Inhibition of hydroxycinnamic acid sulfation by flavonoids and their conjugated metabolites

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Abstract.

Hydroxycinnamic acids and flavonoids are dietary phenolic antioxidants that are abundant in our diet. Hydroxycinnamic acids are highly sulfated *in vivo*, and sulfotransferases (SULTs), in particular SULT1A1, play a major role in their metabolism. Flavonoids are potent inhibitors of human SULTs. In this study, the potential metabolic interaction between dietary hydroxycinnamic acids and flavonoids was investigated. Flavonoids, such as luteolin, quercetin, daidzein and genistein, are identified as potent inhibitors of hydroxycinnamic acid sulfation in human liver S9 homogenate with IC_{50} values less than 1 μM. The inhibitory activity was less potent in the human intestinal S9 homogenate. We also demonstrate that quercetin conjugates found *in vivo* (quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide, and quercetin-3′-*O*-sulfate) moderately inhibited the sulfation of hydroxycinnamic acids in human liver S9. In an intact cellular system, human HepG2 cells, caffeic acid and ferulic acid sulfation was inhibited by luteolin and quercetin (IC_{50}: 1.6-3.9 μM). Quercetin-3′-*O*-sulfate weakly inhibited sulfation. Quercetin glucuronides, limited by their low cellular uptake, were ineffective. These data suggest that the inhibition of SULTs by flavonoids and *in vivo* flavonoid conjugates may modify the bioavailability of dietary hydroxycinnamic acids by suppressing their conversion to sulfated metabolites.

Keywords: hydroxycinnamic acids, flavonoids, flavonoid conjugates, sulfation

Running title: Flavonoids inhibit hydroxycinnamic acid sulfation

1. Introduction

In humans, the first-pass metabolism of hydroxycinnamic acids plays a major role in limiting their bioavailability. Human studies and *in vitro* mechanistic studies showed that hydroxycinnamic acids are predominantly sulfated in the intestine and liver [1-6]. Characterization with recombinant human SULTs demonstrated that SULT1A1 and SULT1E1 are the major isoforms involved in the sulfation of hydroxycinnamic acids [4]. SULT1A1, highly expressed in both human liver and intestine, is the most active in the sulfation of all the major hydroxycinnamic acids found in the diet. SULT1E1 catalyzes the sulfation of the 4-hydroxyl groups of ferulic and dihydroferulic acids. Therefore, inhibition of SULTs may be a possible strategy to increase the proportion of unconjugated hydroxycinnamic acids, which, in turn, could lead to a modified or even enhanced bioefficacy [7].

Flavonoids are natural inhibitors of SULTs. Quercetin, the most abundant flavonol in foods, is a potent inhibitor of SULT1A1 with IC_{50} value of <100 nM [8-10]. Flavonoids from different subgroups including flavones, flavonols, flavanone and isoflavones also show highly effective inhibition of SULTs, especially SULT1A1. Given the importance of SULT1A1 in the sulfation of hydroxycinnamic acids, the concomitant consumption of flavonoids and hydroxycinnamic acids may inhibit the metabolism of the latter. In particular, human intestinal SULTs may be inhibited by dietary doses of flavonoids, since the local concentrations of flavonoids in the gut is much higher than that in plasma [11]. Human liver is another important

site of hydroxycinnamic acid sulfation. However, flavonoids are extensively metabolized *in vivo* into conjugates that are present in much lower concentrations in the circulation (<10 μM). It is thus critical to evaluate the inhibitory effect, if any, of the biologically relevant flavonoid conjugates on the sulfation of hydroxycinnamic acids.

In this study, we investigated the inhibitory effects of flavonoids and their conjugates on the sulfation of five major dietary hydroxycinnamic acids (caffeic acid, dihydrocaffeic acid, dihydroferulic acid, ferulic acid and isoferulic acids) using human intestine and liver homogenates. Furthermore, we examined the inhibitory effect of luteolin, quercetin and quercetin conjugates (gluucronides and sulfate) on hydroxycinnamic acid sulfation in the human hepatoma cell line, HepG2, as a model for human liver metabolism.

