ascorbic acid stabilized quercetin in both media. The presence of HCT-116 cells had no effect on the stability of quercetin in McCoy's 5A culture medium.

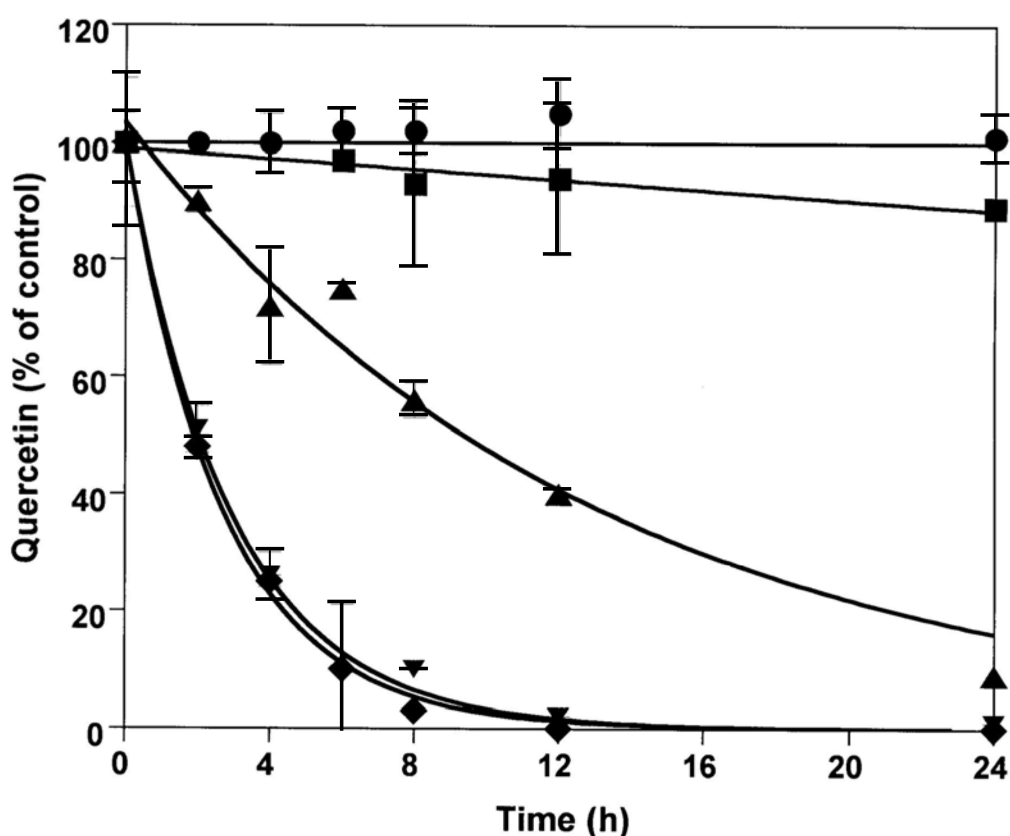

Figure 1 Stability in time of 50 µM quercetin solutions (0.5% DMSO v/v) in 0.1 M potassium phosphate pH 7.4 without (▲) and with (•) 1 mM ascorbic acid, and in McCoy's 5A medium without (♦) and with (!) 1 mM ascorbic acid or with (5) HCT-116 cells. Quantification of quercetin is based on HPLC peak area expressed as a percentage of the t = 0 samples. Data are presented as mean ± SD.

Quercetin solubility

Figure 2 presents the results of static light scattering studies on the solubility of quercetin in different media. To prevent quercetin degradation, experiments were all performed in the presence of 1 mM ascorbic acid. An increase in the relative intensity of scattered light reflects

the start of quercetin precipitation. The results obtained reveal that in 0.1 M potassium phosphate pH 7.4, quercetin precipitation starts at 80 μM. In McCoy's 5A medium without as well as with HCT-116 cells, maximum solubility is 100 μM. The addition of 10% fetal calf serum to McCoy's 5A medium enhances quercetin solubility significantly up to a concentration of 250 μM, possibly through extensive binding to serum proteins (*13*).

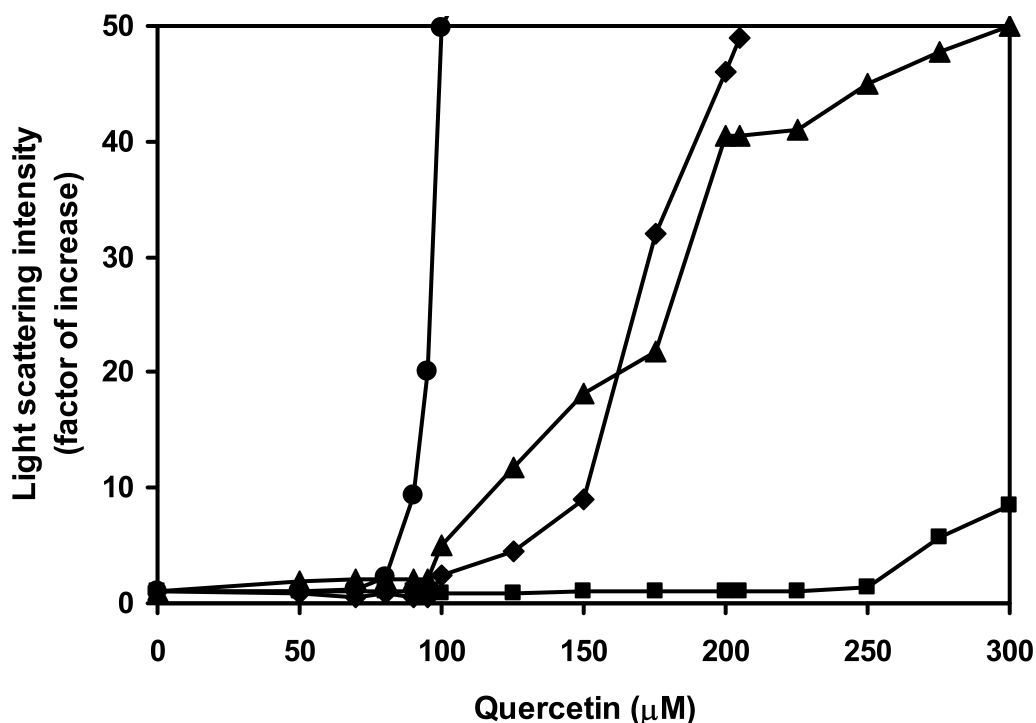

Figure 2 Solubility of quercetin in various media containing 1 mM ascorbic acid measured using static light scattering: McCoy's 5A medium (♦), McCoy's 5A medium in the presence of HCT-116 cells (▲), McCoy's 5A medium containing 10% fetal calf serum (■) and 0.1 M potassium phosphate pH 7.4 (•). The intensity of scattered light is expressed as a factor of increase relative to the corresponding control samples.

Cell viability and proliferation

Using the optimized conditions for quercetin stability and solubility, the effect of quercetin on cell proliferation was re-evaluated. Figure 3 shows the effect of quercetin, stabilized by the presence of ascorbic acid, on the proliferation of HCT-116, HT29 and MCF-7 cells after 24-hour exposure. The concentration range of quercetin used did not affect cell viability, as measured by LDH-leakage (data not shown). Quercetin appeared to inhibit the proliferation of colon cell lines, but this effect was only observed at relatively high concentrations, above 30 μM for HCT-116 and 80 μM for HT29 cells. Surprisingly, upon exposure of all cell types to

relatively low concentrations of quercetin, a subtle but significant increase in cell proliferation was observed, up to 20% for colon cell lines and up to 100% for MCF-7. These results suggest that quercetin modulates cell proliferation in a concentration-dependent biphasic way.

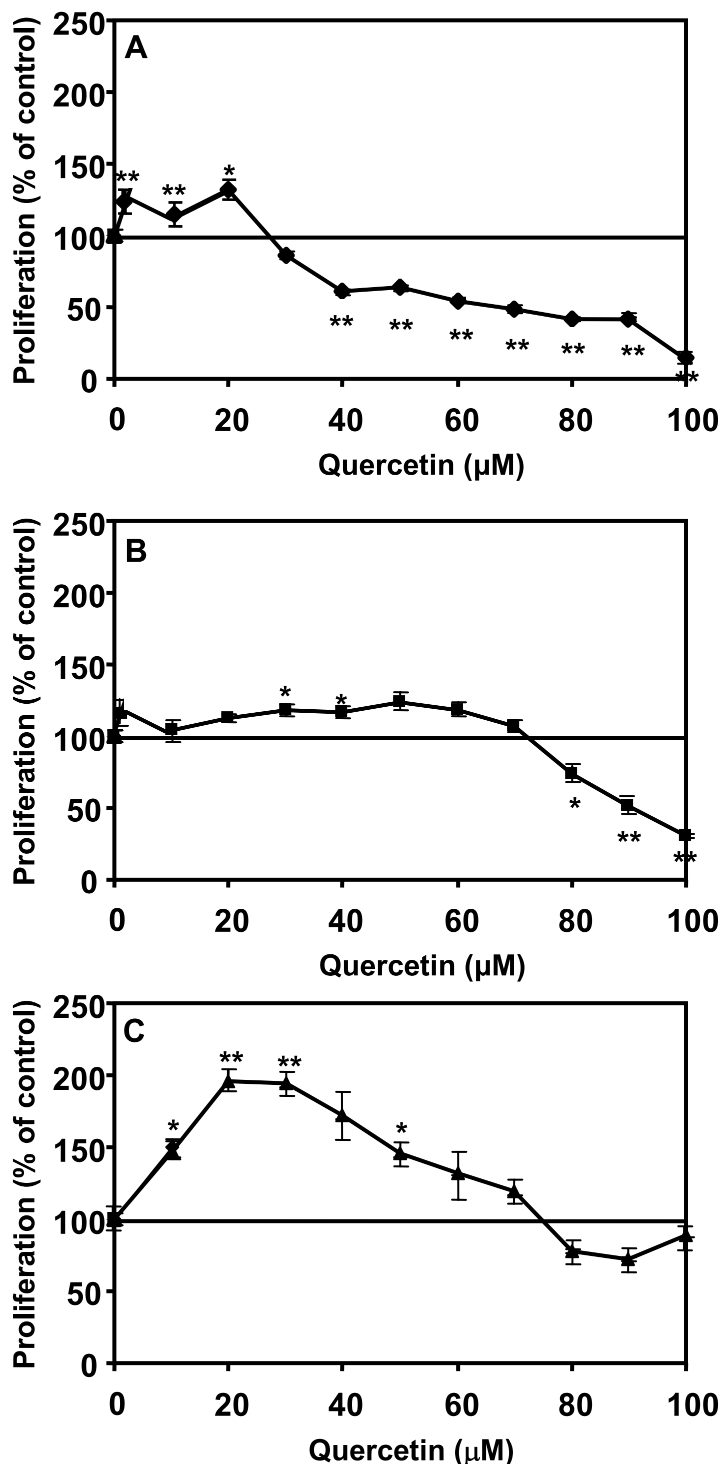

Figure 3 Effect of quercetin in the presence of ascorbic acid on proliferation of HCT-116 (A), HT29 (B) and MCF-7 (C) cells after 24-hour exposure. Proliferation is expressed as a percentage of control cells exposed to 0.5% DMSO in culture medium with ascorbic acid. Data are presented as mean ± SEM (n=6). Significantly different from control: *P<0.05 and ** P<0.01.

Discussion

In this study, analytical evidence is presented for the low stability and solubility of quercetin in aqueous media relevant for in vitro experiments. The poor stability of quercetin in aerobic aqueous media is due to its fast auto-oxidation (*6*)and could be prevented by the addition of 1 mM ascorbic acid. These results are in line with literature, reporting a half-life of 10 hours for quercetin in PBS pH 7.2 (*14*) and stabilization of quercetin by ascorbic acid (*9, 14, 15*). Degradation of quercetin was five times faster in McCoy's 5A medium, which is remarkable in view of the antioxidant components present, including vitamins and amino acids. Generally, conclusions in the literature on effects of quercetin have been drawn from in vitro exposure times varying from 12 hours to several days (*4, 5, 7-10*), in most cases without addition of stabilizing factors (*8, 16*). This implies that these literature results may have to be ascribed to lower concentrations of quercetin than originally added and/or by biologically active products resulting from the chemical decomposition of quercetin, like protocatechuic acid (*17, 18*)and phloroglucinol carboxylic acid (*18*). Furthermore, results of the present study provide evidence that the solubility of quercetin in the media tested is limited to 100 and 250 μM depending on the absence or presence of serum. The evaluated media are thought to be representative for many of the media currently in use for tissue culture purposes.

Using the thus defined optimal conditions for in vitro experiments with quercetin, the effect of quercetin on cell proliferation was re-evaluated in the colon cell lines HCT-116 and HT29 and in the mammary adenocarcinoma cell line MCF-7. A significant biphasic effect of quercetin on cell proliferation was found for the colon cell lines, whereas a mainly stimulating effect on cell proliferation was observed for the MCF-7 cell line within the concentration range tested. This result is of importance because in the majority of publications on the effects of quercetin on cell proliferation, only an antiproliferative effect is reported (*4, 7, 9, 10*). Several mechanisms for the antiproliferative effect of quercetin have been proposed, including DNA strand breakage (*4*), cell cycle arrest (*8*) and/or the induction of apoptosis (*8, 9*), possibly caused by inhibition of enzymes associated with signal transduction, including phosphatidyl-inositol-3-kinase (*19*), protein kinase C and protein tyrosine kinase (*20*). Furthermore, the antiproliferative effect of quercetin has been linked to its interaction with the type II estrogen binding sites (EBS) (*21-23*). This was supported by the fact that the sensitivity of HT29 for the antiproliferative effect of quercetin appeared to correlate with the number of type II EBS per cell (*10*).

In the present study, surprisingly, results obtained at lower concentrations of quercetin point at a subtle but significant stimulating effect on cell proliferation. So far, a dualistic effect of quercetin on cell proliferation has been reported only for the human oral squamous carcinoma cell line SCC-25, which showed growth stimulation after a 72-hour exposure to 1 and 10 μM, but growth inhibition at 100 μM quercetin (*24*). Several mechanisms for stimulation of cell proliferation by quercetin can be proposed. First, analogous to the mechanism suggested for the biphasic effect of the iso-flavonoid genistein on cell proliferation (*25*), the estrogen receptor (ER) might be involved in the slight stimulation of proliferation by quercetin seen in HT29, HCT-116 and MCF-7, all known to express ER-mRNA (*26, 27*). The fact that the quercetin induced increase in cell proliferation was more pronounced in the MCF-7 cells, known to express a different type of ER (ERα instead of ERβ) (*26, 27*)can be seen in favor of this explanation. However, contradictory data have been published on binding of quercetin to the ER as well as on subsequent ER-mediated gene expression (*25, 28*). Second, a biphasic effect on cell proliferation, generally called growth hormesis, might be explained by regulatory over-corrections by biosynthetic control mechanisms to low levels of growth inhibiting challenge (*29*), presenting another possible mechanism for the observed biphasic modulation of cell proliferation by quercetin. Finally, quercetin is a powerful antioxidant (*30*) capable of decreasing oxidative stress in in vitro cell systems (*31*). The balance between the antioxidant and the prooxidant activity of quercetin has been suggested to be concentration-dependent (*32*), providing a third possible mechanism for the concentration-dependent differential effects of quercetin on cell proliferation seen in this study.

The biphasic effect of quercetin on cell proliferation found in this in vitro study is important in view of the use of quercetin as bioactive functional food ingredient and/or food supplement. Indeed, blood serum levels in humans after oral quercetin ingestion from onions or supplements vary around 1 μM (*33, 34*), concentrations found in this study to enhance cell proliferation in HCT-116 cells. Higher quercetin concentrations, however, are expected in the intestine: following the ingestion of a standard quercetin supplement (250 to 500 mg) free quercetin concentrations inside the intestine lumen may vary within the limits of solubility shown in the present study to be between 0 and 100 μM. According to the findings in this study, this concentration range of quercetin could have promoting as well as inhibiting effects on cell proliferation, depending on the cell type.

Together, the results of the present study point at a dualistic effect of quercetin on cell proliferation at concentrations that are physiologically relevant. This observation may affect present views on its supposed beneficial antiproliferative effect.

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7

The stimulation of cell proliferation by quercetin is mediated by the estrogen receptor

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Abstract

Quercetin causes biphasic modulation of the proliferation of specific colon and mammary cancer cells. In this study, the possible involvement of the estrogen receptor (ER) in the stimulation of cell proliferation by quercetin was investigated. For this purpose, the effect of quercetin on cell proliferation was tested in ER-positive MCF-7 and T47D cells, and in ER-negative HCC-38 and MDA-MB231 cells. Quercetin stimulated proliferation of ER-positive cells only, suggesting this effect to be ER-dependent.

In support of these results, quercetin induced ER-ERE-mediated gene expression in a reporter gene assay using U2-OS cells transfected with either ERα or ERβ, with 10^5-10^6 times lower affinity than 17β-estradiol (E2) and 10^2-10^3 times lower affinity than genistein. Quercetin activated the ERβ to a 4.5 fold higher level than E2, whereas the maximum induction level of ERα by quercetin was only 1.7 fold that of E2. These results point at the relatively high capacity of quercetin to stimulate supposed 'beneficial' ERβ responses as compared to the stimulation of ERα, the receptor possibly involved in adverse cell proliferative effects. Altogether, the results of this study reveal that physiologically relevant concentrations of quercetin can exert phyto-estrogen-like activity similar to that observed for the isoflavonoid genistein.

Introduction

Quercetin (Figure 1A) is a well-known flavonoid, which can be found in a variety of fruits, vegetables, seeds and nuts where it is mainly present as glycoside. The Western diet contains approximately 16 mg/day of quercetin (*1*). The interest in quercetin as a functional food ingredient or food supplement is growing due to the presumed beneficial health effects of flavonoids and quercetin in particular, that include prevention of cancer and cardiovascular diseases (*2, 3*). These conclusions are mainly based on *in vitro* studies reporting quercetin to be a strong antioxidant (*4*) and inhibitor of cancer cell proliferation (*5, 6*).

