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In vitro biological properties of flavonoid conjugates found *in vivo*

G. WILLIAMSON[1], D. BARRON[1], K. SHIMOI[2,†], & J. TERAO[3,‡]

[1]*Nestlé Research Center, Vers-Chez-Les-Blanc, PO Box 44, CH-1000 Lausanne 26, Switzerland,* [2]*Institute for Environmental Sciences, Graduate School of Nutritional and Environmental Sciences, University of Shizuoka, 52-1 Yada, Shizuoka 422-8526, Japan, and* [3]*Department of Food Science, Graduate School of Nutrition and Biosciences, The University of Tokushima, Kuramoto-cho 3-18-15, Tokushima 770-8503, Japan*

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Abstract

For some flavonoids such as quercetin, isoflavones and catechins, the pathways of absorption and metabolism are now reasonably well characterised and understood. By definition, for biological activity of flavonoids to be manifest, the target tissue, which includes the blood and vascular system, must respond to the form(s) of flavonoid *that it encounters*. Bioavailability studies have shown that the circulating form of most flavonoids is as conjugates, with a few notable exceptions. There have been several recent papers on the *in vitro* biological properties of conjugates that have been found *in vivo*. This paper reviews the properties of these conjugates. Most of the information currently available is on quercetin glucuronides, but also on isoflavone and catechin conjugates. In addition to the biological properties of the conjugates, the partition coefficients and methods of synthesis are also presented.

Keywords: *Quercetin, glucuronide, genistein, sulfate, conjugates, biological property*

Abbreviations: *EGCG, (−)-epigallocatechin gallate; TBARS, thiobarbituric acid reactive substances; COX, cyclo-oxygenase; DMSO, dimethylsulfoxide; LDL, low density lipoprotein*

Introduction

There have been almost 100 reports on the bioavailability of flavonoids in humans [1] and a similar number on the biological activity of flavonoids in human clinical and intervention studies [2]. It is now clear that flavonoids are absorbed into the plasma and most of the absorbed flavonoids are present in the blood as conjugates, but that the extent of absorption, bioavailability and biological activity is highly dependent on the nature of the flavonoid. Isoflavones (from Soya) are the best absorbed, and typically a dose of 50 mg gives a maximum plasma concentration of 2 μM for genistein. Quercetin from onions, when normalised to a 50 mg dose, gives 1.5 μM in plasma. On the other hand, intact procyanidins (from cocoa, apples and other fruits) are apparently the most poorly absorbed, with maximum concentration of the parent compound (probably in conjugated form) in the plasma of ≤10 nM (normalised to a 50 mg dose). However, all of these plasma concentrations above were determined after treatment with deconjugating enzymes, and represent the total concentration of non-methylated flavonoid.

Since flavonoids exert biological effects *in vivo* in human intervention studies, then one hypothesis to explain the efficacy and bioavailability data is that flavonoid conjugates retain some biologically active properties. An example of a drug is morphine, which is more active after conjugation with glucuronic acid at

Correspondence: G. Williamson, Nestlé Research Center, Vers-Chez-Les-Blanc, PO Box 44 CH-1000, Lausanne 26, Switzerland. Tel: 41 21 785 8546. Fax: 41 21 785 8544. E-mail: gary.williamson@rdls.nestle.com

[†]Tel: 81 54 264 5787. Fax: 81 54 264 5787. E-mail: shimoi@smail.u-shizuoka-ken.ac.jp

[‡]Tel: 81 88 633 7087. Fax: 81 88 633 7089. E-mail: terao@nutr.med.tokushima-u.ac.jp

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the C(6) position than the parent molecule [3–5]. This report reviews the current knowledge on the biological properties *in vitro* of the flavonoid conjugates that are found *in vivo*. We do not cover the properties of flavonoids that are at least partially present in plasma in an unchanged form, such as anthocyanin glucosides and EGCG.

What are the forms of flavonoids in plasma or urine?

A major fraction of absorbed flavonoids are metabolised to conjugates, which circulate in the blood and are excreted into bile and urine (Figure 1). For some compounds, such as quercetin, there is no free aglycone in the plasma [6] and in fact the exact nature of circulating