2. Methods and materials

2.1. Chemicals

Caffeic acid, ferulic acid, isoferulic acid, quercetin, (+)-catechin and (-)-epicatechin were purchased from Sigma-Aldrich (St. Louis, MO). Apigenin, daidzein, eriodictyol, genistein, hesperetin, kaempferol, isorhamnetin, luteolin and phloretin were obtained from Extrasynthèse, (Genay, France). Dihydrocaffeic acid and dihydroferulic acid were purchased

from Alfa Aesar (Lancashire, UK). The conjugates caffeic acid-3-*O*-glucuronide, caffeic acid-4-*O*-glucuronide, caffeic acid-3-*O*-sulfate, caffeic acid-4-*O*-sulfate, dihydroferulic acid-4-*O*-sulfate, ferulic acid-4-*O*-glucuronide, ferulic acid-4-*O*-sulfate, isoferulic acid-3-*O*-glucuronide, isoferulic acid-3-*O*-sulfate were synthesized as described elsewhere [12] and kindly provided by Prof Denis Barron, Nestle Research Center, Lausanne, Switzerland. Quercetin-7-*O*-glucuronide and quercetin-3-*O*-glucuronide were synthesized enzymatically and purified by HPLC [13, 14]; quercetin-3′-*O*-sulfate was chemically synthesized as described [15, 16]. Identity of the compounds was further confirmed by comparing the retention time and absorption spectra of the authentic standards kindly provided by Dr. Paul Kroon and Dr Paul Needs. The purities were checked by HPLC to be over 95 %. 3'-phosphoadenosine-5'-phosphosulfate (PAPS) was from MP Biochemicals (London, UK). Pooled Liver S9 homogenate was purchased from Sigma-Aldrich, while pooled intestinal (duodenum/jejunum) S9 homogenate was from Xenotech (Lenexa, Kansas). All other chemicals, unless otherwise stated, were purchased from Sigma-Aldrich.

2.2. Inhibition of hydroxycinnamic acid sulfation by flavonoids in human liver and intestinal S9

The incubation mixture, in a final volume of 50 µL, consisted of 100 mM potassium phosphate buffer (pH 7.4), with 100 µM vitamin C, 100 µM PAPS and 1 mM DTT. Human

liver S9 and intestinal S9 homogenates were used at 1 mg/mL and 0.4 mg/mL, respectively. The flavonoids were added from a 5 mM stock solution dissolved in DMSO, with final DMSO concentration equalized to 0.2 %. Quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide and quercetin-3′-*O*-sulfate were dissolved in water. After a 15 min pre-incubation period, the reaction was initiated by adding 10 µM cinnamic acids and 25 µM dihydrocinnamic acids from a 50 mM stock solution in DMSO. To inhibit hydrolysis of quercetin glucuronides, 5 mM saccharolactone was added. After 30 min incubation in a 37 ^{0}C water bath, the reaction was stopped by the addition of 10 µL ice-cold acetonitrile containing 500 mM HCl. Controls were treated under identical conditions and consisted of samples with 0.2 % DMSO (final concentration) added to the buffer. Samples were stored at -70 ^{0}C until analysis. Due to the co-elution of dihydrocaffeic acid sulfates with unknown components in the S9 homogenates, an extraction procedure was necessary only for samples from dihydrocaffeic acid. After stopping the reaction, the buffer was evaporated *in vacuo*. Residue was extracted first with 60 µL of methanol, and then with 60 µL of acetonitrile. The supernatants of the extracts were combined and evaporated to dryness. Finally, the extracted material was re-suspended in 60 µL of the initial mobile phase and analyzed by HPLC. Extraction efficiency was 92 ± 4 % for dihydrocaffeic acid conjugates (*n*=3).

2.3. Hydrolysis of flavonoids conjugates in human liver S9

Quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide, and quercetin-3′-*O*-sulfate, at 20 µM, were added to 100 mM potassium phosphate buffer (pH 7.4), with 100 µM vitamin C, 1 mM DTT and 1 mg/mL liver S9 homogenates, in a final volume of 50 µL. After 30 min incubation at 37 ^{0}C, the reaction was stopped by the addition of 10 µL ice-cold acetonitrile containing 500 mM HCl.