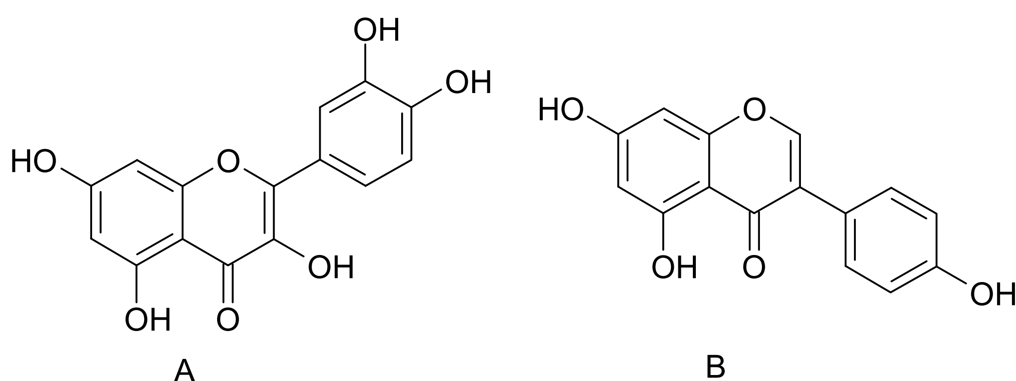

Figure 1 Molecular structure of quercetin (A) and genistein (B).

Recently, however, it was shown that quercetin causes a biphasic modulation of the proliferation of specific human colon cancer and breast cancer cell lines, known to express estrogen receptor (ER) mRNA (*7, 8*), including a stimulation of cell proliferation at the lower, physiologically relevant concentrations (*9*). Analogous to quercetin, the isoflavone genistein (Figure 1B) also causes a biphasic effect on cell proliferation (*10, 11*). This stimulation of cell proliferation by genistein, known to be a ligand of the ER (*11, 12*) and to stimulate ER-mediated gene expression, is only found in cells expressing the estrogen receptor (ER) (*13, 14*). The objective of the present study was to investigate whether, analogous to the mechanism underlying stimulation of cell proliferation by genistein, the ER is also involved in the stimulation of cell proliferation by quercetin.

The ER is a member of the superfamily of nuclear hormone receptors (*15*). To date, three different subtypes of the ER are known; ERα and ERβ are the most studied ones and recently, ERγ was discovered (*16*). ERα and ERβ are expressed in many tissues, including liver, brain, bone, and especially in reproductive tissues (*17*). Based on the finding that the ratio ERα / ERβ is higher in breast tumors than in normal tissue (*18*) and that by the formation of

heterodimers ERβ modulates ERα transcriptional activity (*19*), the hypothesis arose that ERα might mediate the proliferative effects of estrogenic compounds, whereas ERβ might have inhibiting effects on this process (*18*).

Whether quercetin can be classified as a phyto-estrogen is a matter of debate. Controversial findings have been reported on the binding affinity of quercetin for the ER (*10-12, 14*). Furthermore, although quercetin appeared to affect processes following ER activation, including binding of the ER to the ERE in the DNA and translocation of the ER from the cytoplasm to the nucleus (*20*), quercetin appeared to have no (*20-22*) or very little effect (*11*) on ER-mediated gene expression in cell lines. However, luciferase reporter gene assays in which the activation of the ER was studied generally pointed at quercetin as an estrogen agonist, although the induction at a concentration of 1 μM was approximately a factor 30-50 times lower than by 17β-estradiol (*12*). Finally, some studies using ER-positive cells showed no stimulation of cell proliferation mediated by quercetin (*20, 23*), whereas significant proliferation of ER-positive cell lines was reported in other studies (*9, 21*), the latter indicating the possible involvement of the ER in effects of quercetin on cell proliferation.

To obtain more insight into the possible role of the ER in the effects of quercetin on ER-mediated gene expression and cell proliferation, the ability of quercetin to induce ER/ERE mediated gene expression was studied in reporter gene assays. For this purpose, two clones of the human osteosarcoma cell line U2-OS were used, transfected with ERα or ERβ, expressing physiological levels of either of these receptors (*24*). Furthermore, the effect of quercetin on ER-mediated cell proliferation was investigated using the ER-positive cell lines MCF-7 and T47D (*25*), as well as the ER-negative cell lines MDA-MB231 (*26*) and HCC-38 (*27*). To validate the conclusions, the effects of quercetin on cell proliferation obtained in all these cell systems were compared with the effects obtained for the model phyto-estrogen genistein.

Materials and Methods

Materials

Dimethyl sulfoxide (DMSO) and quercetin dehydrate were purchased from Acros Organics (New Jersey, USA). Magnesium carbonate ($(MgCO_3)_4Mg(OH)_2 \cdot 5H_2O$) was obtained from Aldrich (Saint Louis, MO, USA). Hygromycin and D-luciferin were obtained from Duchefa (Haarlem, The Netherlands). Trans-1,2-diaminocyclohexane-N,N,N',N'-tetra-acetic acid monohydrate (CDTA) was obtained from Fluka (Buchs, Germany). Ascorbic acid, sodium bicarbonate ($NaHCO_3$), sodium hydroxide (NaOH), ethylenedinitrotetra-acetic acid

(EDTA·$2H_2O$; Titriplex), magnesium sulfate ($MgSO_4 \cdot 7H_2O$) and 1,4-dithiothreitol (DTT) were obtained from Merck (Darmstadt, Germany). Tris was obtained from Invitrogen Life Technologies (Paisley, Scotland). The cell culture media Dulbecco's Modified Eagle's Medium (DMEM), Ham's F12, RPMI-1640, non-essential amino acids, gentamicin, geneticin, L-glutamine, sodium pyruvate, fetal calf serum (FCS), phosphate buffer solution (PBS), Hank's Balanced Salt solution (HBSS) were obtained from Gibco (Paisley, Scotland). Adenosine triphosphate (ATP) was obtained from Roche (Mannheim, Germany). 17β-Estradiol, genistein and tricine were obtained from Sigma (Saint Louis, MO, USA).
The human breast cancer cell lines MCF-7, T47D, MDA-MB-231 and HCC-38 were purchased from the American Type Culture Collection (Manassas, VA, USA). The human osteosarcoma cell line U2-OS transfected with an expression vector encoding either human ERα or ERβ as well as with the estrogen-responsive reporter gene plasmid 3xERE-tata-Luc was made as previously described (*24*).

Cell culture

The human breast cancer cell lines T47D and HCC-38 were cultured in RPMI-1640 medium, supplemented with 10% FCS, 1 mM sodium pyruvate, 2 mM L-glutamine and 50 μg/mL gentamicin. MCF-7 and MDA-MB-231 cells were cultured in DMEM, supplemented with 10% FCS and 50 μg/mL gentamicin. Cells were cultured at 37°C, 5% CO_2 in a humidified atmosphere.
The human osteosarcoma cell lines U2-OS-ERα and U2-OS-ERβ were cultured in a 1:1 mixture of DMEM and Ham's F12 medium, buffered with 1260 mg/l $NaHCO_3$, supplemented with 7.5% FCS and 0.5% non-essential amino acids. In addition, medium for U2-OS-ERβ medium was supplemented with 200 μg/ml geneticin as a selection marker and medium for U2-OS-ERα was supplemented with both 200 μg/ml geneticin and 50 μg/ml hygromycin. Cells were cultured at 37°C and 7.5% CO_2 in a humidified atmosphere.

Luciferase assay

Confluent U2-OS cells were trypsinized and seeded in transparent 96 well plates (Nunclon, NUNC, Rochester, NY, USA) at a density of $10x10^4$ cells/ml (U2-OS-ERα) or $7.5x10^4$ cells/ml (U2-OS-ERβ) in a 1:1 mixture of DMEM and Ham's F12 medium without phenol red, buffered with 1260 mg/l $NaHCO_3$ and supplemented with 5% dextran-charcoal-treated FCS (DCC-FCS) and 0.5% non essential amino acids (100 μL/well). DCC-FCS was prepared

by heat inactivation (30 minutes at 56°C) of FCS, followed by two 45-minute DCC-treatments at 45°C as previously described (*28*). Culture medium was refreshed after 24 hours. Forty eight hours after seeding, cells were exposed in triplicate to quercetin or genistein in the presence of 600 µM ascorbic acid for stabilization of the test compounds (*9*) at the indicated concentrations (final DMSO concentration 0.1%) for 24 hours at 37°C, 5% CO_2 in a humidified atmosphere. Ascorbic acid in a concentration of 600 µM proved to have no cytotoxic effects on U2-OS cells. To guarantee stability of the test compound for the whole incubation period under these conditions, the exposure medium was refreshed after 8 hours. In addition, on each plate, cells were exposed in triplicate to a calibration curve of 17β-estradiol in the presence of 600 µM ascorbic acid, at the indicated concentrations (final DMSO concentration 0.1%). After 24 hours, the medium was removed and cells were washed with 100 µL/well 0.5x PBS. Cells were lysed with 30 µL of a hypotonic low salt buffer, pH 7.8, consisting of 10 mM Tris, 2 mM DTT and 2 mM CDTA in nanopure. Plates were put on ice for 10 minutes and subsequently frozen at –80°C.

Before analysis, plates were thawed on ice and shaken to reach room temperature. Analyses were performed at room temperature in a Luminoskan (RS, Labsystems) as follows: first, background light emission of each plate was measured for two seconds. Then, 100 µl/well flashmix was added, after which light emission was measured for another two seconds and extinguished with 50 µl 0.2 M NaOH. Flashmix consisted of 20 mM tricine buffer pH 7.8, supplemented with 1.07 mM $(MgCO_3)_4Mg(OH)_2.5H_2O$, 2.67 mM $MgSO_4$, 0.1 mM $EDTA{\cdot}2H_2O$, 2 mM DTT, 0.47 mM D-luciferin and 5 mM ATP. Luciferase induction by quercetin and genistein was compared with the luciferase induction by the natural ligand for the ER, 17β-estradiol.

To determine EC_{50} values of the compounds, curves were fitted using Slidewrite 6.10 for Windows. The EC_{50} was defined as the concentration of compound at which 50% of the maximum luciferase activity was reached. EC_{50} values were expressed as mean ± standard error. The estradiol equivalency factor (EEF) was calculated using equation 1:

$$EEF = \frac{EC50(estradiol)}{EC50(compound)} \qquad \textbf{(1)}$$

Cell proliferation

Confluent cells were washed with HBSS, trypsinized and seeded in transparent 96-wells plates (100 µL/well; Greiner, Frickenhausen, Germany) in plate medium. The composition of

plate medium was similar to the culture medium described above, with the following modifications: phenol red or antibiotics were omitted, and instead of FCS, 5% DCC-FCS was added. The cells were incubated for 24 hours at 37°C in a humidified atmosphere and 5% CO_2, as indicated above, to allow attachment. Plates having 60-80% confluent wells were used for experiments.

After attachment, culture medium was removed and 100 μL/well exposure medium was added. The composition of the exposure media was similar to the culture media described above, only without phenol red, FCS and antibiotics. Exposure media were supplemented with quercetin or genistein from stock solutions in DMSO (final concentration 0.5%) at the indicated concentration range, proven to give no cytotoxic effects in the LDH-leakage test (data not shown), the latter performed essentially as previously described (*29*). Furthermore, 200 μM (MCF-7 cells), or 1 mM ascorbic acid (T47D, MDA-MB-231 and HCC-38 cells) was added for flavonoid stabilization (*9*). Ascorbic acid in a concentration of 200 μM proved to have no cytotoxic effects on MCF-7 cells. To guarantee presence of the test compound for the whole incubation period under these conditions, the exposure medium was refreshed after 8 hours.

After 24 hours of incubation, the extent of proliferation was determined with the BrdU-labeling method using the Cell Proliferation ELISA, BrdU (colorimetric) kit from Roche Diagnostics GmbH (Mannheim, Germany). Results were expressed as percentage of the proliferation of control cells exposed to the solvent DMSO in culture medium containing ascorbic acid.

Statistical analysis

Statistical analysis of data was carried out using the Dunnett or Dunnett T3 test (ANOVA). Differences were significant if at least $P<0.05$.

Results

Effect of quercetin and genistein on ER-ERE-mediated luciferase activity in transfected U2-OS cells

To investigate whether quercetin and genistein can induce gene expression by interaction with ERα and/or ERβ, the effect of the two compounds was studied in reporter gene systems consisting of human osteosarcoma U2-OS cells, transfected with ERα or ERβ, and a luciferase reporter gene with an ERE-containing promoter region. Figure 2A and 2B show the

effect of quercetin, genistein and 17β-estradiol (E2) on luciferase activity mediated by ERα and ERβ, respectively

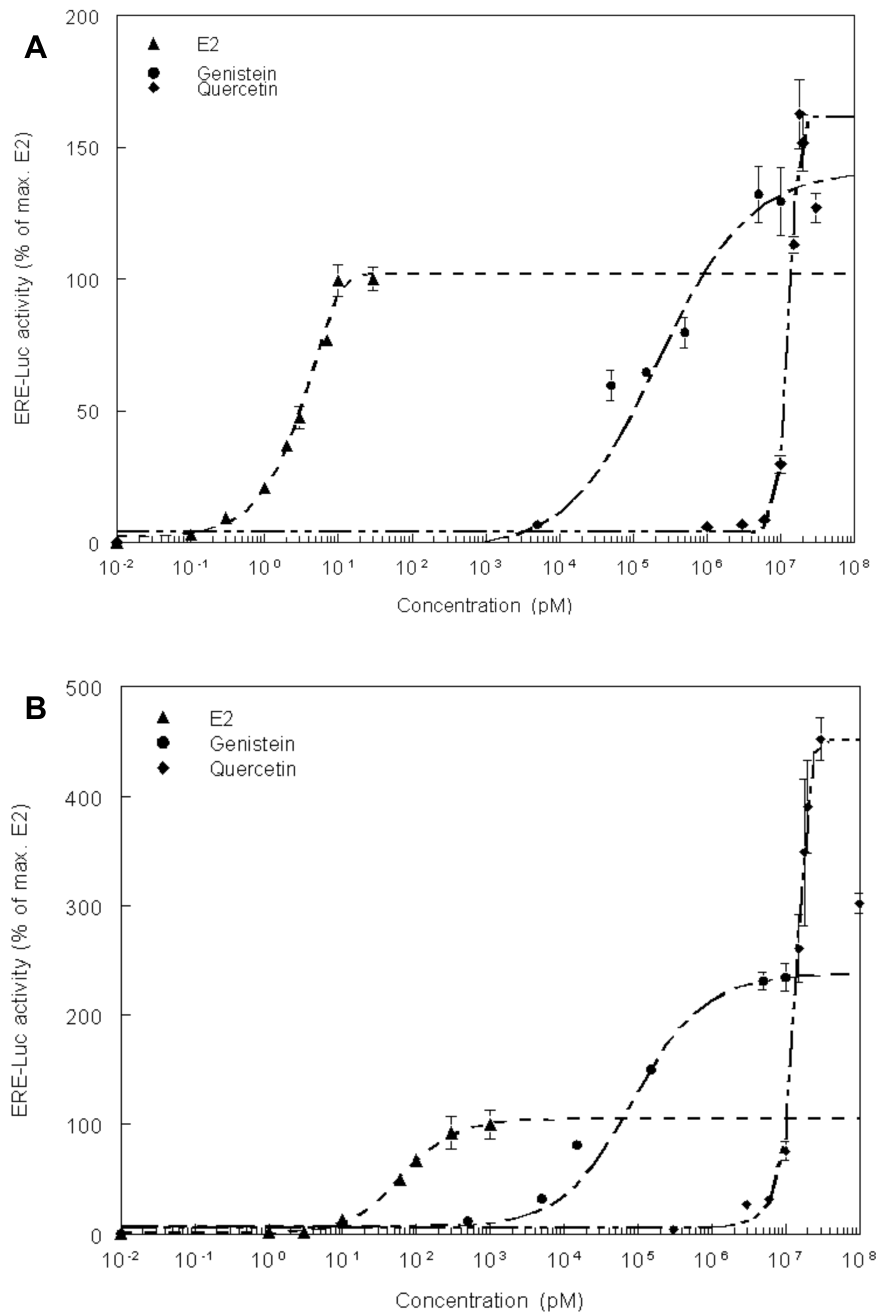

Figure 2 ER-ERE mediated luciferase activity in U2-OS-ERα (A) and U2-OS-ERβ (B) cells exposed to quercetin or genistein for 24 hours in the presence of ascorbic acid, compared to a reference curve of 17β-estradiol (E2).

Comparing the affinities of quercetin, genistein and E2 for ERα and ERβ, it appeared that the EC_{50} of quercetin was approximately similar for ERα and ERβ, amounting to 11 ± 0.1 and 8

± 0.7 μM, respectively. The affinity of genistein for the ERs appeared to be higher than that of quercetin. Genistein showed a higher affinity for ERβ than for ERα, with EC_{50} values of 9 ± 0.1 and 58 ± 6 nM respectively. Previously, EC_{50}-values of 6 and 20 nM were determined (*12*). For E2, EC_{50} values of 3 ± 0.3 pM and 58 ± 2 pM were found for ERα and ERβ respectively, which is comparable to values from earlier studies, amounting to 5 and 50 pM respectively (*12*). Consequently, the EEFs of quercetin for ERα and ERβ were $2x10^{-7}$ and $7x10^{-6}$, respectively whereas the EEFs of genistein for ERα and ERβ were $5x10^{-5}$ and $7x10^{-3}$, respectively. Thus, the affinities of both genistein and quercetin for the ERs were lower than that of E2, the affinity of quercetin for the ERα being a factor 10^7 lower, that for ERβ a factor 10^6 lower. The affinity of genistein for ERα and ERβ was a factor 10^5 and 10^3 lower respectively than that of E2.

Although the affinities of quercetin and genistein for both ERs were lower than that of E2, the maximum induction reached was higher. For ERα, maximum induction by quercetin and genistein were respectively 1.7 and 1.4 times higher than that of E2. For ERβ, the difference with E2 was even higher, with maximum induction of quercetin and genistein being respectively 4.5 and 2.4 times higher than that of E2. At quercetin and genistein concentrations above respectively 1.8 to $5x10^7$ pM and $1x10^7$ pM, ERE-Luc activity decreased, which was probably due to cytotoxicity of the test compounds. Altogether, the results show that both quercetin and genistein can induce ER-mediated gene expression in the presence of ERα or ERβ, although with lower affinity than E2. The maximum induction that can be reached with these compounds is however significantly higher than with E2.