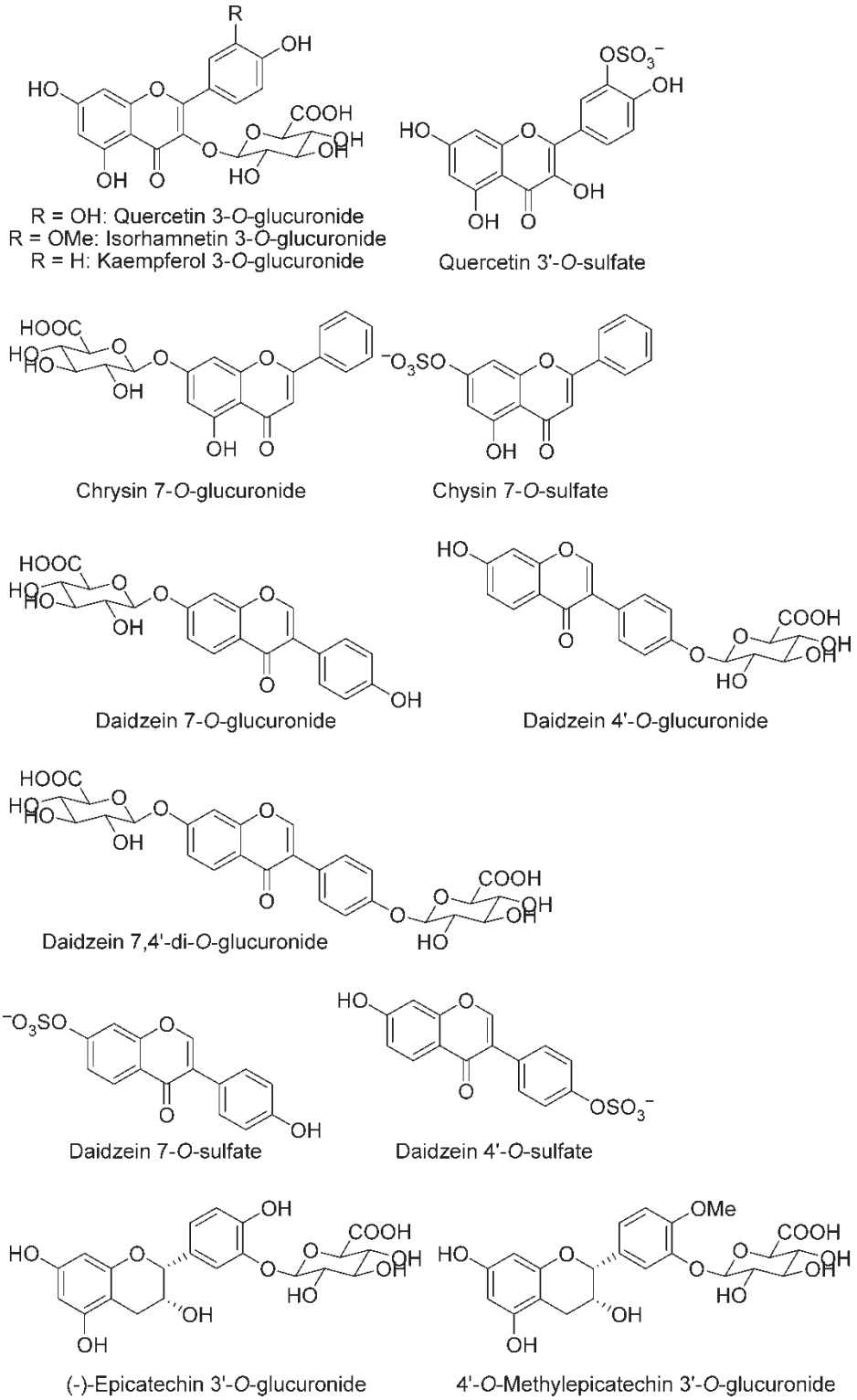

Figure 1. Chemical structures of some flavonoid conjugates.

conjugates of quercetin after consumption of onions in the plasma at 2 h has been reported [7]. For others, such as EGCG, a substantial amount is present in plasma as the unconjugated aglycone; the exact amount varies between laboratories, but is up to 90% [8]. For isoflavones, about 10% in the plasma is unconjugated. The circulating main metabolite of luteolin is the monoglucuronide, but the free form of luteolin is also present in plasma [9].

Some studies have measured the exact nature of the conjugated forms of flavonoids in plasma or urine after oral consumption. Table I shows the nature of conjugates that have been positively identified *in vivo* in human intervention studies. Quercetin is the best characterised, and exists in plasma conjugated with glucuronide, sulfate and methyl groups. The (−)-epicatechin is also found as conjugates, although these are less extensively characterised. On the other hand, anthocyanins are found as the original glucoside form, although a recent report suggests that they may also be additional glucuronidated and sulfated forms [10]. EGCG is found substantially in an unconjugated form in plasma, but a summary of the many properties of EGCG is outside the scope of this review. However, strictly speaking, EGCG is one of the most bioavailable flavonoids, since it is one of the very few that is found in substantial amounts in the aglycone form; this situation allows real pharmacokinetic parameters to be estimated.

Table I. Nature of the flavonoid conjugates found in human studies *in vivo*.

Conjugate	Found in:	Reference
Quercetin 3-*O*-*β*-D-glucuronide	Human plasma	[7]
Quercetin 3′-*O*-sulfate	Human plasma	[7]
Isorhamnetin 3-*O*-*β*-D-glucuronide	Human plasma	[7]
Kaempferol 3-*O*-*β*-D-glucuronide	Human plasma	[65]
Chrysin 7-*O*-*β*-D-glucuronide	Human plasma and urine	[66]
Chrysin 7-*O*-sulfate	Human plasma and urine	[66]
Daidzein 7-*O*-*β*-D-glucuronide	Human urine	[67]
Daidzein 4′-*O*-*β*-D-glucuronide	Human urine	[67]
Daidzein 4′, 7-di-*O*-*β*-D-diglucuronide	Human urine	[67]
Daidzein 7-*O*-sulfate	Human urine	[67]
Daidzein 4′-*O*-sulfate	Human urine	[67]
(−)-Epicatechin	Human plasma	[68]
3′-*O*-*β*-D-glucuronide	Human urine	
4′-*O*-methylepicatechin	Human plasma	[68]
3′-*O*-*β*-D-glucuronide	Human urine	
4′-O-methylepicatechin	Human plasma	[68]
5- or 7-*O*-*β*-D-glucuronide	Human urine	
Anthocyanin glucosides	Human urine and plasma	[69–73]
EGCG	Human plasma	[8]

Pharmacokinetics of other flavonoids are measured after deconjugation, but these values only represent apparent pharmacokinetic parameters since the administered parent compound is metabolised before reaching the blood.