2.5. HepG2 cell culture and metabolism

HepG2 cells (ATCC) were routinely cultured in 75 cm^2 cell culture flasks (Corning Costar Corp., Cambridge, MA) at 37 ^{0}C under a humidified 5 % CO_2 / O_2 atmosphere. The culture media consisted of Eagle's Minimum Essential Medium media (ATCC) supplemented with 10% fetal bovine serum and 100 U/ml penicillin- streptomycin. All experiments were performed with HepG2 cells between passages 80 to 95. For metabolic studies, HepG2 cells were seeded into 12-well plates (Corning Costar Corp., Cambridge, MA) at a cell density of 2 x 10^5 per well. The cell monolayers were allowed to grow over 96 h before they were used for experiments. Hydroxycinnamic acid metabolism experiments were carried out in serum-free media with 100 µM vitamin C and 1.8 mM $CaCl_2$, adjusted to pH 7. Caffeic acid and ferulic acid (10 µM) were added from a 50 mM stock solution in DMSO. Quercetin and luteolin (5 mM) were also dissolved in DMSO and added to the media to give a final DMSO concentration of 0.25 %. 0.4 mL of hydroxycinnamic acids, with or without the inhibitors, were added to the

HepG2 cells and incubated for 4 h at 37 ^{0}C. The incubation media were then collected, acidified with 1 mM vitamin C and dried under vacuum. The residue was extracted by sonication for 5 min and vortex for 1 min, first with 500 µL acetonitrile, followed by 500 µL methanol. The extracts were combined and evaporated under vacuum. Prior to HPLC analysis, the dried residue was re-dissolved in 100 µL of initial mobile phase.

2.6. Uptake of quercetin metabolites into HepG2 cells.

For the uptake study, HepG2 cells were seeded into 12-well plates (Corning Costar Corp., Cambridge, MA) at a cell density of 2 x 10^5 per well. The cell monolayers were allowed to grow over 96 h before they were used for uptake experiments. Uptake experiments were carried out using serum-free media with 100 µM vitamin C and 1.8 mM $CaCl_2$, adjusted to pH 7. After removal of media, the cells were washed twice with 0.4 mL transport buffer and incubated for 10 min. Preincubation buffer was aspirated and replaced with 0.4 mL transport buffer containing quercetin metabolites. After 4 h, 2 mL ice-cold transport buffer containing 0.2 % bovine serum albumin (BSA) was added. This was quickly aspirated and further washed t

wice with 0.5 mL ice-cold transport buffer with 0.2 % BSA. Finally, the cells were rinsed with 0.5 mL ice-cold transport buffer without BSA. Cells were collected with 0.4 mL 50 % methanol and stored at -80 ^{0}C. Extraction was performed by sonication for 5 min followed by the addition of 1 mL of ice-cold acetone. The samples were placed in a -20 ^{0}C freezer for 1 h and centrifuged at 17,000 g for 5 min. The supernatant was collected and evaporated to dryness *in vacuo* at 30 ^{0}C and stored at -20 ^{0}C until analysis. The protein pellet was re-dissolved in 0.1 N NaOH and the protein contents were determined by the Bradford assay. All the uptake values were corrected against protein content.

2.7. HPLC methodology

HPLC analyses were carried out using the Agilent 1200 series liquid chromatography system. For the analysis of caffeic acid, ferulic acid, isoferulic acid, dihydroferulic acid and their conjugates, chromatography was performed with a Zorbax XDB-C18 column (4.6 x 150 mm, 5 μm). The mobile phase consisted of 20 mM ammonium formate, pH 2.8 (A) and methanol (B). For the analysis of caffeic acid and conjugates, samples were eluted at 1 mL/min with 5 % to 25 % B in 20 min, followed by 80 % B in 2 min and back to 5 % B for 3 min. For the analysis of ferulic and isoferulic acid and their conjugates, the gradient was from 10 % to 20 % B in 10 min, to 60% B in 15 min, then set at 80 % B for 2 min and back to 10 % B for 3 min, at 1 mL/min. Dihydroferulic acid and conjugate analysis was carried out at 1 mL/min, from 10