Effect of quercetin and genistein on the proliferation of ER-positive and ER-negative cell lines

In order to validate the results obtained in the reporter gene systems, an *in vitro* system with a physiologically more relevant endpoint was used to investigate whether the ER is involved in the stimulating effect of quercetin on cell proliferation. Because no functional ER was detected in original U2-OS cells (*24*) and because E2 proved to have no effect on proliferation of original U2-OS cells, or U2-OS cells transfected with ERα or ERβ (*30*), these types of U2-OS cells were not used for proliferation experiments because these observations point at the possible absence of an ER-dependent proliferation machinery. Figure 3 and Figure 4 show the effects of quercetin and genistein, respectively, on the proliferation of the ER-positive cell

lines T47D (A) and MCF-7 (B), and of the ER-negative cell lines MDA-MB-231 (C) and HCC-38 (D). The concentration range tested proved to have no effect in the LDH-leakage assay for all cell lines tested (data not shown). In the ER-negative cell lines, quercetin inhibited proliferation over the entire concentration range tested (Figure 3). From these results, it can be concluded that the inhibition of cell proliferation caused by quercetin is not dependent on the presence of an ER. However, a biphasic effect on cell proliferation was found for the cell lines expressing an ER, with a stimulating effect of quercetin on cell proliferation up to approximately 150% at relatively low concentrations. At concentrations higher than 45 or 55 μM for T47D and MCF-7 cells, respectively, quercetin inhibited cell proliferation (Figure 3).

In contrast with quercetin, genistein (Figure 4) showed no significant effect on the proliferation of ER-negative cell lines in the concentration range tested. However, genistein stimulated cell proliferation in the ER-positive cell lines with a maximum of approximately 200% in T47D and 120% in MCF-7. In the higher concentration range, genistein had no significant effect on cell proliferation. In view of the fact that the stimulating effect of both quercetin and genistein on cell proliferation was only seen in cell lines expressing the ER, these results suggest that the stimulating effect of quercetin and genistein on cell proliferation is mediated by the ER.

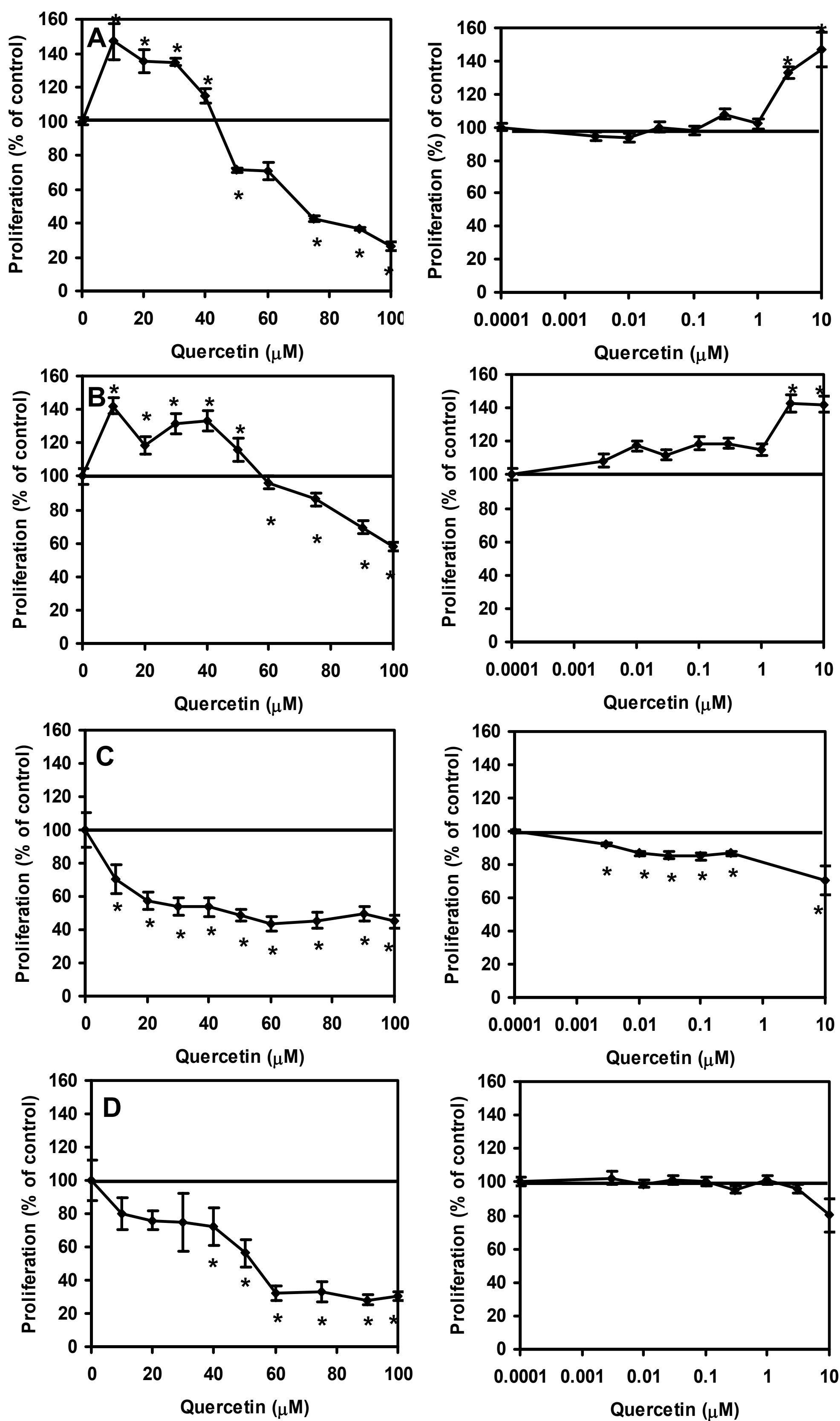

Figure 3 Effect of quercetin in the presence of ascorbic acid on the proliferation of T47D (A), MCF-7 (B), MDA-MB-231 (C) and HCC-38 (D). Graphs on the right are results obtained at the lower concentration range for the same cell line. * Significantly different from control at P<0.05.

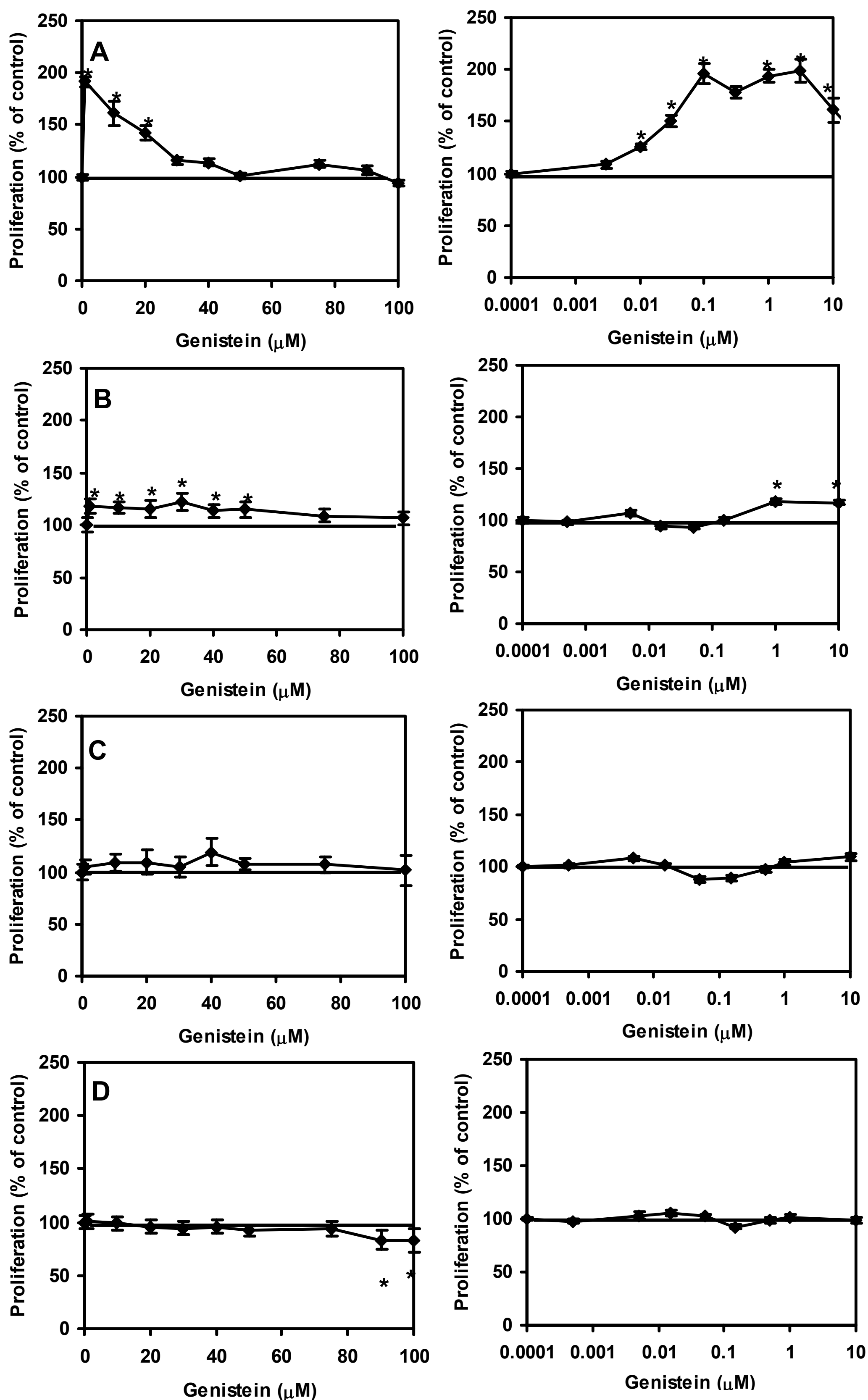

Figure 4 Effect of genistein in the presence of ascorbic acid on the proliferation of T47D (A), MCF-7 (B), MDA-MB-231 (C) and HCC-38 (D). Graphs on the right are results obtained at the lower concentration range in the boxes in the graphs on the left. * Significantly different from control when P<0.05.

Discussion

Recent studies showed that quercetin has a biphasic effect on the proliferation of the mammary carcinoma cell line MCF-7 and on the colon carcinoma cell lines HCT116 and HT29 (*9*). Because these cell lines express ER-mRNA (*7, 8*) and because the ER is involved in the stimulation of cell proliferation by the phyto-estrogen genistein (*11, 12*), the hypothesis that the ER mediates the stimulating effect of quercetin on cell proliferation was investigated in this study.

First the capacity of quercetin to activate the ER and to induce subsequent gene expression was investigated using a reporter gene assay in U2-OS cells transfected with either ERα or ERβ. Quercetin, and for comparison also genistein, both induced ER/ERE mediated luciferase activity in both ERα and ERβ receptor systems, although with higher EC_{50} values than E2, which is in line with previous studies (*12, 22*). It is important to note that both compounds have a higher induction factor in both cell systems than E2. This result was reported before for genistein but claimed to be absent for quercetin (*12*). The fact that in our study quercetin was stabilized by the presence of ascorbic acid (*9*) might account for the enhanced quercetin-induced gene expression seen in the present study. The control incubation, relative to which proliferation was expressed, excludes possible effects on cell proliferation of ascorbic acid itself.

To validate the results with a physiologically more relevant endpoint than luciferase activity, the effects of quercetin and genistein on cell proliferation were tested in human breast cancer cell lines, including the ER-positive cell lines MCF-7 and T47D and the ER-negative cell lines HCC-38 and MDA-MB-231. In the ER-positive cell lines E2 has been reported to modulate cell proliferation in a biphasic manner (*25*). Similar to E2, low concentrations of quercetin enhanced the proliferation of the ER-positive cell lines only, thereby showing that the stimulating effect of quercetin on cell proliferation is ER-dependent. The isoflavone genistein, used in this study as model phyto-estrogen, also displayed stimulating effects on cell proliferation in ER-positive but not in ER-negative cell lines. The fact that quercetin showed similar effects in both the reporter gene assay and the proliferation assay as the well-established phyto-estrogen genistein (*11-14*) indicates that quercetin may also be considered a phyto-estrogen.

The present study suggests that the ER is involved in the stimulation of cell proliferation by quercetin. The mechanism behind ER-mediated stimulation of cell proliferation by quercetin is still unclear. However, the results of the reporter gene assay showed that quercetin is

capable of activating ER-ERE mediated gene expression in a concentration range comparable to the range in which stimulating effects of quercetin on cell proliferation were seen. Nevertheless, controversial findings have been reported on the binding affinity of quercetin for the ER (*11, 12, 14*), which raises the question whether quercetin can activate the ER by acting as a ligand of the receptor (*22*), or whether a different mechanism is involved. Another possibility is that quercetin affects the phosphorylation state of various components in the signal transduction cascade involved in ER activation, by interference with the activity of kinases (*31-34*).

The results of the present study also show that quercetin inhibits cell proliferation at the higher concentrations in all cell lines studied, which suggests that the inhibition of cell proliferation by quercetin is not dependent on the ER. Different mechanisms underlying the inhibition of cell proliferation by quercetin have been proposed, including DNA strand breakage (*35*), cell cycle arrest (*36*) and/or the induction of apoptosis (*6, 36*), possibly by influencing the activity of various kinases, including phosphatidylinositol-3-kinase, tyrosine protein kinase and protein kinase C (*33, 34*). Another plausible mechanism of inhibition of cell proliferation, which is also in line with the estrogenic character of quercetin, is the interaction of quercetin with so-called type II estrogen binding sites (EBS) (*37, 38*), probably involved in the inhibition of estrogen-stimulated growth *in vivo* (*38*). In contrast with previous data (*11*), genistein, contrary to quercetin, did not inhibit cell proliferation at concentrations up to 100 μM in the present study. However, similar to quercetin, genistein is also known as a protein kinase inhibitor (*39*) and a ligand for type II EBS with subsequent inhibitory effect on cell proliferation (*11*). Up to now, however, no differences in efficiency of kinase activity inhibition or type II EBS-mediated inhibition of cell proliferation between quercetin and genistein have been reported that might account for the differences in effects seen (*36, 40*).

Phyto-estrogens are generally associated with health-promoting effects, including the inhibition of breast and endometrial cancer (*41*), cardiovascular diseases and osteoporosis (*42*). The present data however suggest that quercetin and also genistein have a stimulating effect on cell proliferation of ER-containing cells at the lower physiologically relevant concentrations (*43, 44*). Of importance to note is the fact that quercetin and genistein appeared able to activate the ERβ to a 4.5 and 2.4 fold higher level respectively than E2, whereas the maximum induction levels of ERα were only 1.7 and 1.4 fold higher than that of E2. This is especially interesting given the hypothesis that ERα might mediate the proliferative effects of estrogenic compounds, whereas ERβ might have protective effects on

this process (*18*) by the formation of heterodimers of ERα and ERβ (*19*). Thus, the results of the present study point at the relatively high capacity of the flavonoids to stimulate 'beneficial' ERβ responses as compared to their stimulation of ERα, the receptor possibly involved in adverse cell proliferative effects. This might be an explanation for the inverse correlation between cancer incidence and consumption of fruits and vegetables, rich in this type of bioactive compounds (*45, 46*). Whether the extent of cell proliferation in the ER-containing cells also matches their relative level of ERα as compared to ERβ, remains an interesting topic for further research and awaits identification and quantification of the ER types present in MCF7 and T47D cells used in the present study.

Nevertheless, the fact that quercetin has estrogenic potency suggests that it might interact with the endocrine function. Although phyto-estrogens generally have a lower affinity for ER activation than E2 as reflected by their EC_{50} values, their respective physiological concentrations are such that values in the order of the EC_{50} values can be expected for both types of compounds (*43, 47*). The present results show that the maximum induction by quercetin and genistein is even 2 to 5 fold higher than that by E2 at these physiologically relevant concentrations. Because in general, phyto-estrogens other than quercetin or genistein may also reach concentrations that are 1000-fold higher than E2 concentrations *in vivo* (*48*) and because additive effects of estrogenic compounds are known (*49*), the cumulative effect of several compounds with weak estrogen agonism may result in significant effects *in vivo* (*50*), especially because phyto-estrogens may interfere with the development of the fetus (*51*). Several animal studies revealed possible adverse health effects of phyto-estrogens, including stimulation of tumor growth in mice (*52*) and neurobehavioral effects in primates resulting in altered patterns of agonistic and social behavior (*53*). In view of these effects and the fact that consumption of large amounts of food supplements may dramatically increase the dietary load of quercetin, careful attention should be paid to the balance between beneficial and potentially adverse effects of this flavonoid.

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8

The definition of hormesis and its implications for *in vitro* to *in vivo* extrapolation and risk assessment

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Abstract

The present paper comments on some of the basic questions put forward in state-of-the-art discussions on hormesis. There seems to be a need for a better definition of the concept itself and reconsideration of whether all biphasic dose-response curves should be considered representative for hormesis. Hormesis may be restricted to phenomena that proceed by mechanisms that are broadly generalizable and represent possibly beneficial overcompensation in response to an adverse stimulus. Using the concept that hormesis is defined as such, the biphasic effect of quercetin on cell proliferation, but also several other receptor-mediated biphasic dose-response phenomena should not be related to hormesis.

Taking into account hormesis in the procedures for risk assessment on compounds characterised by a threshold for the adverse effect is another matter for considerable debate. To our opinion, this would require the reduction of safety factors, providing the possibility for beneficial hormesis-type effects for some people, at the cost of increased chances on adverse effects for other parts of the population. Whether this is a proper way forward remains to be discussed. Improvement of risk assessment strategies may include taking into account biphasic dose-response curves, but should rather start with the consideration of proper physiology-based pharmaco-kinetic (PBPK) models for better extrapolation of differences in toxicokinetics going from high to low dose exposure, as well as taking into account kinetics for gene repair systems. Without considering in vivo toxicokinetics in the in vitro models, extrapolation from in vitro biphasic dose-response curves on cell proliferation to in vivo cell proliferation are difficult to make. Altogether it is concluded that hormesis is an important phenomenon especially from the scientific point of view, but that its consequences for risk assessment and the possibilities for in vitro to in vivo extrapolation may remain limited without additional mechanistic insight.