Biological properties of conjugates

Quercetin

For conjugate properties, the most studied flavonoid is quercetin. Table II shows the activities that have been measured *in vitro* for various quercetin conjugates. The quercetin conjugate with glucuronic acid at the 3-position is the most studied, as this has been purified from green beans and is more readily available [11]. The activity of the conjugates is very dependent on the position of substitution. For example, xanthine oxidase catalyses the oxidation of hypoxanthine and xanthine to uric acid and generates superoxide radicals. Quercetin glucuronides inhibited bovine xanthine oxidase in the order $4' > 3' > 7 > 3$ [12]. This immediately implies that the quercetin glucuronide that is in plasma (the 3-*O*-*β*-glucuronide) is inactive. However, the other major conjugate is quercetin 3′-*O*-sulfate, which although not tested, is likely to also be very active, since the inhibition seems to depend primarily on the position of substitution and less on the nature of the substitution. The inhibition constant (K_i) of the most potent conjugate (4′ position) was 0.2 μM. Lipoxygenase from soybean was also inhibited with a similar efficacy order to that of xanthine oxidase, except that the inhibition was weaker. Quercetin 3-*O*-*β*-D-glucuronide was a weak inhibitor with $K_i = 60$ μM [12], but this is unlikely to be physiologically relevant.

The interpretation of results that do not show a dose response should be viewed with caution. For example, quercetin glucuronides inhibited 2-aminofluorene acetylation in human acute myeloid HL-60 leukaemia cells [13], and a low acetylation phenotype leads to increased risk of bladder cancer in humans. Quercetin glucuronides were prepared from rabbit serum fed quercetin, and so contained a mixture of physiologically-produced glucuronides—but also sulfated and methylated forms. Experiments in the presence of 1% DMSO showed a cytotoxic effect on HL-60 leukaemia cells of quercetin metabolites. About 1% DMSO showed a small effect after 24 h, similar to the effect of the quercetin metabolites after 24 h. The problem in interpretation is that 1 nM has almost the same effect as 10 μM (the range of concentrations tested), suggesting that the DMSO (1% DMSO is ~130 mM) could be producing the effect.

Isoflavones

Although a few percent of circulating isoflavones are as free aglycone, the majority is conjugated. These conjugates show some biological activities. Genistein

Table II. Activities of quercetin conjugates.

Quercetin conjugate	Effective or tested concentration	Activity	Ref
Quercetin 3-*O*-β-D-glucuronide	$K_i = 160\,\mu M$	Weak inhibition of xanthine oxidase	[12]
	5 μM, dose-dependent	12% inhibition of superoxide generation by phenazine methosulfate-NADH	[89]
	$K_i = 60\,\mu M$	Weak inhibition of soybean lipoxygenase	[12]
	10 μM, dose-dependent	~50% inhibition of COX-2 mRNA in unstimulated caco-2 cells	[90]
	10 μM	~75% inhibition of COX-2 mRNA in IL-1β-stimulated caco-2 cells	[90]
	10 μM	No inhibition of COX-2 enzymic activity	[90]
	1 μM, dose-dependent	2.4-fold increase in lag time of copper-induced oxidation of human LDL	[91]
	$K_q = 8.6 \pm 0.3\,\mu M$	Tight binding to human serum albumin in physiological conditions	[91]
	1 μM	No increase in lag time of copper-induced oxidation of human LDL in the presence of human serum albumin	[91]
	20 μM	~10% inhibition of iron ion induced lipid peroxidation in phosphatidylcholine large unilamellar vesicles as assessed using TBARS	[75]
	20 μM	~50% inhibition of 2,2′-azobis(2-amidino-propane) dihydrochloride-induced lipid peroxidation in hosphatidylcholine large unilamellar vesicles as assessed using TBARS	[75]
	10 μM	~70% inhibition of 3-morpholinosydnoni-minnine-HCl-induced lipid peroxidation in phosphatidylcholine large unilamellar vesicles as assessed using phosphatidyl-choline hydroperoxides	[75]
	10 μM	~50% inhibition of 15-lipoxygenase-induced lipid peroxidation in phosphati-dylcholine large unilamellar vesicles as assessed using phosphatidylcholine hydroperoxides	[75]
	5 μM	Inhibition of copper ion catalysed oxidation of human low density lipoprotein and 1,1-diphenyl-2-picrylhydrazyl radical scavenging activity	[92]
	50 μM	Protection against peroxynitrite-induced antioxidant (lycopene, β-carotene and α-tocopherol) consumption in human plasma low density lipoprotein	[93]
	10 μM	40% inhibition of hydrogen peroxide-induced peroxidation in mouse fibroblast 3T3 cells	[94]
	10 μM	~20% inhibition of hydrogen peroxide induced peroxidation in mouse fibro-blast 3T3 cells when pre-treated with quercetin-3-*O*-β-D-glucuronide (quercetin was inactive under these conditions)	[94]
	1 μM	Prevention of vascular smooth muscle cell hypertrophy by angiotensin II via effects on JNK and AP-1, but no effect on ERK1/2 or p38.	[95]
	Not stated	Quercetin-3-*O*-β-D-glucuronide prevents angiotensin II-induced vascular smooth muscle cell hypertrophy	[96]
	20, 40 μM	No effect of quercetin-3-*O*-β-D-glucuronide on expression of adhesion molecules in human aortic endothelial cells	[36]
	10 μM	Inhibition (~25%) of non-enzymic superoxide generation	[89]