% to 20 % B in 15 min, to 60 % B in 10 min, up to 80 % B for 2 min and finally to 10 % B for 3 min. For dihydrocaffeic acid and conjugates, the analyses were performed with a Zorbax XDB-C18 column (4.6 x 50 mm, 1.8 μm) with 20 mM ammonium formate, pH 4.5 (A) and methanol (B) as the mobile phase. The gradient started at 3 % (B) kept for 15 min, followed by an increase to 40 % B in 5 min, and then returned to 3 % B for 5 min. Samples were centrifuged and 25 μL of the supernatant were injected into the column. UV-detection carried out at 280 nm and 310 nm using a photodiode array detector. Caffeic, ferulic and isoferulic acids were quantified at 310 nm, dihydroferulic acid and dihydrocaffeic acid at 280 nm. The on-column limits of quantification of this HPLC method for the quantification of caffeic acid, ferulic acid and isoferulic acid were ~1 pmol, and ~5 pmol for dihydrocaffeic acid and dihydroferulic acid. All conjugates were positively identified by comparing the retention times and UV absorption spectra with those of the authentic synthetic standards. Conjugates were quantified using calibration curves of the authentic standards of glucuronides and sulfates. Standard curves were linear over the range loaded (0.1 to 2 nmol, $R^2 > 0.99$). Slope of peak area (AU) / amount (nmol): caffeic acid-3-*O*-sulfate: 1120; caffeic acid-4-*O*-sulfate: 512; dihydrocaffeic acid-3-*O*-sulfate: 113; dihydroferulic acid-4-*O*-sulfate: 157; ferulic acid-4-*O*-sulfate: 922; isoferulic acid-3-*O*-sulfate: 872 (corrected for purity). For quercetin conjugates, analyses were performed with a Zorbax XDB-C18 column (4.6 x 50 mm, 1.8 μm) with 20 mM ammonium formate, pH 4.5 (A) and methanol (B) as the mobile phase. Elution was performed at 1 mL/min

and the gradient started at 30 % (B) and increased linearly to 50 % in 10 min, and equilibrated at 30 % for 2.5 min. The limit of detection for the quantification of quercetin was 0.02 µM. Quantification was based on the peak area at 370 nm.

2.8. Data analysis

Data are shown as mean ± S.D. Statistical differences were determined using analysis of variance using the student's t-test. Differences were considered significant when $p<0.05$.

3. Results

3.1. Inhibition of hydroxycinnamic acid sulfation by flavonoids in human intestinal S9

The inhibitory effect of 11 flavonoids on hydroxycinnamic acid sulfation was evaluated using human intestinal S9; caffeic acid and ferulic acid were chosen as representative substrates for SULT1A1 and SULT1E1, respectively. It was found that flavonoids inhibited the sulfation of caffeic acid and ferulic acid in a concentration-dependant manner. At 10 µM, all flavonoids, with the exception of epicatechin, inhibited sulfation of both caffeic acid and ferulic acid by >50 %. IC_{50} values are shown in Table 1. Sulfation of caffeic acid appeared to be more susceptible to inhibition, as indicated by lower IC_{50} values. Isoflavones genistein and

daidzein were the most potent inhibitors in the human intestinal S9, and they inhibited the sulfation of caffeic acid and ferulic acid with IC_{50} values of < 1 μM and 1.5-2.3 μM, respectively. Other good inhibitors included the flavones apigenin and luteolin, as well as phloretin, but for the flavanols, catechin and epicatechin, the inhibitory effect was relatively moderate. The inhibitory effect of isoflavones genistein and daidzein on dihydrocaffeic acid, dihydroferulic acid and isoferulic acid sulfation was further examined (Figure 1). Isoflavones were found to be potent inhibitors of caffeic and dihydrocaffeic acid sulfation, while they are less effective in inhibiting sulfation of methylated forms, in particular dihydroferulic acid.

3.2. Inhibition of hydroxycinnamic acid sulfation by flavonoids in human liver S9

Sulfation of caffeic acid and ferulic acid in liver S9 was more sensitive to inhibition by flavonoids. Inhibitory activities of the flavonoids are shown in Table 2. The most potent inhibitors were luteolin and quercetin. Luteolin, in particular, was found to strongly inhibit caffeic acid sulfation with IC_{50} = 0.08 μM. Quercetin, on the other hand, inhibited sulfation of caffeic acid and ferulic acid with IC_{50} values of 0.41 and 0.64 μM, respectively. Isoflavones genistein and daidzein were also potent inhibitors (IC_{50} < 1 μM), while catechin, epicatechin and hesperetin were weak inhibitors. The inhibitory activity of luteolin and quercetin was further evaluated with isoferulic acid, dihydrocaffeic acid and dihydroferulic acid (Figure 2). Luteolin was a highly effective sulfation inhibitor for all the hydroxycinnamic acids, with > 50

% inhibition at 1 μM. Sulfation of dihydrocaffeic acid and dihydroferulic acid were less strongly inhibited compared to their cinnamic acid counterparts.