Introduction

Within the past years there has been growing interest in the topic of hormesis, with some papers even stating that the topic is expected to have profound impact on the practice of risk assessment (*1-3*). However, based on our work on biphasic quercetin-mediated effects on cell proliferation (*4*) it seems relevant to ask the following questions: 1) is the principle of hormesis well enough defined, and 2) what are the implications of hormesis for risk assessment? The present paper gives some views on these matters.

What is hormesis and is it well defined?

Hormesis has been defined as a dose-response phenomenon characterised by a stimulatory response at low doses and an inhibitory response at high doses (*5-7*). The hormetic response may also consist of a low-dose inhibitory response and stimulation at high doses (*1*), but to provide a clear overview, the former definition will be used throughout this paper. The accompanying paper on cancer biology and hormesis (*8*) states that 'hormetic dose-responses are broadly generalizable, being independent of biological model, endpoint measured and stressor agent, and represent a basic feature of biological responsiveness to chemical and physiological stress'. Furthermore, hormesis has been presented to the general public by stating (*9*) '… hormesis appears to be primarily an adaptive response to stress. The stress triggers cellular repair and maintenance systems. A modest amount of overcompensation then produces the low-dose effect, which is often beneficial'. Examples include a single dose of ionizing radiation that stimulates DNA repair, delaying the onset of cancer in mice (*10*), and reduced tumor incidence in rats at low doses of cadmium chloride (*11*). These and many other examples have led to the conclusion that hormesis originates from activated defence mechanisms that result in overcompensation leading to possibly beneficial low dose effects (*5, 8*).

Recently we described a biphasic dose-response curve for the proliferation of MCF7, HT29, and HCT-116 cells exposed to quercetin (*4*) (Figure 1). In the discussion of this observation it was pointed out that the biphasic effect of quercetin on cell proliferation could be explained by regulatory over-corrections by biosynthetic control mechanisms to growth inhibiting challenge at low doses and an adverse inhibitory effect on cell proliferation at high doses of quercetin. Thus, in line with the definitions given above, the biphasic effect would be due to what is defined as hormesis. Another mechanism was considered as well, and suggested to be related to the estrogen receptor (ER), since MCF7, HT29 and HCT-116 cells are all known to

express ER-mRNA (*12*). More recent data reveal the absence of the increase in cell proliferation induced by low levels of quercetin, but still a decrease in cell proliferation at higher levels of quercetin in ER-deficient (*13*). These results further illustrate a role for the ER in the increase in cell proliferation at low doses of quercetin. The inhibition of cell proliferation, observed at relatively higher concentrations seems to be ER-independent, since it was observed also in cells known to be devoid of the ER. The antiproliferative effect of quercetin is not ER-mediated or may be linked to its interaction with a different type of estrogen-binding protein, namely the type II estrogen binding sites (EBS) (*14-16*).

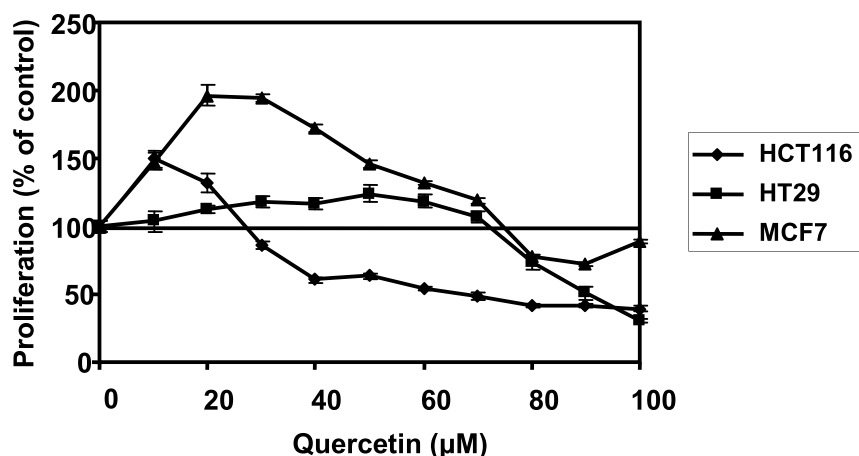

Figure 1 Biphasic dose-response curve for quercetin-mediated effects on proliferation of HCT116, HT29 and MCF7 cells (*4*).

The fact that the quercetin-mediated inhibition of cell proliferation was observed in all cell lines and proved to be ER-independent, but that the increase in proliferation was only observed for ER-containing cells, leads to the conclusion that the increase in cell proliferation does not seem to represent an overcompensation in response to an adverse effect leading to decreased cell proliferation by the same stressor at higher concentrations. This conclusion is corroborated by considering the normal physiological role of the ER. Figure 2 presents a schematic overview of the functioning of the ER, illustrating how binding of an estrogen-like compound to the receptor initiates gene expression, leading to cell proliferation. The suggestion that an ER-mediated increase in cell proliferation represents overcompensation of a disruption in homeostasis does not seem to be in line with the concept of the function of this receptor in various cellular processes (*17-20*). So, based on the regular function of the ER, the ER-mediated increase in cell proliferation cannot be qualified as “overcompensation”. Furthermore, the biphasic dose-response curve of quercetin and the ER-mediated increase in cell proliferation at low quercetin concentrations seem to be restricted to ER-containing cells,

and can therefore not be described as “broadly generalizable”. Altogether, this leads to the conclusion that the ER-mediated increase in cell proliferation by low doses of quercetin may not represent a case of hormesis. Hormesis should rather be restricted to those cases where the low dose stimulatory response occurs following initial disruption in homeostasis and appears to represent a modest overcompensation response.

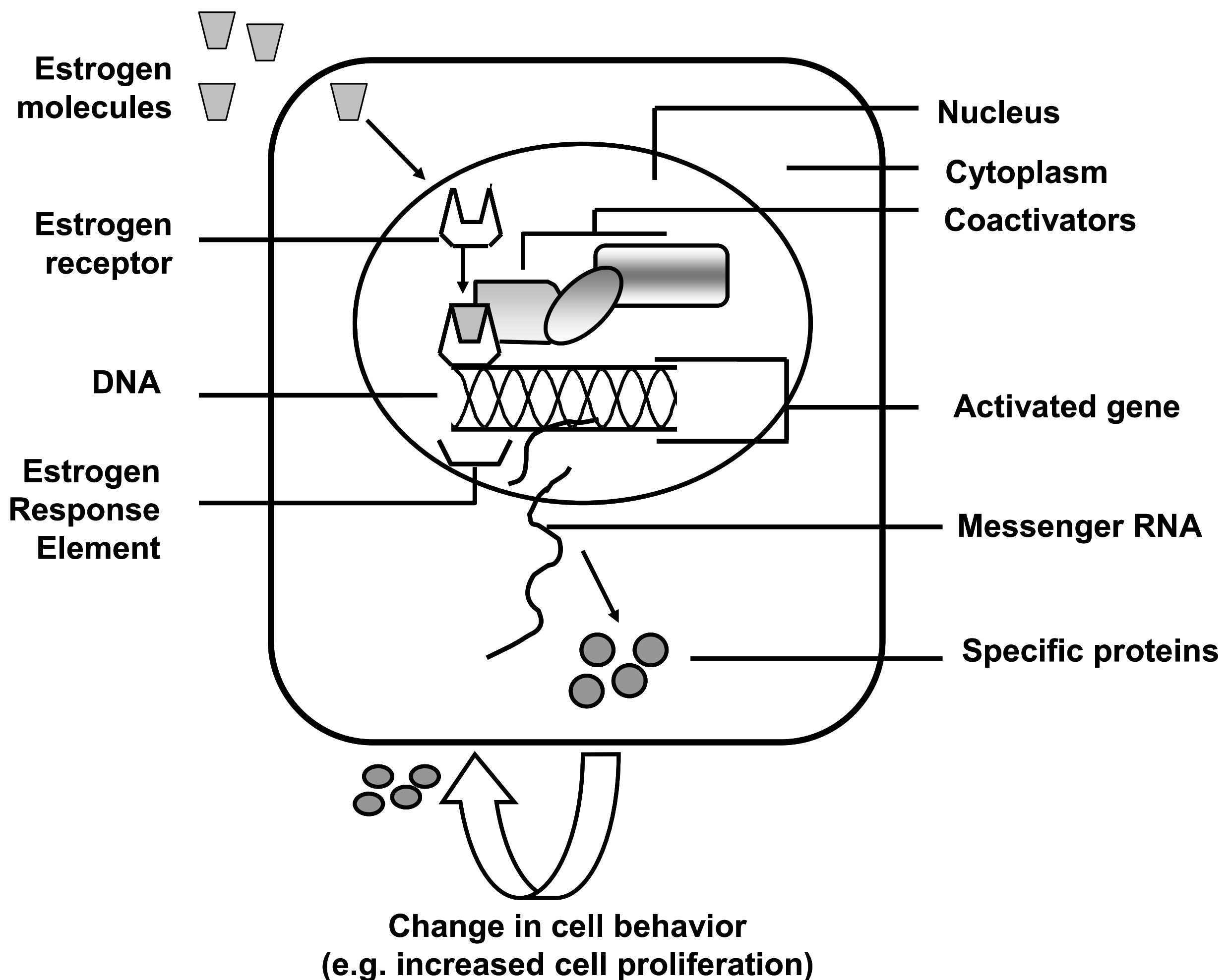

Figure 2 Schematic presentation of the effect of estrogens on cell behavior, mediated via the ER, which acts as a transcription factor in the nucleus.

Several other examples of biphasic dose-response curves exist that represent cases of activation of different stimulatory and inhibitory regulatory pathways by receptors with different agonist affinities (*8, 21, 22*). One example is the biphasic dose-response curve for dexamethasone-mediated effects on cell growth, showing stimulation at the lower concentration range in glucocorticoid receptor (GR) positive cells, no stimulatory effect on the growth of GR-negative cells and at higher concentrations, inhibition of cell growth that appeared independent of the presence of the receptor (*21*). Furthermore, thirteen phyto-estrogens caused a biphasic effect on DNA synthesis in ER-positive cells whereas they

inhibited cell proliferation in ER-negative cells (*22*). A final example relates to the stimulation of cell proliferation mediated by high affinity epidermal growth factor receptor (EGFR) at the lower concentrations of epidermal growth factor, and inhibition of cell proliferation being mediated by low affinity EGFR at the higher concentration range (*23*). These examples of biphasic dose-response curves point at the need for a better definition of hormesis, since receptor-mediated responses as described in the examples above cannot be considered as 'overcompensation' and 'broadly generalizable'.

What are the implications of hormesis for in vitro to in vivo extrapolation and risk assessment?

Another important issue to discuss in the field of hormesis relates to how risk assessment paradigms need to be modified to take hormesis into account. It is claimed that recognition of the phenomenon of hormesis should lead to markedly improved integrative assessments of whole animal/human responses to toxic substances in general (*5-7*). Some extreme opinions even state that the regulatory agents may be damaging our health by not taking into account the beneficial effects of low dose exposure to toxins. Therefore it is worth considering the impact the concept of hormesis could have on regular risk assessment strategies. As far as knowledge reaches today, there seems to be no need to discriminate in this discussion between non-genotoxic and genotoxic compounds, because the genotoxic compounds showing hormetic dose-response relationships generally seem to have a threshold for the adverse effect(*24-26*). Therefore, one can focus on compounds characterised by a dose-response curve with a threshold for the adverse effect. The threshold in the dose response curves of these compounds can be characterised as a NOAEL (No Observed Adverse Effect Level), also called ZEP (zero equivalent point). Figure 3 shows the hormetic dose-response curve for this type of compounds, with the NOAEL being the concentration below which the stimulatory response is observed. The NOAEL threshold dose-response model considers all responses below this dose to be zero. In risk assessment procedures the NOAEL is used for definition of safe exposure limits, including for example ADI (Acceptable Daily Intake) values, TDI (Tolerable Daily Intake) values, and RfD (Reference Dose) values. To convert NOAEL data from animal experiments into these safety levels requires the use of safety factors that take into account inter- and intraspecies differences and in some cases additional safety factors to take into account for example differences in exposure regimen between the animal experiment and the human situation (p.e. subacute in the animal experiment versus

chronic human exposure). Generally these safety or assessment factors amount all together to a factor of at least 100 (10 for interspecies and 10 for intraspecies differences), resulting in a NOAEL divided by at least 100 to reach for example the ADI or TDI. A detailed study on probabilistic assessment factors for human health risk assessment has confirmed that an overall assessment factor of at least 100 is required to protect 95 % of the general population(*27*). This safety factor should be compared to the dose range leading to the hormetic stimulating response below the NOAEL. It is generally stated that most stimulatory ranges for an hormetic effect are less than 100-fold measuring back from the ZEP dose/concentration(*1, 8*). This implies that taking into account hormesis in risk assessment would require the reduction of safety factors providing the possibility for hormesis-type beneficial effects at low doses for part of the population at the cost of increased chances on adverse effects for another part of the population. Whether this is a proper way forward remains to be discussed.

Improvement of risk assessment strategies may include taking into account biphasic dose-response curves and hormesis, but should rather start with the consideration of proper physiology-based pharmaco-kinetic (PBPK) models for better extrapolation of differences in toxicokinetics going from high to low dose exposure, as well as taking into account kinetics for gene repair systems. For our model compound quercetin, for example, it is well known that quercetin concentrations in human plasma are generally amounting to a few micromolar (*28*), with most of the quercetin being present in the conjugated form (*29*). Thus, extrapolation of the low dose stimulatory effect on cell proliferation can only be done adequately upon PBPK modelling revealing expected plasma and tissue concentrations of the aglycon at low dose, taking also into account the processes of biotransformation. This may lead to personified risk assessment, discriminating sensitive from insensitive groups within the population. Furthermore, in vitro studies should also focus on possible biphasic effects of the phase II metabolites of quercetin on cell proliferation since these may be more relevant for in vivo exposure than the stimulation of cancer cell proliferation by low doses of the aglycon. Without considering in vivo toxicokinetics in the in vitro models, extrapolation from in vitro biphasic dose-response curves on cell proliferation to in vivo cell proliferation are difficult to make.

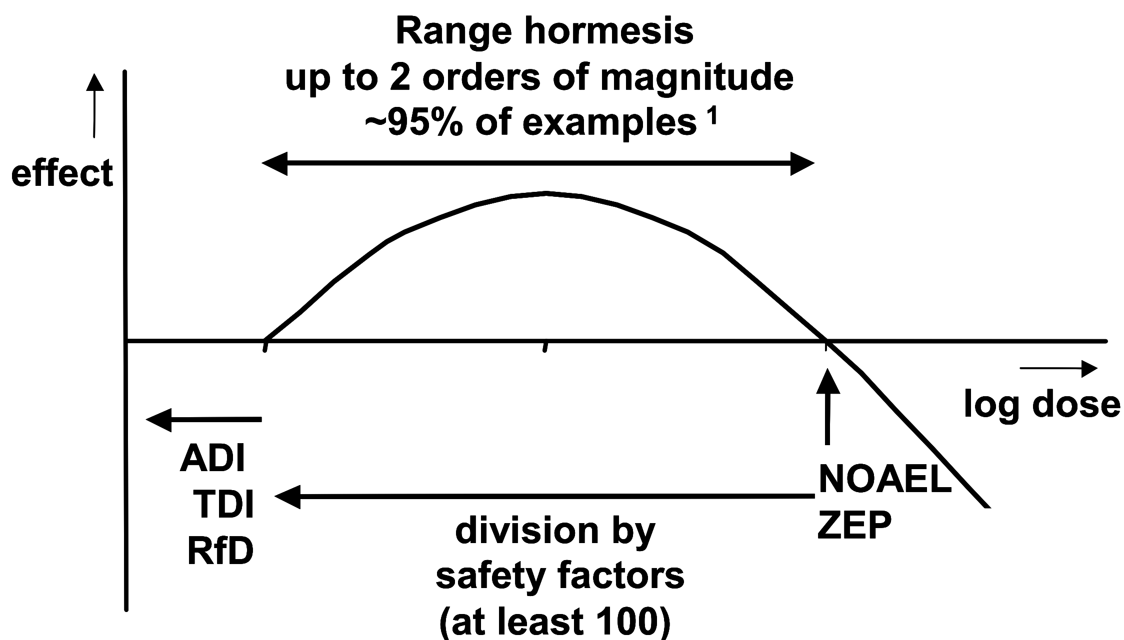

Figure 3 Schematic presentation of the dose range of hormesis that is about 2 orders of magnitude below the NOAEL or ZEP in 95% of the cases(*1*), as compared to the consequences of applying assessment safety factors to derive safe exposure levels (TDI, ADI or RfD values) from the NOAEL or ZEP. The diagram illustrates that taking hormesis into account in risk assessment of compounds with a threshold for the adverse effect requires the lowering of safety factors below the factor of 100 (10 for inter- and 10 for intraspecies differences). This would result in possibilities for beneficial effects for part of the population, but increased chances on adverse effects for another part of the population.

Conclusion

Altogether it is concluded that hormesis is an important phenomenon especially from the scientific point of view but that its consequences for risk assessment and the possibilities for in vitro to in vivo extrapolation may remain limited without additional mechanistic insight.

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9

Summary and future perspectives

1. Summary

Over the course of the years, a large number of beneficial health effects have been ascribed to the flavonoid quercetin, including protection against various forms of cancer, neurodegenerative diseases, cardiovascular diseases and the process of ageing (See Chapter 1). The scientific evidence supporting these effects has however mainly been based on epidemiological studies showing associations between fruit and vegetable intake and health effects, as well as on *in vitro* studies with quercetin focussing on its antioxidant activity, its capacity to modulate the activity of phase I and II biotransformation enzymes, and its effects on cell proliferation (reviewed in Chapter 1). These *in vitro* studies have in general been performed with the aglycon, not taking into consideration that physiologically relevant forms of quercetin include glycoside conjugates in the pre-absorption phase if quercetin originates from plant foods, and a mixture of phase II conjugates in the post-absorption phase. In general, such conjugates are known in general to have less potent biological activities than the aglycon (*1-8*). Besides the relatively weak evidence for possible beneficial health effects of quercetin, various *in vitro* but also *in vivo* studies have pointed at possible adverse health effects of quercetin. Quercetin is genotoxic in a variety of bacterial and mammalian *in vitro* systems, forms covalent adducts with cellular protein and DNA and under certain conditions increases cancer incidences in experimental animals (Table 5, Chapter 1). However, these studies also have not taken into account the extensive phase II metabolism of quercetin and a large number of *in vivo* animal studies contradict the stimulating effects of quercetin on cancer and report no effect or an inhibitory effect of quercetin on cancer incidences (Tables 3 and 4, Chapter 1).