Table II – *continued*

Quercetin conjugate	Effective or tested concentration	Activity	Ref
	5 μM	~5-fold increase in lag time of copper-induced oxidation of human LDL as measured by cholesterol hydroperoxide levels (quercetin aglycone was 8-fold, quercetin-4′-*O*-β-D-glucuronide had no effect)	[92]
	0.1 and 1 μM, but not 4 μM	Decreased expression of COX-2 in human lymphocytes *ex vivo*, but poor dose response	[97]
Quercetin 3′-*O*-sulfate	10 μM, dose-dependent	~50% inhibition of COX-2 mRNA in unstimulated caco-2 cells	[90]
	10 μM	~75% inhibition of COX-2 mRNA in IL-1β-stimulated caco-2 cells	[90]
	10 μM	~50% inhibition of COX-2 activity	[90]
	1 μM	Small (10-20%) increase in lag time of copper-induced oxidation of human LDL	[91]
	K_q ~4 μM	Tight binding to human serum albumin	[91]
	1 μM	~30% increase in lag time of copper-induced oxidation of human LDL in the presence of human serum albumin	[91]
	10 μM	Stimulation (15%) of non-enzymatic superoxide generation	[89]
	4 μM	Small decrease in expression of COX-2 in human lymphocytes *ex vivo*	[97]
	1 μM	Suppression of formation of 3-nitro-tyrosine in human serum albumin	(K. Shimoi, unpublished results)
Isorhamnetin 3-*O*-β-D-glucuronide	2 μM	No change in lag time of copper-induced oxidation of human LDL	[91]
	K_q ~ 15 μM	Tight binding to human serum albumin	[91]
	2 μM	No change in lag time of copper-induced oxidation of human LDL in the presence of human serum albumin	[91]
	10 μM, dose-dependent	~60% inhibition of COX-2 mRNA in unstimulated caco-2 cells	[90]
	10 μM	Small inhibition of COX-2 mRNA in IL-1β-stimulated caco-2 cells	[90]
	10 μM	No inhibition of COX-2 activity	[90]
	0.1, 1 μM, and 4 μM	Decreased expression of COX-2 in human lymphocytes *ex vivo*, with good dose response	[97]
Quercetin conjugates made by feeding quercetin to rats and collecting the plasma, as total sulfate and glucuronide conjugates	glucuronide 6.1 μM, sulfate 0.4 μM, sulfoglucuronide 3.1 μM after 1 h dose of 2 mg aglycone	Quercetin metabolites inhibit copper-ion induced lipid peroxidation in rat plasma as estimated by accumulation of cholesteryl ester hydroperoxides and the consumption of α-tocopherol.	[98]
Enzymically-synthesized quercetin glucuronide mixture	0.5 μM	Inhibition of copper ion catalysed oxidation of rat very low density lipoprotein by 2-fold prolonged lag phase	[99]
Quercetin glucuronides prepared from rabbit serum fed quercetin	0.001 to 10 μM	Quercetin glucuronides inhibited 2-amino fluorene acetylation in human acute myeloid HL-60 leukemia cells.	[13]
Quercetin glucuronides prepared from rabbit serum fed quercetin	0.001 to 10 μM	No effect on NAT-1 mRNA levels in human acute myeloid HL-60 leukemia cells	[13]
Quercetin glucuronides prepared from rabbit serum fed quercetin	0.001 to 10 μM	No inhibition of IL-1β-stimulated U937 cell adhesion (although quercetin aglycone was active)	[20]
Quercetin 3-*O*-sulfate	10 μM	Prevention of hydrogen peroxide-induced chromosomal damage to lympho-blastoid WIL2-NS cells	[100]
	K_i = 16 μM	Inhibition of soybean lipoxygenase	[12]