3.3. Inhibition of hydroxycinnamic acid by quercetin conjugates in liver S9

Next, we evaluated the inhibitory effect of *in vivo* quercetin conjugates, quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide and quercetin-3′-*O*-sulfate, towards the sulfation of caffeic acid and ferulic acid in human liver S9. These conjugates significantly inhibited sulfation of caffeic acid and ferulic acid, albeit with IC_{50} values 1-2 orders of magnitude higher than that of the quercetin aglycone (Table 3). The overall potency of inhibition was in the order of quercetin-3′-*O*-sulfate > quercetin-7-*O*-glucuronide > quercetin-3-*O*-glucuronide. The human liver contained β-glucuronidase and sulfatase that may hydrolyze conjugates to quercetin, which in turn inhibits SULTs. We examined the hydrolysis of quercetin conjugates (20 μM) in human liver S9 homogenates and found that quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide were hydrolyzed significantly (27.2 and 26.1 pmol / min per mg, respectively), whereas the hydrolysis of quercetin-3′-*O*-sulfate was slower (9.0 pmol / min per mg). To evaluate the potential role of hydrolysis by β-glucuronidase in the sulfation inhibitory activity of quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide, they were incubated with in the presence of saccharolactone, a β-glucuronidase inhibitor. HPLC analysis showed that saccharolactone completely inhibited

glucuronide hydrolysis. Inhibition of hydrolysis resulted in a marked reduction of the ability of quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide (20 µM) to inhibit the sulfation of caffeic acid (Figure 3). In particular, the inhibitory activity of quercetin-3-*O*-glucuronide was reduced from > 90% to only 20 %, and less than 50 % inhibition was observed at 50 µM. Quercetin-7-*O*-glucuronide maintained over 50 % inhibition at 20 µM, with IC_{50} value of 17.6 µM. Hydrolysis of glucuronide conjugates thus plays an important role in their sulfation inhibitory activity.

3.4. Metabolism of hydroxycinnamic acids in HepG2 cells and inhibition of sulfation by quercetin and conjugates

Although flavonoids and their conjugates inhibited sulfotransferase activity in the cell-free preparations *in vitro*, their inhibition in intact cells may be limited by their access to the enzyme [8]. Thus, the inhibitory activity of flavonoids was further examined in human HepG2 cells. We first investigated the nature of metabolites of hydroxycinnamic acids produced by HepG2 cells. Caffeic and ferulic acids (10 µM) were separately incubated with the HepG2 cells for 4 h, and the media was analyzed by HPLC-UV. Sulfated metabolites were the major metabolites identified, while glucuronides were not formed to a significant extent. This is in good agreement with the metabolic profile found in *in vitro* incubations with human liver S9. For caffeic acid, we found that there was also significant methylation to form ferulic acid.

Next, the effect of flavonoids and their conjugates on hydroxycinnamic acid metabolism by HepG2 cells was investigated.

As shown in Figure 4, quercetin and luteolin were potent inhibitors of sulfation in the HepG2 cells. Luteolin and quercetin significantly inhibited caffeic acid sulfation at 1 µM, with IC_{50} values of 1.6 and 1.9 µM respectively; they also inhibited ferulic acid sulfation with IC_{50} values of 3.9 and 3.6 µM, respectively. On the other hand, the tested quercetin metabolites were weak inhibitors of sulfation. Among the quercetin conjugates, only quercetin-3′-*O*-sulfate showed significant inhibition of sulfation with IC_{50} value of ~20 µM. Quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide did not significantly inhibit sulfation even at 40 µM. Since quercetin-3-*O*-glucuronide, quercetin-7-*O*-glucuronide and quercetin-3′-*O*-sulfate all inhibited sulfation in liver S9 incubations, we propose that it is the uptake of quercetin conjugates that limits their inhibitory activity. Indeed, we found that the uptake of quercetin-3′-*O*-sulfate was 8-fold greater than quercetin-3-*O*-glucuronide and quercetin-7-*O*-glucuronide in HepG2 cells (Figure 3). While quercetin-3-O-glucuonide and quercetin-7-O-glucuronide can be hydrolyzed, and re-conjugated in HepG2 cells to form quercetin-3′-*O*-sulfate [22], the rate of conversion is very low (<10 % after 48 h). Thus, the contribution of formed quercetin-3′-*O*-sulfate to cellular uptake and SULT-inhibitory effect of quercetin glucuronides is expected to be minimal over 4 h.