In spite of the limited and sometimes contradictory knowledge on the biological effects of quercetin and the uncertainty about the eventual health effects of long-term consumption of high daily doses of quercetin, quercetin is available in health shops and on the Internet as ingredient of functional foods and food supplements. Upon consumption of such products at the recommended dosage, the daily dose may increase to 15 or even 1000-times the intake from natural dietary sources. Therefore, the objective of this thesis was to investigate the mechanisms of toxicity of quercetin, with special emphasis on its extensive phase II metabolism, the effect of this phase II metabolism on its anti- and pro-oxidant behaviour, on its covalent addition to cellular macromolecules and on its effects on cell proliferation. As a first step in this process, Chapter 2 focuses on the phase II metabolism of quercetin. Although it is known that quercetin mainly circulates *in vivo* as glucuronidated, sulphated and/or

methylated conjugates, the regioselectivity of these conjugations has often not been determined. Because the biological activity of a flavonoid is associated with the number and position of free hydroxyl groups, the regioselectivity of phase II metabolism of quercetin is expected to be an important determinant of the ultimate biological effects of quercetin *in vivo*. To obtain more insight into this matter, Chapter 2 describes a set of analytical methods based on HPLC-DAD, LC-MS and ^{1}H NMR, with which HPLC, UV, MS and ^{1}H NMR characteristics of 14 different phase II mono- and mixed conjugates of quercetin were determined. This knowledge proved to be a useful tool in the identification of quercetin phase II metabolite patterns in various biological systems. Using these data, the phase II metabolite pattern of a variety of rat and human liver and intestinal *in vitro* model systems, including cell lines, S9 homogenates and hepatocytes, was elucidated. This revealed that the 7-, 3-, 3'- and 4'-hydroxyl groups were important targets for phase II biotransformation of quercetin. Because the catechol group (3',4'-dihydroxybenzene group) of quercetin is generally considered one of the key elements in the structure of quercetin contributing to its biological activity and because these hydroxyl groups proved to be targets for methylation, glucuronidation and sulfation, the effect of methylation of the 3'- or 4'-hydroxyl groups on the antioxidant and the pro-oxidant activity of quercetin was investigated in Chapters 3 and 5.
In Chapter 3, the consequences of methylation of the catechol hydroxyl groups for pH-dependent radical-scavenging properties of quercetin were characterised both experimentally using the TEAC assay, and theoretically with quantum-mechanical calculations. The pH-dependent TEAC curves together with the computer-calculated electronic parameters, including bond dissociation energy and ionisation potential, revealed that methylation of the catechol group of quercetin decreases the radical scavenging capacity and thus the antioxidant capacity of quercetin by both increasing the pK_a for deprotonation and by reducing the electron and hydrogen atom donating properties of the neutral and the anionic form of the molecule. Comparison of the pH-dependent TEAC profiles of 3'-O-methylquercetin with the profile of kaempferol, which lacks the 3'-OH moiety of quercetin, showed that methylation of a catechol hydroxyl group has a similar effect on radical scavenging capacity as replacement of the hydroxyl group by a hydrogen atom.

Because similar structural elements appear to be of importance for both the antioxidant and the pro-oxidant activity of quercetin (*7, 9*), catechol-O-methylation of quercetin may also be expected to influence its pro-oxidant activity. In Chapter 5, this was investigated in more detail by studying the formation of covalent adducts of quercetin or methylated conjugates of

quercetin with glutathione and cellular DNA. Oxidation of 3'- and 4'-O-methylquercetin with horseradish peroxidase in the presence of glutathione yielded two major metabolites for each substrate, identified as 6- and 8-glutathionyl conjugates of 3'- and 4'-O-methylquercetin using HPLC-DAD, LC-MS and ^{1}H NMR . This indicated that catechol-O-methylation of quercetin does not eliminate its pro-oxidant chemistry. Furthermore, the formation of these A-ring glutathione conjugates indicates that quercetin-*ortho*-quinone is probably not an intermediate in the formation of covalent adducts of quercetin with glutathione, protein and/or DNA. Based on these findings, an alternative mechanism for the pro-oxidant chemistry of quercetin was proposed, eliminating the requirement for the formation of quercetin-*ortho*-quinone and explaining why methylation of quercetin does not fully abolish the formation of reactive DNA-binding metabolites. In additional studies, it was demonstrated that covalent DNA adduct formation by a mixture of radio-labeled 3'- and 4'-O-methylquercetin in HepG2 cells amounted to only 42% of the level of covalent adducts formed by a similar amount of radio-labeled quercetin. These results show that, similar to the effects of methylation on the radical scavenging properties of quercetin, methylation of the catechol group of quercetin does not eliminate but considerably attenuates the implications of the pro-oxidant activity of quercetin. This attenuating effect of methylation of quercetin on its pro-oxidant activity might be a reason contributing to the apparent lack of *in vivo* carcinogenicity of this *in vitro* genotoxic compound.

The objective of this thesis was also to obtain more insight into possible toxic effects of quercetin, by studying various *in vitro* mechanisms that might be relevant in the context of carcinogenesis. Chapter 4 describes the covalent adduct formation by quercetin resulting from its pro-oxidant chemistry in some more detail. In this chapter, the role of cellular tyrosinase and /or peroxidase-like oxidative enzyme activity in the covalent binding of quercetin to glutathione, protein and DNA was investigated. Cells with elevated tyrosinase or peroxidase levels contained approximately two times higher levels of covalent quercetin adducts than cells without detectable levels of these oxidative enzymes. However, this difference was smaller than might be expected based on the differences in tyrosinase and/or peroxidase levels, indicating that these types of oxidative enzyme activities do not play a major role in the cellular pro-oxidant activity of quercetin. This might be explained by the intracellular compartementalization of such enzymes, known to be sequestered in compartments surrounded by membranes (*10-12*). As a result, oxidative metabolites of quercetin, formed in the compartments containing these types of oxidative enzyme activities, may have limited

access to DNA or protein. This may explain why only a limited increase in DNA and protein adducts is found in cell types containing elevated levels of tyrosinase or peroxidase activities. A second possible reason for the relative low contribution of these types of oxidative enzymes to the cellular pro-oxidant activity of quercetin could be that pro-oxidant quinone-type metabolites may be very efficiently scavenged by glutathione and thus detoxified (*13, 14*). Temporarily elevated intracellular quercetin quinone metabolite levels may be conjugated to glutathione quickly after their formation, even before covalent binding to protein or DNA can occur. In support of this, the present study shows that glutathionyl quercetin adducts could only be detected in the medium of cell lines with elevated tyrosinase or peroxidase activities.

The results obtained in Chapter 4 thus indicate that the formation of covalent adducts of quercetin to protein and DNA can be expected in all cells, independent of their tyrosinase and/or peroxidase levels. Additional experiments showed that quercetin DNA adducts were of transient nature, independent of the presence of a nucleotide excision repair system, suggesting that covalent DNA adduct formation by quercetin is chemically reversible. Whether this transient nature of quercetin DNA adducts reflects full reversibility of the adduct formation and thus detoxification, or formation of genotoxic depurinated sites, requires additional investigation of the transient nature of quercetin DNA adducts at the molecular level. Full reversibility of quercetin DNA adducts may limit or cause the ultimate biological impact of the adducts formed and may provide an explanation for the apparent lack of *in vivo* carcinogenicity of this *in vitro* genotoxic compound.

In addition to the pro-oxidant activity of quercetin as a possible mechanism of importance in the context of carcinogenesis, the effect of quercetin on cell proliferation was studied in Chapters 6 and 7. First, the effect of quercetin on the proliferation of the colon carcinoma cell lines HCT-116 and HT29, as well as the mammary adenocarcinoma cell line MCF-7 was investigated. Quercetin inhibited the proliferation of the colon carcinoma cell lines at concentrations above 30-80 μM, an effect also seen in the majority of proliferation studies available (*15-26*). However, in the lower, physiologically relevant concentration range, quercetin caused a subtle but significant stimulation of the proliferation of these three cell lines. These results point at a dualistic influence of quercetin on cell proliferation that may affect present views on its supposed beneficial antiproliferative effect. It should, however, be considered, that these effects were obtained in tests with the aglycon, not taking into account that *in vivo* exposure of mammary cells as well as of intestinal cells from the plasma side will

occur to a mixture of phase II metabolites of quercetin, known to have altered biological activities (*1-8*).

The mechanism underlying the stimulation of cell proliferation by quercetin was investigated in some more detail in Chapter 7. From the results described in Chapter 6, the hypothesis arose that, analogous to the isoflavonoid genistein, the stimulation of cell proliferation by quercetin might be caused by interaction of quercetin with the estrogen receptor (ER), known to be expressed by the cell lines used in Chapter 6. To study this hypothesis, the effect of quercetin on cell proliferation was tested in ER-positive and in ER-negative breast cancer cell lines. Quercetin stimulated the proliferation of ER-positive cells only, suggesting this effect to be ER-dependent. In support of these results, similar concentrations of quercetin induced ER-ERE-mediated gene expression in a reporter gene assay using U2-OS cells transfected with either ERα or ERβ as well as the Estrogen Responsive Element (ERE), with 10^5-10^6 times lower affinity than 17β-estradiol (E2) and 10^2-10^3 times lower affinity than genistein. However, quercetin activated the ERβ to a 4.5 fold higher level than E2, whereas the maximum induction level of ERα by quercetin was only 1.7 fold that of E2. This is especially interesting given the hypothesis that ERα might mediate the proliferative effects of estrogenic compounds, whereas ERβ might have inhibiting influences on this process by the formation of heterodimers of ERα and ERβ (*27, 28*). Therefore, the results of this study point at the relatively high capacity of quercetin to stimulate supposed 'beneficial' ERβ responses as compared to the stimulation of ERα. Altogether, the results of this study reveal that physiologically relevant concentrations of quercetin can exert phyto-estrogen-like activity similar to that observed for the isoflavonoid genistein. The meaning of these findings in the context of carcinogenesis awaits identification and quantification of ER-types present in tissues affected by hormone-dependent cancers.

The dose-response relationship obtained for the effects of quercetin on the proliferation of ER-positive cells could be qualified as hormetic (Chapter 6). Hormesis designates the phenomenon that includes stimulation of an endpoint at lower doses and inhibition of that same endpoint at the higher concentration range (or vice-versa) (*29*). Although the shape of the dose-response curve obtained for the effect of quercetin on the proliferation of ER-positive cells (Chapters 6 and 7) may point at hormesis, there seems to be a need for a better definition of the concept of hormesis and reconsideration of whether all biphasic dose-response curves should be considered representative for hormesis. Chapter 8 presents a discussion on the definition of hormesis and of the applicability of hormesis to our results on

the biphasic effect of quercetin on cell proliferation. The term hormesis may be restricted to phenomena that proceed by mechanisms that are broadly generalisable and represent possibly beneficial overcompensation in response to an adverse stimulus. Using the concept that hormesis is defined as such, the biphasic effect of quercetin on cell proliferation, demonstrated to be ER-dependent for the increase in cell proliferation, but also several other receptor-mediated biphasic dose-response phenomena, should not be referred to as hormesis.

2. Future perspectives

Taking into consideration the new results and insights obtained from the studies described in this thesis, the question remains to what extent the expected increased consumption of quercetin, due to the intake of functional foods and/or food supplements, may significantly affect human health. It is always difficult, if not impossible, to extrapolate results obtained from *in vitro* studies to expectations regarding *in vivo* behaviour of a compound. Nevertheless, altogether, the studies presented in this thesis provide indications for the dualistic character of quercetin regarding its role in the process of cancer development.

On the one hand, various results revealed possible stimulating effects of quercetin in the development of cancer. This is based on the following argumentation.

1. Quercetin appeared to stimulate the proliferation of various cell lines at concentrations physiologically relevant in human (Chapter 6 and 7) and this effect was dependent on the estrogen receptor (Chapter 7). This might pose questions regarding the presumed protecting role of quercetin in the development of cancer (*30, 31*), especially hormone-dependent cancers. In addition, quercetin appears to inhibit the differentiation of Caco-2 cells, which is also an effect that is not expected from a compound with putative cancer-inhibiting properties (*32*).
2. The covalent binding of quercetin to DNA appeared to be of reversible nature. In theory, the loss of covalently bound quercetin may be due to either full reversibility of the adduct formation, as seen with quercetin adducts with glutathione and other thiol reagents (*33*) or to depurination of the DNA base involved. In general, apurinic sites formed in the DNA due to the formation of such depurinating adducts can lead to mutations by error-prone base excision repair or misreplication, which substantially increases the risk for cancer and other diseases (*34, 35*). When the transient nature of quercetin DNA adducts would be due to depurination of the DNA adducts, the

apparent lack of *in vivo* carcinogenicity should rather be ascribed to the fact that quercetin is a weak carcinogen, for which adequate *in vivo* models are hard to define.

3. Although the rapid phase II metabolism of quercetin facilitates its excretion (*36*) and may contribute to limitation of the biological effects of quercetin *in vivo*, methylation of the catechol group of quercetin did not fully eliminate the cellular implications of the pro-oxidant chemistry of quercetin. As demonstrated in Chapter 5, methylated conjugates of quercetin still form approximately 42% of the levels of covalent DNA adducts, as compared with the aglycon. It remains to be investigated to what extent covalent DNA adducts formed by phase II metabolites of quercetin share similar characteristics as quercetin DNA adducts, i.e. transient nature and possible dupurinating effects. Thus phase II metabolism of quercetin does not fully eliminate the risks intrinsically associated with quercetin.
4. In addition to the possible direct risks associated with quercetin, putative indirect health risks associated with increased consumption of quercetin deserve some attention because this may increase the risk for carcinogenic effects of other compounds. For example, due to extensive phase II metabolism of quercetin, an overload of quercetin may lead to depletion of co-factors essential for the biotransformation and detoxicification of estrogens and other carcinogens. Indications for this phenomenon were obtained from studies in hamsters treated with estradiol showing that co-administration of quercetin in the diet for approximately 6 months considerably increased the number of tumors and metastases as compared to hormone treatment alone, probably because of depletion of the cofactor for methylation, essential for the detoxification of reactive estrogen metabolites (*37*).

The fact that quercetin may have a dualistic influence on the process of carcinogenesis is illustrated by several other results presented in this thesis, pointing at limitation of potential risks or even protecting effects of quercetin in the process of cancer development. This is based on the following arguments.

1. Quercetin is extensively metabolised by the phase II biotransformation system *in vivo*, which facilitates its rapid excretion via urine and bile (*36*). This decreases the risk for harmful biological effects *in vivo*. In addition, the biological activity of the phase II metabolites of quercetin is reduced, relative to the parent compound. This was demonstrated in Chapter 5 for the pro-oxidant chemistry of quercetin resulting in covalent adduct formation with DNA.

2. Although phase II metabolism of quercetin attenuates its biological activity and thus also the beneficial biological effects of phase II metabolites of quercetin, methylation of quercetin was shown to attenuate but not eliminate the radical scavenging properties of quercetin (Chapter 3). The latter is supposed to be one of the mechanisms underlying protection against cancer by quercetin (*7*). In addition, quercetin is known to induce the expression of phase II enzymes *in vitro* (*38*), which may support the claim that quercetin protects against carcinogenic effects of other compounds. Figure 1 shows the effects of methylated conjugates of quercetin on the EpRE-mediated gene expression, as compared to quercetin (Van der Woude *et al.* unpublished results). Although the results show that conjugation of the 3'- or 4'-hydroxyl group of quercetin with a methyl group decreases the capacity of quercetin to induce EpRE-mediated gene expression, catechol-O-methylated conjugates of quercetin still retain approximately 20% of the biological activity of the aglycon. Thus, upon phase II conjugation, quercetin may retain part of its putative beneficial effects *in vivo*.

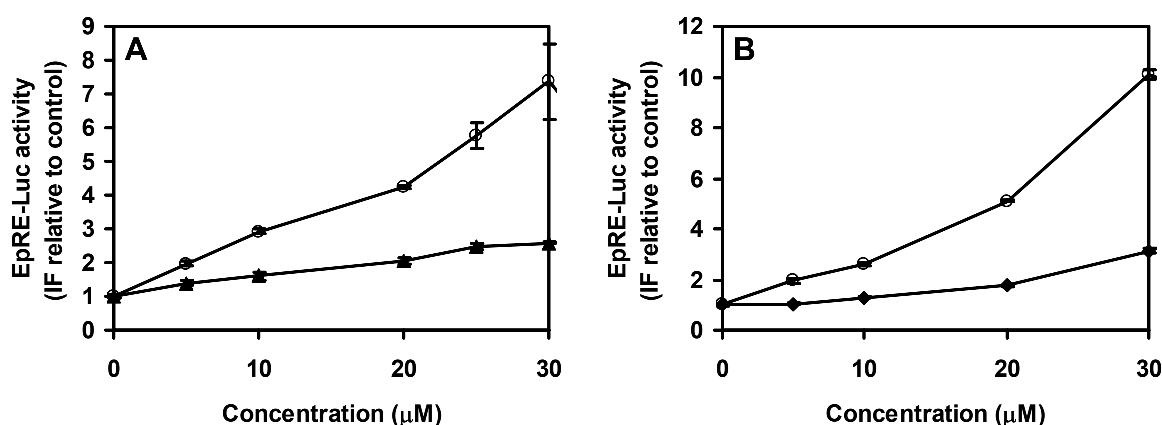

Figure 1 Effect of quercetin and (A) 3'-O- and (B) 4'-O-methylquercetin on EpRE-mediated gene expression (open circles: quercetin; triangles: 3'-O-methylquercetin; diamonds: 4'-O-methylquercetin; IF: induction factor) (Van der Woude *et al.* unpublished results).