Table II – *continued*

Quercetin conjugate	Effective or tested concentration	Activity	Ref
	0.5 μM	Inhibition of copper ion catalysed oxidation of rat very low density lipoprotein by prolonging lag phase (2.5 fold)	[99]
Quercetin 7-*O*-*β*-D-glucuronide	30 μM	No effect on neuronal toxicity or neuronal signalling, in contrast to quercetin aglycone	[101]
	30 μM	No protection of dermal fibroblasts from hydrogen peroxide-induced oxidative damage and caspase-3 activation	[102]
	$K_i > 100$ μM	No significant inhibition of bovine xanthine oxidase	[12]
	$K_i = 6$ μM	Inhibition of soybean lipoxygenase	[12]
Quercetin 3′-*O*-*β*-D-glucuronide	$K_i = 1.4$ μM	Potent inhibition of bovine xanthine oxidase	[12]
	$K_i = 6.5$ μM	Inhibition of soybean lipoxygenase	[12]
Quercetin 4′-*O*-*β*-D-glucuronide	$K_i = 0.25$ μM	Potent inhibition of bovine xanthine oxidase	[12]
	$K_i = 8.4$ μM	Inhibition of soybean lipoxygenase	[12]
Quercetin 7,4′-di-*O*-sulfate	100 μM	77% inhibition of aggregation of pig platelets induced by thrombin owing to changes in calcium metabolism	[103]

4′-*O*-sulfate and 4′,7-di-*O*-sulfate were compared to genistein, and ranked genistein >> genistein 4′-*O*-sulfate > genistein 4′,7-di-*O*-sulfate for: *In vitro* antioxidant activity (measured using the FRAP and TEAC assays); inhibition of collagen-induced platelet aggregation; inhibition of NO production by murine RAW 264.7 macrophages; and modulating secretion by primary human endothelial cells of monocyte chemoattractant protein-1 (MCP-1), intercellular adhesion molecule 1 (ICAM-1), and vascular cell adhesion molecule 1 (VCAM-1). Concentrations used were 5–100 μM for acute exposure [14]. Isoflavone conjugates bound to cytosolic oestrogen receptors of B6D2F1 mouse uterine cells with a IC_{50} to displace estradiol of 14.7 and 7.3 μM for daidzein and genistein 7-*O*-*β*-D-glucuronides, respectively [15]. Isoflavone sulfate showed weak effects on cell growth of MCF-7 cells and weak binding to human ER α and *β*, but some glucuronosyl conjugates exhibited moderate effects [16].

Catechins

Ungalloylated catechins such as (−)-epicatechin and (+)-catechin are almost 100% conjugated in plasma; glucuronide conjugates are at position 5 or 7. The (−)-Epicatechin glucuronide was detected in rat brain after administration of 100 mg/kg [17]. An (−)-epicatechin 5-*O*-*β*-D-glucuronide and (−)-epicatechin 7-*O*-*β*-D-glucuronide mixture showed no effect on hydrogen peroxide-induced-cell death in neurones and fibroblasts, and did not prevent caspase-3 activation in these cells [18]. Glucuronide conjugates of (−)-epigallocatechin gallate and (−)-epigallocatechin inhibit the release of arachidonic acid from HT-29 human colon cancer cells [19]. At 2 and 10 μM, (−)-epigallocatechin 3′-*O*-*β*-glucuronide was less effective than (−)-epigallocatechin 7-*O*-*β*-D-glucuronide or (−)-epigallocatechin at inhibiting release of arachidonic acid and its metabolites from HT29 cells [19]. In the same assay, EGCG 7-*O*-*β*-D-glucuronide was weaker than EGCG, although EGCG 3′-, 3″- and 4″-*O*-*β*-D-glucuronides were equivalent to EGCG [19]. The (−)-Epicatechin 3′-*O*-*β*-D-glucuronide and 4′-*O*-methyl (−)-epicatechin 3′-*O*-*β*-D-glucuronide have minimal activities on superoxide scavenging and inhibiting peroxyl radical-induced oxidation of human plasma and low-density protein. In contrast, (−)-epicatechin 7-*O*-*β*-glucuronide has considerable activities (Natsume *et al.* unpublished results). Pre-treatment of human aortic endothelial cells with (+)-catechin metabolites inhibited monocyte U937 cell adhesion to, and reactive oxygen species generation by, IL-1*β*-stimulated cells, whereas (+)-catechin had no effect. Hydrogen peroxide stimulated stress was inhibited by both (+)-catechin and metabolites [20].

Hesperetin

Hesperetin glucuronides were synthesized using rat liver UDP glucuronosyl transferase activity and a mixture of 5- and 7-*O*-*β*-D-glucuronides obtained after purification. Human foreskin derived fibroblasts (FEK4) did not take up the glucuronides, but did metabolise some of the added hesperetin into glucuronides. At 30 μM, the hesperetin glucuronides gave a 25% protection against UV-A-induced necrotic cells death, whereas hesperetin

had no effect [21]. Using *in vitro* mouse brain endothelial b. END5 and rat mouse brain endothelial RBE4 cell models, it was shown that hesperetin 5-*O*-*β*-D-glucuronide, hesperetin 7-*O*-*β*-D-glucuronide, naringenin 5-*O*-*β*-D-glucuronide, and naringenin 7-*O*-*β*-D-glucuronide poorly traverse the blood brain barrier, whereas the corresponding aglycones pass more freely [22].

Baicalin

Baicalin is baicalein 7-*O*-*β*-D-glucuronide, which is found in several Chinese herbs. Baicalein inhibited proliferation of DU145 cells, via apoptosis, but a high concentration (150 μM) was required to give 50% inhibition [23]. Baicalin at 50–200 μg/ml also induced apoptosis in various systems by acting as a pro-oxidant and inducing caspase-3 activation via a mitochondrial pathway [24], but had no effect on inducing apoptosis in PBMC and fibroblasts [25]. Shosaiko-to derived phenolic metabolites, including baicalin, induced apoptosis in human lung fibroblasts and peripheral lymphocytes *in vitro* [25].

Others

Morin is found in fruits and Chinese herbs. Morin sulfates and glucuronides at 1.5 μM gave 50% inhibition of NO production and anti-inflammatory activity on LPS-activated RAW 264.7 macrophages [26]. Some anti-inflammatory activity has been demonstrated for myricetin 3-*O*-*β*-D-glucuronide [27], but this study was unusual in that it gave glucuronides in the diet. There is some *β*-glucuronidase activity in the bile, but it is generally very low since it is inhibited by components of the bile itself such as bile salts (Ho *et al.* 1979). Most of the *β*-glucuronidase activity in the gut lumen originates from microflora. In addition, myricetin 3-*O*-*β*-D-glucuronide is not likely to be absorbed intact, but deconjugated in the lumen of the intestine followed by reconjugation in the enterocyte or liver; probably a certain proportion will be newly-synthesized myricetin 3-*O*-*β*-D-glucuronide. Myricetin is unstable under physiological conditions, and therefore the glucuronide conjugation may act to stabilise the myricetin, delivering it intact to the blood. There was a potent, marked and dose-dependent effect in acute and chronic models of inflammation in rats *in vivo* [27]. In addition, myricetin 3-*O*-*β*-D-glucuronide was a potent inhibitor of human platelet COX-1 with an IC_{50} of 0.5 μM [27].

Flavonoid glucosides

There have been several publications on the properties of flavonoid glucosides, especially quercetin glucosides (for example [28]). Most of the properties measured were free radical scavenging and antioxidant. However, with the exception of anthocyanins, it is now accepted that glucosides are not found in plasma [6,7,29–31].

Metabolism and entry into tissues and cells

Based on their partition coefficients (Table III) and molecular size, many flavonoid aglycones are sufficiently hydrophobic to diffuse through membranes. However, conjugation with a polar group decreases the lipid solubility, decreases the partition coefficient and decreases the likelihood of passage across cell membranes. In contrast, conjugation with methyl groups can increase the lipid solubility, although generally methylated flavonoids are also conjugated with glucuronic acid or sulfate groups.

For measurement of biological activities in cell free systems *in vitro*, the conjugate can be added to the assay system and its effect measured directly. For intact cell based *in vitro* assays, the experimental design becomes more complicated. If the conjugate present in the plasma cannot enter cells by passive diffusion (or at least only slowly), then for the conjugate to exert activity, one or more of the following must occur: (a) deconjugation outside of the cell followed by passive diffusion of the resulting aglycone; (b) interaction of the conjugate with a receptor on the cell surface eliciting a signalling response inside the cell; (c) transport of the conjugate into the cell; (d) facilitated diffusion into the cell via a concentration gradient generated by intracellular deconjugation or (e) a combination of one or more of the above.

The processes have not been extensively studied, but some information is available in the literature from cellular studies. Metabolism by HepG2 cells has given some insight into the process [32]. In these cells, which are a model for hepatic metabolism, quercetin 7-*O*-*β*-D-glucuronide and quercetin 3-*O*-*β*-D-glucuronide were both metabolised, either by an additional methylation, or by deconjugation followed by reconjugation. In marked contrast, quercetin 4′-*O*-*β*-D-glucuronide was not metabolised, even though the activity of isolated intracellular *β*-glucuronidase was comparable for all three conjugates [33]. The uptake into cells was at least partially active, since transport inhibitors decreased the rate of metabolism. The pattern of inhibition implied an organic anion transporter protein (OATP)-like transport.

In addition to intracellular deconjugation, *β*-glucuronidase is also present at sites of inflammation. For example, luteolin glucuronide is deconjugated by *β*-glucuronidase during inflammation [34,35]. Further, injection of LPS caused an increase in the concentration of *β*-glucuronidase leading to deglucuronidation of luteolin glucuronide. These studies imply that glucuronidation does not always equal inactivation, and in fact glucuronidation may even stabilise the flavonoid in the biological milieu. For example, at pH 7.4, quercetin aglycone breaks down totally in less than one hour, but

Table III. Partition coefficients of some flavonoid conjugates compared to ascorbate, lutein and aglycones.