3. Although quercetin, similar to genistein, appeared to stimulate cell proliferation of cancer cell lines *in vitro* at concentrations physiologically relevant in humans (Chapters 6 and 7), indications were obtained that quercetin stimulates 'beneficial' ERβ-mediated processes to a larger extent than ERα-mediated processes. This is especially interesting in view of the hypothesis that ERα might mediate the proliferative effects of estrogenic compounds, whereas ERβ might have protective effects on this process by the formation of heterodimers of ERα and ERβ (*27, 28*). The

relevance of these findings in the context of carcinogenesis awaits identification and quantification of ER-types present in tissues affected by hormone-related types of cancer.

4. The covalent binding of quercetin to DNA, but also to protein, is of transient nature, as demonstrated in Chapter 4. At present, it is unknown whether the transient nature reflects the depurination of the DNA bases upon formation of depurinating quercetin adducts. This might present a mechanism underlying a possible risk for genotoxicity and initiation of cancer by quercetin, as discussed previously. However, in case of full reversibility of the addition reaction, as also observed for quercetin glutathione adducts (*33*), the DNA damage may be only temporary, providing another explanation for a limited risk on cancer.
5. The well-controlled *in vivo* carcinogenicity study directed by the National Toxicology Program (*39, 40*) only provided weak evidence for carcinogenic effects of quercetin. Moreover, there are indications that the increased numbers of kidney tumours observed in male rats only, may not be caused by a quercetin-specific mechanism, but may rather be caused by a non-specific mechanism involving α_{2u}-globulin nephropathy (*39, 40*). This phenomenon is often observed in male rats because they express high quantities of α_{2u}-globulin. Because similar to female rats in which no significant effects were observed, humans express much lower amounts of this protein, carcinogenic effects in the kidney as observed in male rats may not be representative for the human situation. In addition, Table 1 presents results obtained from an animal study carried out within Task 4 of the overall project in which the present thesis work was embedded entitled “Benefit-risk evaluation of flavonoids in foods and their use as functional food ingredients” (project no. 014-12-012) funded by the Netherlands Organisation for Health Research and Development (see also Chapter 1, Section 5). Male F344 rats in which colon cancer was induced with azoxymethane were fed 0-10000 ppm quercetin in the diet for 38 weeks. Some initial results are presented in Table 1 (*41*) and reveal that after 38 weeks, quercetin induced a dose-dependent decrease in the incidence and the multiplicity of tumours detected in the AOM-exposed rats. This indicates that quercetin may in inhibit the formation of chemically induced colorectal tumours in rats and may suppress the number and the size of newly formed tumours. These findings present additional evidence for protecting effects of quercetin against cancer, although the present findings were

obtained at doses that amount for the 1000 and 10000 ppm groups respectively to about 10 and 100-fold the dose usually recommended for the consumption of quercetin supplements.

As illustrated by the argumentations presented above, the risk-benefit evaluation of the increased consumption of quercetin aglycon and cancer is far from straight-forward. As far as we know now, *in vivo* biological activity of quercetin is attenuated by limited bioavailability of quercetin due to restricted uptake of quercetin into the enterocytes as well as by possible efflux of phase II metabolites of quercetin from the enterocyte back into the intestinal lumen (*42*), and by the extensive phase II metabolism of quercetin facilitating its excretion (*36*) and decreasing its biological activity (*1, 3-8*). Nevertheless, although generally less potent than the parent compound, the phase II metabolites of quercetin appear to possess similar biological characteristics of quercetin, amounting to 30-50% of the potency of quercetin depending on the biological effect under study. This holds for both possible harmful effects of quercetin, such as the pro-oxidant chemistry and covalent binding to DNA, and for possible beneficial effects such as the antioxidant activity and induction of EpRE-mediated gene expression. Furthermore, it is also important to note that the effects seen for quercetin aglycon may still be relevant in case of consumption of a quercetin supplement causing exposure to relatively high levels of the aglycon at the luminal side of the intestine.

Table 1 Effect of quercetin on tumour data in rats fed quercetin for 38 weeks. Colon cancer was induced in the rats with the colon carcinogen azoxymethane (*41*)

	Quercetin (ppm)			
	0	**100**	**1000**	**10000**
Nr. of rats per group	22	22	22	20
Nr. of tumour bearing rats per group	11	9	8	4
Tumour incidence per group (%) [1)]	50	41	36	20
Total nr. of tumours per group	17	13	11	4
Tumour multiplicity [2)]	1.55 ± 0.93	1.44 ± 1.01	1.38 ± 0.74	1.00 ± 0.0
Tumour size (mm) [2)]	5.94 ± 3.83	5.85 ± 4.22	5.64 ± 3.98	5.00 ± 1.83

[1)] Tumour incidence was significantly (P<0.05) decreased by increasing dietary quercetin, as assessed by the Jonckheere-Terpstra trend analysis test.
[2)] Tumour multiplicity and size were inversely associated with increasing dietary quercetin (*r* = 0.98, P<0.05), as assessed by Pearson's correlation coefficient.

Besides the fact that phase II metabolites of quercetin represent the major biologically active forms of quercetin *in vivo* (*43-45*) and that more knowledge on the biological effects of these quercetin forms is necessary, more insight on the uptake and processing mechanisms of quercetin phase II metabolites by cells exposed from the plasma side are necessary for a well-funded estimation of the biological activity of phase II metabolites of quercetin. There are indications that cells may be equipped with enzymes capable of the hydrolysis of glucuronide and/or sulfate conjugates (i.e. β-glucuronidases) or of the demethylation of the catechol group of 3'- or 4'-O-methylquercetin (i.e. certain P450 enzymes) (*46, 47*). Various cell types, including hepatocytes, most blood cells, kidney cells and intestinal cells are known to contain this type of enzyme activity with in general large inter-individual variability in its activity and expression (*48*). Blood cells such as macrophages are known to excrete β-glucuronidase activity into the plasma upon stimulation of the cell by an antigen (*49*). Therefore, enhanced exposure to quercetin aglycon may be expected inside these cell types and at sites of inflammation.

In conclusion, its remains a matter of debate whether increased consumption of quercetin will eventually lead to an improvement or a deterioration of the health status of the consumer. Because functional foods and food supplements, unlike regular foods, are intended to provide health benefits for the consumer that go beyond the normal health benefits of a balanced diet, they fall into a grey/undefined regulatory area between food and medicine. The increasing use of such enriched foods has raised important concerns within scientific and regulatory communities. In principle, consumers should be able to expect a same degree of safety for regular food and functional foods or food supplements. Generally, it is expected that the dose intervals at which beneficial health effects are expected may be relatively close to the dose intervals at which possible adverse health effects may occur. Furthermore, the large inter-individual variability among humans may imply the existence of particularly sensitive population groups, which may be at risk for certain adverse health effects upon consumption of functional foods and/or food supplements. The expert group of the Natural Toxin Task Force of the European Branch of International Life Sciences Institute – ILSI Europe – suggested that safety assessment of botanical ingredients should focus on specific safety margins for specific groups of the population taking into account the duration of the exposure as well as the expected adverse health effects (*50*). Given the fact that quercetin is a botanical ingredient, these considerations may apply to the safety assessment of quercetin-based functional foods and supplements as well. The ILSI expert group proposed a decision tree to

assist in the determination of information that needs to be considered for the safety of a botanical ingredient, such as quercetin (*50*). According to this decision tree, a broad package of toxicity studies should be conducted with quercetin, before this ingredient may be considered as safe for the consumer. Aspects such as bioavailability and also human clinical data with special attention for the variability of response, adverse effects and contra-indications should be taken into consideration in the safety assessment of quercetin. The toxicity tests that are needed should be selected on a case-by-case basis. The studies described in this thesis identified several end points that are of interest in the case of such a risk assessment of quercetin that should precede its use as ingredient of functional foods or food supplements, including cell proliferation (Chapters 6 and 7), estrogenic potency (Chapter 7) and covalent binding to cellular macromolecules including DNA (Chapters 4 and 5). As long as quercetin remains on the market without proper safety assessment according to for example the guidelines proposed by the expert group (*50*), it is up to the consumer to decide whether the possible limited risks regarding cancer, especially hormone-related cancers, in addition to the high costs involved in food supplement consumption, counterbalance the probably limited beneficial health effects of the consumption of this flavonoid.

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List of abbreviations

ABTS	2,2'-azinobis(3-ethylbenzothiozoline-6-sulphonic acid) diammonium salt
ACF	Aberrant Crypt Foci
ADI	Acceptable Daily Intake
ADME	Absorption, Metabolism, Distribution, Excretion
AOM	Azoxymethane
BDE	Bond Dissociation Energy
Bcrp1	Breast cancer resistance protein 1
BHBN	N-Butyl-N-(4-hydroxybutyl)-nitrosamine
CO	Croton Oil
COMT	Catechol-O-Methyltransferase
DAD	Diode Array Detection
DCC	Dextran Coated Charcoal
DE	Deprotonation Energy
DFT	Density Functional Theory
DMBH	Dimethylbenza-anthracene
DMEM	Dulbecco’s Minimum essential medium
DMSO	Dimethylsulfoxide
DNA	Deoxyribonucleic acid
E2	17β-Estradiol
EBS	Estrogen Binding Site
EDTA	Ethylene-diamine-tetra-acetic acid
EEF	Estradiol Equivalency Factor
EGFR	Epidermal Growth Factor Receptor
EGTA	Ethylene-bis-(oxyethylenenitrolo)-tetra-acetic acid
EMEM	Eagle’s Minimum Essential Medium
EpRE	Electrophile Responsive Element
ER	Estrogen Receptor
ERE	Estrogen Responsive Element
FAD	Focal Area of Dysplasia
FCS	Fetal Calf Serum
GR	Glucocorticoid Receptor

GSH	Glutathione
GLUT2	Glucose Transporter Isoform 2
HBSS	Hank's Balanced Salt Solution
HDO	Deuterated water
HPLC	High Pressure Liquid Chromatography
HRP	Horseradish Peroxidase
IP	Ionisation Potential
IV	Intravenous
LC-MS	Liquid Chromatography – Mass Spectrometry
LDH	Lactate Dehydrogenase
LPH	Lactase Phlorizin Hydrolase
LPO	Lactoperoxidase
MCA	Methylcholanthrene
MEF	Mouse Embryo Fibroblast
MPO	Myeloperoxidase
MRP2	Multidrug Resistance Protein 2
NER	Nucleotide Excision Repair
NIEHS	National Institute for Environmental Health Sciences
NMR	Nuclear Magnetic Resonance
NOAEL	No Observed Adverse Effect Level
NTP	National Toxicology Program
PAPS	3'-phosphoadenosine-5'phosphosulfate
PB	Phenobarbital
PBPK	Physiology-Based Pharmacokinetic
PBS	Phosphate Buffered Saline
RfD	Reference Dose
RNA	Ribonucleic acid
ROS	Reactive Oxygen Species
SAM	S-Adenosyl Methionine
SGLT-1	Sodium-dependent Glucose Transporter 1
SQ	Semi-quinone anion radical
ST	Sulfotransferase
TDI	Tolerable Daily Intake

TEAC	Trolox Eqiuvalent Antioxidant Capacity
Trolox	6-hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic acid
UDP	Uridine-diphosphate
UDPGA	Uridine-5'-phosphoglucuronic acid
UGT	UDP-glucuronyltransferase
UV	Ultra-Violet
UV-VIS	Ultra-Violet Visible
WME	William's Medium E
ZEP	Zero Equivalent Point

Samenvatting[1]

In de loop van de jaren is een groot aantal gezondheidsbevorderende effecten toegeschreven aan het flavonoïd quercetine, zoals bescherming tegen verschillende vormen van kanker, hart- en vaatziekten, neurodegeneratieve ziekten en het verouderingsproces (zie Hoofdstuk 1). Het wetenschappelijke bewijs voor deze effecten is echter voornamelijk gebaseerd op epidemiologische studies die het verband tussen groente- en fruitinname en gezondheidseffecten aantonen, en op *in vitro* studies met quercetine. De *in vitro* studies hebben verschillende aspecten van deze stof bestudeerd, zoals de antioxidantactiviteit, het vermogen om de activiteit van fase I en fase II biotransformatie-enzymen te beïnvloeden en de effecten op de celproliferatie (een overzicht van deze studies is gegeven in Hoofdstuk 1). Deze *in vitro* studies zijn over het algemeen uitgevoerd met het zogenaamde aglycon. Hiermee wordt het quercetinemolecuul bedoeld, waaraan geen andere groepen, zoals suikergroepen, gebonden zijn. Dit betekent dat in deze studies geen rekening gehouden is met het feit dat quercetine *in vivo* na absorptie in de darm aanwezig is als een mengsel van de fase II conjugaten. Bovendien bestaat quercetine van plantaardige oorsprong voornamelijk uit glycoside conjugaten. Over het algemeen hebben dergelijke geconjugeerde vormen van quercetine een minder sterke biologische activiteit dan het aglycon (*1-8*). Behalve het tamelijk zwakke bewijs voor mogelijke gezondheidsbevorderende effecten van quercetine, hebben verschillende *in vitro* maar ook *in vivo* studies gewezen op mogelijk schadelijke gezondheidseffecten van quercetine. Quercetine is genotoxisch in een groot aantal *in vitro* testsystemen met bacteriën of zoogdiercellen. Verder blijkt quercetine covalent te binden aan eiwitten en DNA in de cel en onder bepaalde omstandigheden in laboratoriumdieren het ontstaan van kanker te bevorderen (Tabel 5, Hoofdstuk 1). Ook de *in vitro* studies naar DNA- of eiwitbinding of genotoxiciteit hebben geen rekening gehouden met het uitgebreide fase II metabolisme van quercetine. Bovendien zijn er een aantal *in vivo* dierstudies bekend die het mogelijk stimulerende effect van quercetine op kanker tegenspreken en juist op een beschermend effect of geen effect op de kankerincidentie wijzen (Tabellen 3 en 4, Hoofdstuk 1).

Ondanks de beperkte en soms tegenstrijdige gegevens over de biologische effecten van quercetine en de onzekerheid over de uiteindelijke gezondheidseffecten als gevolg van langdurig gebruik van deze stof bij hoge dagelijkse dosering, is quercetine te koop in reformzaken en op het internet als ingredient van functional foods en voedingssupplementen.

[1] Voor literatuurreferenties, in deze tekst weergegeven tussen haakjes, zie Hoofdstuk 9.

Wanneer dergelijke producten volgens de aanbevolen dosering worden gebruikt, kan de dagelijkse dosis quercetine oplopen tot hoeveelheden die ongeveer 15 tot zelfs 1000 maal hoger zijn dan de dosis die bereikt wordt bij consumptie van quercetine uit natuurlijke bronnen. Omdat de toxiciteit van een stof samenhangt met de dosis en omdat de gevolgen van langdurige consumptie van hoge doseringen quercetine onduidelijk zijn, was het doel van dit proefschrift om de de toxische effecten van quercetine te onderzoeken, evenals de achterliggende mechanismen. Daarbij is ook aandacht besteed aan het fase II-metabolisme van quercetine en de gevolgen van dit fase II-metabolisme voor de anti- en pro-oxidantactiviteit van quercetine en voor de covalente binding van quercetine aan cellulaire macromoleculen. Ook zijn de effecten van quercetine op de celproliferatie onderzocht.

In Hoofdstuk 2 ligt de nadruk op het fase II-metabolisme van quercetine. Hoewel het al bekend is dat quercetine in het lichaam voornamelijk aanwezig is als glucuronide-, sulfaat- en/of gemethyleerde conjugaten, is de regioselectiviteit van deze conjugaties vooralsnog niet gekarakteriseerd. Omdat de biologische activiteit van een flavonoïd gekoppeld is aan het aantal vrije hydroxylgroepen en de positie daarvan, wordt er verwacht dat de regioselectiviteit van het fase II-metabolisme van quercetine een factor van belang is voor de uiteindelijke biologische activiteit van quercetine *in vivo*. Om meer inzicht in deze materie te verkrijgen, beschrijft Hoofdstuk 2 de bepaling van de structuur van 14 verschillende fase II-mono- en gemengde conjugaten van quercetine aan de hand van een set analytische methoden die gebaseerd zijn op HPLC-DAD, LC-MS en ^{1}H NMR. Deze kennis is vervolgens gebruikt bij de identificatie van fase II-metabolietpatronen van quercetine in verschillende biologische systemen. Zo werd het fase II-metabolietpatroon opgehelderd in verschillende *in vitro* modelsystemen, afkomstig van lever en darm van rat en mens, zoals verschillende cellijnen, S9-homogenaten en primaire hepatocyten. Hieruit bleek dat de 7-, 3-, 3'-, en 4'-hydroxylgroepen belangrijke aangrijpingspunten waren voor het fase II-metabolisme van quercetine. Omdat de catecholgroep (3',4'-dihydroxylbenzeen groep) van quercetine wordt beschouwd als een belangrijk onderdeel in de structuur van quercetine wat betreft de bijdrage aan de biologische activiteit van de stof, en omdat juist deze hydroxylgroepen in grote mate gemethyleerd, geglucuronideerd en gesulfateerd werden, zijn in Hoofdstuk 3 en 5 de gevolgen van methylering van de 3'- en 4'-hydroxylgroepen voor de anti- en pro-oxidantactiviteit van quercetine onderzocht.

In Hoofdstuk 3 zijn de consequenties van methylering van de catecholhydroxylgroepen van quercetine voor de pH-afhankelijke radicaalvangende eigenschappen van quercetine

gekarakteriseerd, zowel experimenteel aan de hand van de TEAC-assay, als theoretisch aan de hand van kwantummechanische berekeningen. De pH-afhankelijke TEAC-curves en de berekende elektronische parameters, zoals de energie voor het verbreken van de O-H-binding en de ionisatiepotentiaal, toonden aan dat methylering van quercetine de radicaalvangende capaciteit en dus de antioxidantcapaciteit doet afnemen door zowel de pK_a voor deprotonering te verhogen, als de electron- en waterstofatoomdonerende eigenschappen te verlagen. Vergelijking van het pH-afhankelijke TEAC-profiel van 3'-O-methylquercetine met het profiel van kaempferol, een flavonoïd waarin vergeleken met quuercetine de 3'-OH groep ontbreekt, toonde aan dat methylering van een catechol hydroxylgroep eenzelfde effect antioxidantactiviteit heeft, als vervanging van de betreffende hydroxylgroep door een waterstofatoom.