	Partition coefficient (logP *n*-octanol:Water/buffer)	Conditions	Reference
L-ascorbic acid (protonated)	−1.64	Experimental	[74]
	−3.98	Calculated	[74]
Quercetin 3-*O*-*β*-D-glucuronide	−2.1	Experimental, pH 7.4	[75]
Quercetin	1.23	Experimental, pH 7.4	[28]
	1.82	Experimental	[76]
Quercetin 3-*O*-*β*-D-glucoside (isoquercitrin)	−0.1	Calculated	[74]
	0.7	Experimental, pH 7.4	[75]
Quercetin 4′-*O*-*β*-D-glucoside	0.43	Calculated	[74]
	1.32	Experimental	[75]
Quercetin 3,4′-di-*O*-*β*-D-glucoside	−1.34	Experimental	[75]
Tamarixetin	2.25	Calculated	Biobyte CogP[2]
Isorhamnetin	2.25	Calculated	Biobyte CogP[2]
Quercetin 3′-*O*-sulfate	0.35	Calculated	Biobyte CogP[2]
Quercetin 4′-*O*-*β*-D-glucuronide	−1.82	Experimental	(J. Terao, unpublished data)
Isorhamnetin 3-*O*-*β*-D-glucuronide	−0.39	Calculated	Biobyte CogP[2]
Rutin (quercetin 3-*O*-*β*-D-rutinoside)	1.3	Experimental, pH 7.4	[77]
	−1.11	Calculated	[74]
Hesperetin	2.44	Calculated	[21]
Hesperetin 5-*O*-*β*-D-glucuronide	−0.01	Calculated	[21]
Hesperetin 7-*O*-*β*-D-glucuronide	0.77	Calculated	[21]
Lutein	14.8	Calculated	[74]

quercetin 3-*O*-*β*-D-glucuronide is much more stable [11]. Some cytokines such as IL-1α (or *β*) and TNFα increase vascular permeability during inflammation. Incubation of human aortic endothelial cells with IL-1α resulted in increased permeability to quercetin 3-*O*-*β*-D-glucuronide. Quercetin conjugates might pass through the endothelium to act on vascular smooth muscle cells during inflammation [36].

Obtaining the conjugates for biological activity estimation

One of the problems in this area of research is obtaining the relevant conjugates and metabolites, even after they have been identified. Several methods have been reported to make conjugates, involving (a) isolation from plants (b) isolation from blood after consumption of the flavonoid (c) chemical synthesis (d) enzymatic synthesis and (e) microbiological transformations. Some of these are addressed below.

Isolation from plants

Methylated derivatives, flavonoid glucuronides and sulfates are all plant products, and their natural occurrence has been regularly reviewed [37–41]. Most mono-methylated derivatives of quercetin (5 = azaleatin; 7 = rhamnetin; 3′ = isorhamnetin; 4′ = tamarixetin; 3-*O*-methyl quercetin), daidzein (4′ = formononetin), genistein (4′ = biochaninA) and naringenin (7 = sakuranetin; 4′ = isosakuranetin) are commercially available and their isolation from plant sources is of little interest. Flavonoid glucuronides are indeed plant products, but their natural structural diversity is quite limited as compared to the wide range of flavonoid glucuronides resulting from mammalian or human metabolism. The natural occurrence of quercetin 4′- and 7-*O*-*β*-D-glucuronides in plants is scarce, whereas the major form of quercetin glucuronide in plants is represented by its 3-*O*-*β*-D-glucuronide, miquelianin (Table IV). Of special interest is the presence of quercetin 3-*O*-*β*-D-glucuronides in edible plants such as strawberry [42], raspberry [43], lettuce [44], blueberry [45] and *Theobroma grandifolium* (Cupuaçu) [46]. In strawberry [42], raspberry [43], some medicinal plants like *Arnica Montana* [47] and other genus's such as *Populus* [48], *Epilobium* [49] or *Tamarix* [50], quercetin 3-*O*-*β*-D-glucuronide coexists with kaempferol 3-*O*-*β*-D-glucuronide. Lettuce accumulates quercetin 3-*O*-*β*-D-glucuronide whereas endives are rich in kaempferol glucuronide [44]. In most plants, quercetin glucuronides are constituents of complex mixtures of polar flavonoids. In a few cases such as in *Arnica* spp. [47,51] or in *Potentilla anserina* [52], quercetin 3-*O*-*β*-D-glucuronide represents the major constituent of the polar flavonoid fraction. However, its preparative isolation from plants, as a source of standard compound, is still of limited application and only one patent has recently described the preparation of quercetin 3-*O*-*β*-D-glucuronide from the leaves of *Rumex aquaticus* [53].

Flavonoid sulfates are also of limited distribution in plants and their natural distribution has been specifically reviewed [54,55]. Their structural variation is wider than that of flavonoid glucuronides and although flavonol sulfation at position 3 is very

Table IV. Selected examples of the occurrence of quercetin glucuronides in plants.

Position of conjugation	Plant species	Organ	Ref
4′	*Psidium guaijava*	Leaves	[78]
7	*Tanacetum spp*	Disk florets	[79]
3	*Lasthenia conjugens*	Whole plant	[80]
3	Strawberry	Fruits	[42]
3	Raspberry	Fruits	[43]
3	Lettuce	Whole plant	[44]
3	*Gutierrezia wrightii*	Leaves & stems	[81]
3	*Arnica montana*	Flowers	[47]
3	*Populus tremuloides*	Leaves	[48]
3	*Alnus sieboldiana*	Leaves	[82]
3	*Heterotheca inuloides*	Flowers	[83]
3	*Arnica longifolia*	Flowers	[51]
3	*Potentilla anserine*	Aerial parts	[52]
3	*Jasonia glutinosa*	Aerial parts	[84]
3	Blueberry	Leaves	[45]
3	*Salvia blepharophylla*	Leaves	[85]
3	*Epilobium hirsutum*	Whole plant	[49]
3	*Tamarix amplexicaulis*	Flowers	[50]
3	*Anethum graveolens*	Aerial parts	[86]
3	*Hypericum perforatum*	Aerial parts	[87]
3	*Theobroma grandiflorum*	Seeds	[46]
3	*Polygonum hydropiper*	Leaves	[88]

common, a number of poly-sulfated flavonoids with sulfation at additional positions are known. Furthermore, sulfated flavonoid glycosides are known as well, the sulfate being either attached to a different phenolic position than the sugar, or directly on the sugar ring. Despite this, plant sulfated flavonoids may differ appreciably from those derived from mammalian metabolism since in the latter case, only mono-sulfated conjugates have been detected so far, and when identified, the conjugates do not carry a 3-sulfate. Furthermore we have considerable evidence that mixed sulfated and glucuronidated flavonoids are derived from metabolism, but in some cases it is still unclear if the sulfate is directly attached to the flavonoid, or on the sugar unit. Flavonoid sulfates are often present in plants as complex mixtures that can be difficult to purify on a preparative scale, due to the poor stability of the sulfate ester linkage and the limited number of suitable non-degrading chromatographic techniques (for a review on the chemistry of flavonoid sulfates, see [55]). For those reasons, isolation of sulfated standards from plant sources can be problematic and the evident lack of reference compounds still limit our knowledge of the sulfation of polyphenols during metabolism.

Other flavonoid metabolites such as methyl ethers, sulfate esters or glucuronides of (+)-catechin, (−)-epicatechin, (−)-epicatechin gallate and (−)-epigallocatechin gallate are not known as plant products. Chemical or enzymatic syntheses represent their sole possible source.

Chemical synthesis

Compared to isolation from plants, the chemical synthesis of flavonoid conjugates offers access to a much larger diversity of compounds. In addition, this is of course the method of choice when the preparation of radiolabelled or stable-isotope-labelled compounds is required.

For a complete overview of the methods that have been developed for the synthesis of flavonoid conjugates, the reader is invited to consult with our recent review on the subject [56]. Thus, only significant papers that have been published after this review are considered here. Two problems are usually encountered in the synthesis of flavonoid sulfates: (i) the incomplete regioselectivity of the sulfation reaction, which implies difficult separation of the products, often associated with appreciable loss of sulfated compound; and (ii) the difficulty of increasing the regioselectivity by the use of protective groups that are compatible with the stability of sulfate esters. An elegant approach in the regioselective synthesis of daidzein sulfates has been recently published [57], making use of the *tert*-butyldimethylsilyl protective group and of chlorosulfonic acid/pyridine as sulfating agent. On the other hand, triethylamine-sulfur trioxide complex in dimethylacetamide has been recently used in the preparation of catechin and (−)-epicatechin sulfates [58]. However, only the fully penta-sulfated esters were synthesized and this reagent has not been applied yet to the preparation of mono-sulfated derivatives.

Enzymatic synthesis and microbiological transformations

Enzymic synthesis using UDP-glucuronosyl transferases, either purified or from cell-free extracts, have been performed to produce glucuronides of several flavonoids, for example quercetin [12], hesperetin [21], EGCG [19] and catechin [59]. These methods are less specific than chemical synthesis and give a mixture of mono-glucuronides. Sulfation of (+)-catechin has been carried out using a sulfotransferase purified from the human intestinal bacterium *Eubacterium* A-44 [60]. The (+)-Catechin 4′-*O*-sulfate and 4′,5-di-*O*-sulfate were the products of this reaction. Subsequent application of the same enzymatic reaction to (−)-epigallocatechin gallate [61] resulted in the formation of (−)-epigallocatechin gallate 4′-*O*-sulfate. Finally, fermentation of naringenin with the fungus *Cunninghamella elegans* yielded naringenin 7-*O*-sulfate [62].

Conclusion and summary

For flavonoids, conjugation has an impact on the biological activity, which is dependent on the system used for investigation. This has been known for some

time *in vitro*; for example, the importance of an unhindered B ring catechol group, and the presence of other structures (2,3-double bonds and 3-hydroxy structure), for antioxidant activity and redox properties, is well documented [63,64]. Based on the available information, structure-function relationships are difficult to decipher at the moment. However, for biological effects in general, it appears that the position of conjugation is more important that the nature of the conjugation in the majority of studies. For inhibition of xanthine oxidase and of lipoxygenase, the presence of the 5,7-dihydroxy structure in the A ring is important. One of the most important effects of conjugation is the change in partition coefficient, which converts the aglycone from a molecule with potential to diffuse passively across membranes, to a conjugate that requires transport across membranes. Thus a cell with a high conjugating potential must also have a high exporting activity in order to avoid build up of the flavonoid. Apparent *in vitro* toxicity can also be confounded by artefactual oxidation, including for example the generation of hydrogen peroxide [104,105]. This *in vitro* phenomenon could be markedly different for aglycones compared to conjugates. When interpreting results from *in vitro* experiments, it is certainly necessary to consider the flavonoid concentration, metabolism and conjugation *in vivo*. Failure to do so can give misleading results and certainly over-emphasise the real biological activities occurring *in vivo*. This applies equally to reported proposed beneficial effects and to putative toxicity studies.

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