Omdat dezelfde structuurelementen van belang lijken te zijn voor zowel de anti- als de pro-oxidantactiviteit van quercetine (*7, 9*), wordt er verwacht dat catechol-O-methylering van quercetine ook de pro-oxidantactiviteit van de stof zal beïnvloeden. Dit is in detail onderzocht in Hoofdstuk 5, waarbij de vorming van covalente adducten van quercetine of van gemethyleerde conjugaten van quercetine met glutathion en met cellulair DNA is bestudeerd. Oxidatie van 3'- en 4'-O-methylquercetine met mierikswortelperoxidase in aanwezigheid van glutathion leidde tot de vorming van twee belangrijke metabolieten van elk van beide substraten. Aan de hand van HPLC-DAD, LC-MS en ^{1}H-NMR zijn deze metabolieten geïdentificeerd als 6- en 8-glutathionylconjugaten van 3'- en 4'-O-methylquercetine. Dit toonde aan dat catechol-O-methylering van quercetine niet de pro-oxidantchemie van quercetine uitschakelt. Verder is de vorming van deze A-ringconjugaten mogelijk een aanwijzing dat het quercetine-*ortho*-chinon waarschijnlijk niet een intermediair is in de vorming van covalente adducten van quercetine met glutathion, eiwit en/of DNA. Op grond van deze resultaten is er een alternatief mechanisme voor de pro-oxidantchemie van quercetine voorgesteld, waarbij het quercetine-*ortho*-chinon geen rol speelt. Dit mechanisme vormt een mogelijk antwoord op de vraag waarom methylering van quercetine de vorming van reactieve DNA-bindende metabolieten niet volledig onderdrukt. In aanvullende studies is aangetoond dat de mate van covalente binding aan DNA door een mengsel van radioactief 3'- en 4'-O-methylquercetine slechts 42% is van de mate van DNAbinding door eenzelfde hoeveelheid radioactief quercetine. Net als bij de antioxidantactiviteit van quercetine, tonen deze resultaten aan dat methylering van de catecholgroep van quercetine de gevolgen van de pro-oxidant activiteit van quercetine niet elimineert maar wel aanzienlijk afzwakt. Het feit dat

methylering van quercetine zijn pro-oxidantactiviteit onderdrukt, zou bij kunnen dragen aan het verschijnsel dat deze stof *in vivo* schijnbaar niet carcinogeen is, maar *in vitro* wel genotoxisch is. De *in vitro* testsystemen die gebruikt worden voor genotoxiciteitsstudies beschikken namelijk niet over een fase II-biotransformatiesysteem waardoor de effecten van fase II-metabolieten niet getest kunnen worden.

Een ander doel van dit proefschrift was om meer inzicht te verkrijgen in mogelijke toxische effecten van quercetine. Dti is gedaan door verschillende *in vitro* methanismen te bestuderen die relevant zouden kunnen zijn in de context van het ontstaan en de ontwikkeling van kanker. Hoofdstuk 4 beschrijft de vorming van covalente adducten door quercetine als gevolg van zijn pro-oxidantchemie in meer detail. In dit hoofdstuk wordt de rol van cellulaire tyrosinase- en/of peroxidase-achtige oxidatieve enzymactiviteit bij de covalente binding van quercetine met glutathion, eiwit en DNA nader onderzocht. Cellen met verhoogde tyrosinase en peroxidase activiteit bevatten een ongeveer tweemaal zo hoge hoeveelheid covalente quercetine adducten dan cellen zonder detecteerbare activiteit van deze oxidatieve enzymen. Echter, dit verschil was kleiner dan op grond van de verschillen in tyrosinase en/of peroxidase nivo's verwacht mocht worden. Dit toont aan dat deze vormen van oxidatieve enzymactiviteit geen grote rol spelen in de pro-oxidantactiviteit van quercetine in de cel. Dit zou verklaard kunnen worden doordat dit soort enzymen in de cel voorkomen in compartimenten die omgeven zijn door membranen (*10-12*). Als gevolg hiervan hebben de oxidatieve metabolieten van quercetine, die gevormd worden in deze compartimenten, mogelijk slechts beperkte toegang to DNA of eiwit. Dit zou kunnen verklaren waarom er slechts een beperkte toename in DNA- en eiwitadducten is gevonden in celtypen met verhoogde tyrosinase- en peroxidaseactiviteit. Een tweede mogelijke reden voor de relatief lage bijdrage van deze typen oxidatieve enzymen aan de pro-oxidantactiviteit van quercetine in de cel is mogelijk dat pro-oxidant chinon-achtige metabolieten zeer efficient weggevangen worden door glutathion en op deze wijze snel ontgift worden (*13, 14*). Op deze wijze zouden intracellulaire quercetine-chinonmetabolieten snel na hun vorming gebonden kunnen worden aan glutathion, nog voordat ze de kans hebben gekregen om covalente DNA- of eiwitadducten te vormen. Glutathionylconjugaten van quercetine konden dan ook alleen gedetecteerd worden in het medium van cellijnen met verhoogde tyrosinase of peroxidaseactiviteit. De resultaten van Hoofdstuk 4 tonen dus aan dat de vorming van covalente adducten van quercetine met eiwit en DNA in alle celtypen verwacht mag worden, onafhankelijk van hun tyrosinase en/of peroxidaseactiviteit. Aanvullende experimenten lieten zien dat de covalente adducten van

quercetine met DNA van tijdelijke aard waren, en dit bleek onafhankelijk te zijn van de aanwezigheid van het zogenaamde nucleotide excision repair (NER) systeem, een enzymatisch DNA-reparatiesysteem dat specifiek nucleotiden verwijdert waaraan relatief grote adducten zitten. Deze resultaten suggereren dat de covalente binding van quercetine aan DNA niet stabiel is. Of dit tijdelijke karakter van quercetine-DNA-adducten zich uit in volledige reversibiliteit van de additiereactie en dus detoxificatie, of juist in de vorming van gedepurineerde nucleotides in het DNA, vereist nader onderzoek van het tijdelijke karakter van quercetine-DNA-adducten op moleculair niveau. Volledige reversibiliteit van quercetine-DNA-adducten zou de uiteindelijke biologische impact van de gevormde adducten kunnen beperken, wat een verklaring zou kunnen vormen voor het schijnbare gebrek aan *in vivo* carcinogene effecten van deze stof, die *in vitro* duidelijk genotoxisch is. Permanente DNA-binding kan mutaties tot gevolg hebben die aan de basis zouden kunnen staan van carcinogeniteit.

Naast de pro-oxidantactiviteit van quercetine als mogelijk mechanisme van belang in het kader van het ontstaan en de ontwikkeling van kanker, is het effect van quercetine op de celproliferatie bestudeerd in Hoofdstuk 6 en 7. Ten eerste is het effect van quercetine op de proliferatie van de darmkankercellijnen HCT-116 en HT29 en de borstkankercellijn MCF-7 onderzocht. Quercetine bleek de proliferatie van de darmkankercellijnen te remmen bij concentraties hoger dan 30-80 μM. Dit effect was al langer bekend uit een groot aantal proliferatiestudies (*15-26*). Quercetine bleek echter bij de lagere, fysiologisch relevante concentraties een subtiele maar significante stimulering van de proliferatie te veroorzaken bij alle drie de bestudeerde cellijnen. Deze resultaten duiden op een dualistische invloed van quercetine op de celproliferatie, wat de huidige kijk op het veronderstelde gunstige antiproliferatieve effect van quercetine op kankercellen zou kunnen beïnvloeden. Er moet desalniettemin wel rekening worden gehouden met het feit dat deze effecten zijn gemeten in tests met het aglycon, terwijl *in vivo* blootstelling van zowel borstcellen als darmcellen aan quercetine vanaf de plasma-zijde plaatsvindt, wat betekent dat er blootstelling plaatsvindt aan een mengsel van fase II-metabolieten van quercetine, met een biologische activiteit die kan verschillen van die van het aglycon (*1-8*).

Het mechanisme achter het stimulerende effect van quercetine op celproliferatie is meer in detail onderzocht in Hoofdstuk 7. Uit de resultaten die beschreven zijn in Hoofdstuk 6 kwam de hypothese naar voren dat, analoog aan het isoflavonoïd genisteïne, quercetine stimulering van celproliferatie kan veroorzaken door interactie met de oestrogeenreceptor (ER), die tot

expressie gebracht wordt door de cellijnen die gebruikt zijn in Hoofdstuk 6. Om deze hypothese te onderzoeken, is het effect van quercetine op celproliferatie bestudeerd in ER-positieve en in ER-negatieve borstkankercellijnen. Quercetine bleek de proliferatie van alleen ER-positieve cellijnen te stimuleren, wat een aanwijzing vormde dat dit effect ER-afhankelijk was. Dit werd ondersteund doordat vergelijkbare quercetineconcentraties ER-ERE-afhankelijke genexpressie bleken te induceren in een reportergenassay bestaande uit U2-OS-cellen die getransfecteerd waren met hetzij ERα, hetzij ERβ alsmede het Estrogen Responsive Element (ERE) gekoppeld aan luciferase als reportergen. De inductie van luciferaseactiviteit door quercetine vond plaats met een affiniteit die 10^5-10^6 maal lager was dan die van 17β-oestradiol (E2) en die 10^2-10^3 maal lager was dan die van genisteïne. Echter, quercetine bleek de ERβ tot een 4.5 maal hoger niveau te activeren dan E2, terwijl het maximale inductieniveau van ERα door quercetine slechts 1.7 maal hoger was dan het inductieniveau door E2. Dit is bijzonder van belang in het licht van de hypothese dat de ERα de proliferatieve effecten van oestrogene stoffen zou mediëren, terwijl ERβ remmende effecten op dit proces zou hebben door de vorming van heterodimeren van ERβ met ERα (*27-28*). Daarom verwijzen de resultaten van deze studie mogelijk naar het relatief grote vermogen van quercetine om veronderstelde ‘gunstige’ ERβ-effecten te stimuleren vergeleken met de stimulering van ERα. Samengevat tonen de resultaten van deze studie aan dat fysiologisch relevante concentraties quercetine fyto-oestrogeenachtige effecten kunnen uitoefenen, vergelijkbaar met de effecten die waargenomen zijn voor het isoflavonoïd genisteïne. Om een uitspraak te kunnen doen over de betekenis van deze bevindingen in het kader van het ontstaan en de ontwikkeling van kanker, moeten onder andere de ER-subtypes geïdentificeerd en gekwantificeerd worden die aanwezig zijn in weefsels die aangetast zijn door hormoon-afhankelijke kankersoorten.

De dosis-responsrelatie die verkregen is voor de effecten van quercetine op de proliferatie van ER-positieve cellen zou aangeduid kunnen worden als ‘hormetisch’ (Hoofdstuk 6). Met hormesis wordt het verschijnsel bedoeld waarbij stimulering van een effect plaatsvindt bij de lagere doses van een stof en remming van datzelfde effect bij de hogere concentraties (of vice-versa) (*29*). Hoewel de vorm van de dosis-responscurve voor het effect van quercetine op de proliferatie van ER-positieve cellen (Hoofdstuk 6 en 7) zou kunnen duiden op hormesis, lijkt er toch behoefte te zijn aan een betere definitie van het hormesisconcept en aan een heroverweging of alle bifasische dosis-responscurves als representatief voor hormesis beschouwd kunnen worden. Hoofdstuk 8 bevat een discussie over de definitie van hormesis

en over de toepasbaarheid van het hormesisconcept op onze resultaten omtrent het bifasische effect van quercetine op celproliferatie. De term hormesis zou beperkt kunnen worden tot verschijnselen die voortkomen uit mechanismen die algemeen geldig zijn en een mogelijk gunstige overcompensatie vormen als reactie op een ongunstige stimulus. Wanneer hormesis als zodanig wordt gedefinieerd zou het bifasisch effect van quercetine op celproliferatie niet als hormesis beschouwd kunnen worden. Er is immers aangetoond dat de stimulering van celproliferatie ER-afhankelijk is en dus niet het gevolg is van overcompensatie door een beschermend mechanisme als reactie op een ongunstige stimulus. Hetzelfde zou gelden voor verscheidene andere receptor-gemedieerde bifasiche dosis-responsverschijnselen.

Toekomstperspectieven

Alle nieuwe resultaten en inzichten van dit proefschrift in ogenschouw nemende, blijft het de vraag in welke mate de verwachte toename van quercetineconsumptie, ten gevolge van inname van functional foods en/of voedingssupplementen, significante gevolgen voor de gezondheid van de mens zal hebben. Het is altijd moeilijk om resultaten van *in vitro* studies te extrapoleren naar verwachtingen over het *in vivo* gedrag van een stof. Desalniettemin geven de studies die in dit proefschrift worden besproken aanwijzingen voor het dualistische karakter van quercetine wat betreft zijn rol in het proces van kankerontwikkeling.

Aan de ene kant duiden verschillende studie-uitkomsten op een mogelijk stimulerend effect van quercetine op de ontwikkeling van kanker. Dit is gebaseerd op de volgende argumenten.

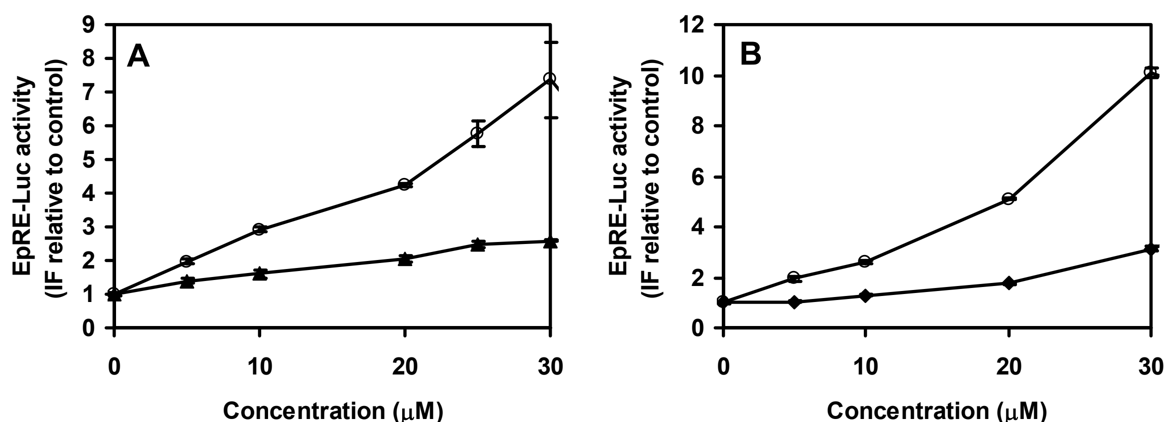
1. Quercetine bleek bij fysiologisch relevante concentraties de proliferatie van verschillende cellijnen te stimuleren (Hoofdstuk 6 en 7). Dit effect was afhankelijk van de aanwezigheid van de oestrogeenreceptor in de betreffende cellen (Hoofdstuk 7). Dit zet vraagtekens bij de veronderstelde beschermende rol van quercetine in de ontwikkeling van kanker (*30, 31*), met name in het geval van hormoonafhankelijke kankersoorten. Bovendien blijkt quercetine de differentiatie van Caco-2 cellen te remmen, wat eveneens een effect is dat niet verwacht wordt van een stof met vermeende kankerremmende eigenschappen (*32*).
2. De covalente binding van quercetine aan DNA bleek van reversibele aard te zijn. In theorie kan het verlies van covalent gebonden quercetine het gevolg zijn van volledige reversibiliteit van de additiereactie, zoals het geval is bij quercetineadducten met glutathion en andere thiolgroep-bevattende stoffen (*33*). Aan de andere kant kan het verlies van covalent gebonden quercetine het gevolg zijn van depurinering van de

DNA-base waaraan quercetine gebonden was. Over het algemeen kunnen gedepurineerde basen aanleiding zijn voor het ontstaan van mutaties, ten gevolge van fouten bij de reparatie of de replicatie van het DNA. Dit verhoogt het risico op kanker aanzienlijk (*34, 35*). Als de tijdelijke aard van de quercetine-DNA-adducten het gevolg is van depurinering van de DNA-basen, dan zou het ontbreken van *in vivo* carcinogene effecten van quercetine kunnen worden toegeschreven aan het feit dat quercetine mogelijk een zwak carcinogene stof is, waarvoor tot op heden geen toereikende *in vivo* diermodellen bekend zijn.

3. Hoewel het snelle fase II-metabolisme van quercetine de uitscheiding van de stof vergemakkelijkt (*36*) en zou kunnen bijdragen aan de beperking van de biologische effecten van quercetine *in vivo*, bleek methylering van de catecholgroep van quercetine de gevolgen van de pro-oxidantchemie van quercetine in de cel niet volledig uit te schakelen. Zoals aangetoond in Hoofdstuk 5 vormen gemethyleerde quercetine-conjugaten nog steeds ongeveer 42% van de hoeveelheid DNA-adducten gevormd door het aglycon. Het moet nog onderzocht worden in hoeverre covalente adducten, gevormd door fase II-metabolieten van quercetine, dezelfde eigenschappen hebben als quercetine-DNA-adducten, zoals het tijdelijke karakter en de mogelijk depurinerende effecten. Het fase II-metabolisme van quercetine schakelt dus niet volledig de risico's uit die intrinsiek verbonden zijn met quercetine.
4. Naast de mogelijke directe risico's die verbonden zijn met quercetineblootstelling verdienen de mogelijke indirecte risico's van een toegenomen consumptie van quercetine ook aandacht. Deze indirecte effecten zijn bijvoorbeeld het effect van quercetin op het risico op carcinogene effecten van andere stoffen. Door het uitgebreide fase II-metabolisme van quercetine, zou een overload aan quercetine bijvoorbeeld kunnen leiden tot depletie van cofactoren die essentieel zijn voor de biotransformatie en detoxificatie van oestrogenen. Aanwijzingen hiervoor zijn verkregen uit studies met hamsters die waren behandeld met oestradiol. In deze dieren bleek co-administratie van quercetine via het dieet gedurende 6 maanden het aantal tumoren en metastases aanzienlijk te verhogen, vergeleken met de dieren die alleen de hormoonbehandeling kregen. De oorzaak zou mogelijk de depletie van de cofactor voor methylering kunnen zijn, die essentieel is voor de detoxificatie van reactieve oestrogeenmetabolieten (*37*).

Het feit dat quercetine een dualistische invloed op het ontstaan en de ontwikkeling van kanker zou kunnen hebben, wordt geïllustreerd door verschillende andere studies die besproken zijn in dit proefschrift, die wijzen op beperking van mogelijke risico's of zelfs beschermende effecten van quercetine in de ontwikkeling van kanker.

1. Quercetine wordt uitgebreid gemetaboliseerd door het fase II-biotransformatiesysteem *in vivo*, wat de snelle uitscheiding via urine en gal vergemakkelijkt (*36*). Dit verkleint het risico op schadelijke biologische effecten *in vivo*. Bovendien is de biologische activiteit van de fase II-metabolieten verminderd, vergeleken met de moederstof. Dit is aangetoond in Hoofdstuk 5 voor de pro-oxidantchemie van quercetine die leidt tot de vorming van covalente adducten met DNA.
2. Hoewel het fase II-metabolisme van quercetine de biologische activiteit afzwakt, geldt dit ook voor de gunstige biologische effecten van fase II-metabolieten van quercetin. Zo bleek dat methylering van quercetine de antioxidanteigenschappen van quercetine wel vermindert maar niet volledig uitschakelt (Hoofdstuk 3). De antioxidantactiviteit van quercetine wordt vaak genoemd als een van de mechanismen voor de bescherming door quercetine tegen kanker (*7*). Verder induceert quercetine de expressie van fase II-enzymen *in vitro* via de inductie van het Electrophile Responsive Element (EpRE), een enhancerelement dat aanwezig is in de promotorregio van genen die coderen voor onder andere fase II-enzymen (*38*). Dit zou de claim kunnen ondersteunen dat quercetine bescherming biedt tegen carcinogene effecten van andere stoffen. Figuur 1 toont de effecten van gemethyleerde conjugaten van quercetine vergeleken met quercetine zelf, op EpRE-afhankelijke genexpressie (Van der Woude *et al.* ongepubliceerde resultaten). Hoewel de resultaten aantonen dat conjugatie van de 3'- of 4'-hydroxylgroep van quercetine met een methylgroep het vermogen van quercetine om EpRE-afhankelijke genexpressie te induceren vermindert, behouden catechol-O-gemethyleerde conjugaten van quercetine ongeveer 20% van de biologische activiteit van het aglycon. Ook na fase II-conjugatie zou quercetine dus een deel van zijn vermeende gunstige effecten *in vivo* kunnen behouden.



Figuur 1 Effect van quercetine en (A) 3'-O- en (B) 4'-O-methylquercetine op EpRE-afhankelijke genexpressie (open cirkels: quercetine; driehoekjes: 3'-O-methylquercetine, ruitjes: 4'-O-methylquercetine; IF: inductiefactor) (Van der Woude *et al.* ongepubliceerde resultaten).

3. Hoewel quercetine, net als genisteïne, de proliferatie van kankercellijnen *in vitro* bleek te stimuleren bij concentraties die fysiologisch relevant zijn in mensen (Hoofdstuk 6 en 7), werden er aanwijzingen verkregen dat quercetine 'gunstige' ERβ-afhankelijke processen sterker stimuleert dan ERα-afhankelijke processen. Dit is van belang in het licht van de hypothese dat ERα mogelijk de proliferatieve effecten van oestrogene stoffen medieert, terwijl ERβ beschermende effecten op dit proces zou kunnen uitoefenen door de vorming van heterodimeren van ERα en ERβ (*27, 28*). Om een uitspraak te kunnen doen over de relevantie van deze bevindingen in het kader van de ontwikkeling van kanker zou onder andere de identificatie en kwantificering van de ER-subtypen bekend moeten zijn die aanwezig zijn in weefsels die aangetast zijn door hormoon-afhankelijke kankersoorten.
4. De covalente binding van quercetine aan DNA, maar ook aan eiwit, is van tijdelijke aard, zoals aangetoond in Hoofdstuk 4. Op dit moment is het onbekend of dit tijdelijke karakter depurinering van de DNA-basen tot gevolg heeft. Het zou een mechanisme kunnen zijn dat bijdraagt aan het mogelijke risico voor genotoxiciteit en initiatie van kanker door quercetine, zoals eerder besproken. Echter, indien er sprake is van volledige reversibiliteit van de additiereactie, zoals ook het geval is voor glutathionylquercetine-adducten (*33*), dan zou de DNA-schade slechts tijdelijk kunnen zijn, wat een verklaring zou kunnen zijn voor het schijnbare gebrek aan carcinogene effecten van quercetine *in vivo* , terwijl de stof *in vitro* duidelijk genotoxisch is.

5. De *in vivo* carcinogeniteitsstudie, uitgevoerd door en volgens de richtlijnen van het National Toxicology Program (*39, 40*), verschafte zwak bewijs voor carcinogene effecten voor quercetine. Er zijn echter aanwijzingen dat de verhoogde aantallen niertumoren die met name gevonden zijn in de mannelijke ratten, mogelijk niet veroorzaakt zijn door een mechanisme specifiek voor quercetine, maar door een niet-specifiek mechanisme waarbij α_{2u}-globuline nephropathy betrokken is (*39, 40*). Dit verschijnsel wordt vaker waargenomen in mannelijke ratten omdat deze grote hoeveelheden α_{2u}-globuline tot expressie brengen. Omdat mensen, evenals vrouwelijke ratten waarin geen significante effecten werden waargenomen, veel lagere hoeveelheden van dit eiwit tot expressie brengen, zouden de carcinogene effecten in de nier, zoals ze zijn waargenomen in mannelijke ratten, mogelijk niet representatief zijn voor de mens. Bovendien toont Tabel 1 resultaten van een dierstudie die uitgevoerd is binnen Taak 4 van het overkoepelende project waar dit proefschrift een onderdeel van uitmaakt, met de titel "Benefit-risk evaluatie van flavonoïden in voedingsmiddelen en hun toepassing als functional food ingredienten" (project no. 014-12-012) gefinancierd door NWO (zie hiervoor ook Hoofdstuk 1, Sectie 5). Mannelijke F344-ratten, waarin dikkedarmkanker was geïnduceerd met azoxymethaan, kregen 0, 100, 1000 of 10000 ppm quercetine in hun dieet gedurende 38 weken. Uit resultaten van deze proef (weergegeven in Tabel 1) blijkt dat quercetine na 38 weken een dosis-afhankelijke afname in de incidentie en de multipliciteit van tumoren veroorzaakt in de dieren die blootgesteld waren aan AOM. Dit is een mogelijke aanwijzing dat quercetine de vorming van chemisch geïnduceerde dikkedarmtumoren in ratten kan remmen en het aantal en de grootte van nieuwe tumoren kan onderdrukken. Deze resultaten verschaffen aanvullend bewijs voor beschermende effecten van quercetine tegen kanker, hoewel deze effecten zijn gevonden bij doses die, wat de 1000- en 10000 ppm-groepen betreft, neerkomen op 10- tot 100 maal de dosis die meestal wordt aanbevolen voor quercetinesupplementen.

Tabel 1 Effect van quercetine op tumordata in ratten die quercetine in hun dieet kregen gedurende 38 weken. Bij de ratten is dikkedarmkanker geïnduceerd met het dikkedarmcarcinogeen azoxymethaan (*41*).

	Quercetine (ppm)			
	0	**100**	**1000**	**10000**
Aantal ratten per groep	22	22	22	20
Aantal tumordragende dieren per groep	11	9	8	4
Tumorincidentie per groep (%) [1)]	50	41	36	20
Totaal aantal tumoren per groep	17	13	11	4
Tumormultipliciteit [2)]	1.55 ± 0.93	1.44 ± 1.01	1.38 ± 0.74	1.00 ± 0.0
Tumoromvang (mm) [2)]	5.94 ± 3.83	5.85 ± 4.22	5.64 ± 3.98	5.00 ± 1.83

[1)] Tumorincidentie was significant ($P<0.05$) verlaagd door toenemende concentraties quercetine, zoals bepaald met de Jonckheere-Terpstra trend-analysetest.
[2)] Tumormultipliciteit en -omvang gaven een negatieve associatie met toenemende concentraties quercetine (r = 0.98, $P<0.05$), zoals bepaald met Pearsons correlatiecoefficient.

Zoals op te maken valt uit bovenstaande uiteenzetting van argumenten is de risk-benefit evaluatie van een verhoogde consumptie van quercetine en kanker verre van recht toe recht aan. Voor zover we nu weten is de *in vivo* biologische activiteit van quercetine afhankelijk van zijn biobeschikbaarheid en biotransformatie en dus van (1) de beperkte opname van quercetine door darmcellen, (2) de mogelijke uitscheiding van (fase II-metabolieten van) quercetine vanuit de darmcel terug in het lumen van de darm (*42*), (3) het uitgebreide fase II-metabolisme van quercetine dat de uitscheiding via urine en gal bevordert en de biologische activiteit reduceert (*1, 3-8*). Desalniettemin, hoewel ze over het algemeen minder potent zijn dan de moederstof, zijn de fase II-metabolieten van quercetine wat biologische effecten betreft vergelijkbaar met het aglycon, oplopend to 30-50% van de sterkte van het effect van quercetine, afhankelijk van het eindpunt dat bestudeerd wordt. Dit geldt voor zowel de mogelijke schadelijke effecten van quercetine, zoals de pro-oxidantchemie en de covalente binding aan DNA, als voor de mogelijk gunstige effecten, zoals de antioxidantactiviteit en de inductie van EpRE-afhankelijke genexpressie. Verder is het van belang te beseffen dat de effecten die gevonden zijn voor het quercetine-aglycon, relevant kunnen zijn in geval van consumptie van een quercetinesupplement. Inname van een supplement met 500 mg quercetin-aglycon per dosering veroorzaakt namelijk blootstelling van de luminale zijde van de darm aan een relatief hoge concentratie van het aglycon.

Behalve dat de fase II-metabolieten van quercetine de belangrijkste biologisch actieve vormen van quercetine *in vivo* zijn (*43-45*) en dat er meer kennis nodig is inzake de biologische effecten van deze quercetine vormen, is er voor een goed gefundeerde schatting van de biologische activiteit van fase II-metabolieten van quercetine ook meer inzicht nodig in de opname- en verwerkingsmechanismen van deze metabolieten door cellen die via het plasma worden blootgesteld. Er zijn aanwijzingen dat bepaalde celtypen uitgerust zijn met enzymen die glucuronide- en/of sulfaatconjugaten kunnen hydrolyseren (vb. β-glucuronidases) of de catecholgroep van 3'- of 4'-O-methylquercetine kunnen demethyleren (vb. bepaalde P450-enzymen) (*46, 47*).

Hepatocyten en bepaalde bloed-, nier- en darmcellen bevatten deze typen enzymen en er is grote interindividuele variatie in de activiteit en expressie ervan (*48*). Verder blijken bloedcellen, zoals macrofagen, β-glucuronidaseactiviteit uit te scheiden in het plasma wanneer deze celtypen gestimuleerd worden door een antigen (*49*). Op grond van deze gegevens mag er in dergelijke celtypen en in ontstekingshaarden verhoogde blootstelling aan het aglycon verwacht worden.

Al met al blijft het dus de vraag of een toename in de consumptie van quercetine uiteindelijk tot een verbetering of juist een verslechtering van de gezondheidstoestand van de consument zal leiden. Omdat het doel van functional foods en voedingssupplementen, in tegenstelling tot reguliere voedingsmiddelen, juist is om een gezondheidsbevorderend effect bij de consument te bewerkstelligen dat verder gaat dan de normale gezondheidbevorderende effecten van een uitgebalanceerd dieet, vallen dergelijke producten in een grijs/ongedefinieerd gebied tussen voeding en geneesmiddelen. De toename in het gebruik van dergelijke verrijkte voedingsmiddelen heeft daarom bezorgdheid gewekt binnen zowel de wetenschap als wetgevende autoriteiten. In principe moeten consumenten namelijk eenzelfde mate van veiligheid kunnen verwachten, of het nu gaat om reguliere voedingsmiddelen of om functional foods en/of voedingssupplementen. Verwacht wordt dat de dosisintervallen waarbij gezondheidsbevorderende effecten optreden relatief dicht bij de dosisintervallen liggen waarbij mogelijke ongewenste gezondheidseffecten kunnen optreden. Bovendien bestaan er door de grote interindividuele variabiliteit tussen mensen waarschijnlijk ook populatiegroepen die extra gevoelig zijn voor bepaalde ongewenste gezondheidseffecten en die dus extra risico lopen bij de consumptie van functional foods en/of voedingssupplementen. Een commissie binnen de Natural Toxin Task Force van de Europese tak van het International Life Science Institute – ILSI Europe – heeft zich over dit probleem gebogen en heeft de aanbeveling

gedaan dat het veiligheidsonderzoek wat betreft botanische ingrediënten zich vooral moet richten op specifieke veiligheidsmarges voor specifieke populatiegroepen. Hierbij moet rekening gehouden worden met zowel de blootstellingsduur als de te verwachten ongewenste gezondheidseffecten (*50*). Aangezien quercetine een botanisch ingrediënt is, zouden deze aanbevelingen ook kunnen gelden voor de bepaling van de veiligheid van functional foods en voedingssupplementen waarin quercetine verwerkt is. De ILSI-commissie heeft een stappenplan voorgesteld dat kan helpen bij de bepaling van de informatie die moet worden ingewonnen om een beslissing te kunnen nemen over de veiligheid van botanische ingrediënten, zoals quercetine (*50*). Volgens dit stappenplan zou een uitgebreid pakket aan toxiciteitsstudies moeten worden uitgevoerd met quercetine, voordat dit ingrediënt beschouwd zou mogen worden als veilig voor de consument beschouwd. Aspecten zoals biobeschikbaarheid en ook klinische studies in de mens met bijzondere aandacht voor variabiliteit in respons, ongewenste effecten en contra-indicaties zouden hierbij in aanmerking genomen moeten worden. De toxiciteitstesten die nodig zijn moeten voor elk geval afzonderlijk worden bepaald. In de studies die in dit proefschrift beschreven zijn, is een aantal eindpunten geïdentificeerd die van belang zouden kunnen zijn bij een risicoschatting van quercetine, die vooraf zou moeten gaan aan de toepassing van de stof in functional foods of voedingssupplementen. Deze eindpunten zouden kunnen zijn: celproliferatie (Hoofdstukken 6 en 7), oestrogene eigenschappen (Hoofdstuk 7) en covalente binding aan cellulaire macromoleculen, zoals DNA (Hoofdstukken 4 en 5). Zolang quercetine op de markt blijft zonder dat een veiligheidsonderzoek is gedaan volgens bijvoorbeeld de richtlijnen die voorgesteld zijn door de ILSI-commissie (*50*), is het aan de consument om te bepalen of de mogelijk nadelige effecten in relatie tot kanker, in het bijzonder voor hormoon-afhankelijke kankersoorten, en de hoge kosten die verbonden zijn met de consumptie van voedingssupplementen, opwegen tegen de waarschijnlijk beperkte gezondheidsbevorderende effecten van het gebruik van dit flavonoïd.

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Het is af! Op naar een mooie toekomst!

Curriculum vitae

Hester van der Woude werd op 28 juni 1978 geboren in Bennekom. Op 8-jarige leeftijd verhuisde zij naar Zuid-West Frankrijk en volgde zij Franstalig onderwijs aan de Ecole Primaire Immaculée Conception en het Collège Saint-Joseph in Lectoure. Dankzij deze ervaring werd Frans een tweede moedertaal. In 1991 keerde zij terug naar Nederland, waar zij in 1996 haar Gymnasium diploma haalde aan het Buys Ballot College in Goes. In datzelfde jaar begon zij aan de opleiding Moleculaire Wetenschappen aan Wageningen Universiteit (WU). Tijdens deze opleiding ontving zijn drie studiebeurzen van de Vereniging van de Nederlandse Chemische Industrie (VNCI) en deed zij afstudeervakken bij Prof. Dr. I.M.C.M. Rietjens (WU, Biochemie en Toxicologie) en bij Dr. M.C. Franssen en Dr. H.J. Bouwmeester (WUR, Plant Research International). Voor de scriptie behorende bij dit laatste afstudeervak ontving zij de C.T. de Wit Scriptieprijs, uitgereikt door het Wagenings Universiteitsfonds. Ze sloot haar studie af met een stage van zes maanden bij de afdeling Toxicology and Drug Disposition van het farmaceutische bedrijf Organon NV, onder begeleiding van Dr. D.J. van den Dobbelsteen. In juni 2001 behaalde zij haar doctoraal diploma met lof. Aansluitend startte zij het promotie-onderzoek aan Wageningen Universiteit, sectie Toxicologie, waarvan de resultaten in dit proefschrift beschreven zijn. Sinds 1 mei 2005 is zij werkzaam als studieleider *in vitro* ADME bij Notox BV te ’s-Hertogenbosch.

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Abstracts

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Training and Supervision Plan

Overview of conferences and courses attended during PhD

International Advanced Course on Chemistry and Biochemistry of Antioxidants (VLAG)	2001
Organising and Supervising Thesis Work (OWU)	2001
Techniques for Writing and Presenting a Scientific Paper (Mansholt)	2001
4th International PK/PD Symposium on Measurement and Kinetics of *in vivo* Drug Effects	2002
Food Toxicology and Food Safety (PET)	2002
Toxicological Risk Assessment (PET)	2002
Conference Antioxidants: Benefits and Risks (EuroFeda), *United Kingdom*	2002
Laboratory Animal Science (PET)	2003
1st International Conference on Polyphenols and Health, *France*	2003
Medical and Forensic Toxicology (PET)	2003
Legal and Regulatory Toxicology (PET)	2004
Epidemiology for Toxicologists (PET)	2004
Environmental Toxicology (WUR)	2004
Pathobiology (PET)	2004
PhD symposium (NVT)	2004
Career Perspectives (WGS)	2005
Scientific Writing English (Centa)	2005

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