4. Discussion

SULTs are important determinants of the bioavailability and bioactivity of hydroxycinnamic acids in humans. On the other hand, dietary flavonoids are natural inhibitors of SULTs. Here, using human S9 homogenates, recombinant human SULT1A1 and HepG2 cells, we demonstrated that flavonoids are potent inhibitors of hydroxycinnamic acid sulfation.

In the intestinal S9 homogenates, all the flavonoids tested significantly inhibited sulfation of hydroxycinnamic acids, with the isoflavones daidzein and genistein being the most potent inhibitors (Table 1). In the human small intestine, SULT1A1 and SULT1A3 are highly expressed and they are both highly active in the conjugation of hydroxycinnamic acids [4]. Of the two isozymes, SULT1A3 activity is known to be relatively resistant to inhibition by flavonoids [17]. Hence, SULT1A3-mediated sulfation may explain the reduced inhibitory effect of flavonoids in the intestinal S9 compared to the liver S9.

Nevertheless, local concentrations of flavonoids in the gut lumen are potentially much higher than that in the human plasma, and thus dietary flavonoids may exert inhibitory activity to intestinal SULTs without being absorbed into circulation. It has been estimated that 500 mg of polyphenols diluted in the digestive bolus would result in concentrations up to 3 mM in the lumen. Daily consumption of total flavonoids varies between individuals, but can easily reach more than 1 g [11]. Factoring in the significant transfer of different flavonoids (40-60 %) into the intestinal wall as shown by several ileostomy studies [18-20], dietary intake of flavonoids

will result in levels in the intestinal epithelial cells sufficiently high to potently inhibit both SULT1A1 and SULT1A3 activity. In our previous study, we showed that intestinal SULTs contribute, at least in part, to sulfation of hydroxycinnamic acids *in vivo* [4] and it may represent a significant site of metabolic interaction between dietary flavonoids and hydroxycinnamic acids.

The liver is an important site of hydroxycinnamic acid metabolism in humans. *In vitro* incubations with liver S9 (Table 2) homogenates showed that human liver SULTs were more susceptible to inhibition compared to the intestine, with IC_{50} values in nanomolar range for flavones (luteolin, apigenin), flavonols (quercetin) and isoflavones (daidzein and genistein). This is in good agreement with several studies which showed flavonoids are potent inhibitors of SULTs in human liver homogenates, in particular SULT1A1 [8, 17]. Strong inhibition of caffeic acid and ferulic acid sulfation was also observed with both luteolin and quercetin in HepG2 cells, albeit with inhibitory IC_{50} values in low-micromolar range (4 to 20-fold higher than that in liver S9). Flavonoid aglycones are thus promising inhibitors of hydroxycinnamic acid sulfation in the human liver.

However, circulating levels of flavonoids are much lower than that in the gut, reaching a maximum of 4 μM in the plasma depending on the dose and the type of flavonoids ingested [21, 11]. Moreover, flavonoids such as quercetin are highly metabolized in the intestine and are conjugated before reaching the liver [22]. Here, we demonstrate that glucuronide and sulfate

conjugates of quercetin also significant inhibited sulfation in human liver S9, with IC_{50} values in the low micromolar range. In HepG2 cells, quercetin-3′-*O*-sulfate showed inhibitory activity, while the glucuronides had no effect (Figure 4). Quercetin conjugates may act as direct inhibitors of SULTs. Quercetin glucuronides and sulfates can also be hydrolyzed by β-glucuronidase and sulfatase, respectively, to release the highly potent inhibitor quercetin, which in turn significantly inhibits SULTs-mediated sulfation of hydroxycinnamic acids. However, given the low sulfatase activity in HepG2 cells [22], it is likely that quercetin -3′-*O*-sulfate act predominantly as a direct inhibitor of SULTs in these cells.

]The lack of inhibitory activity of quercetin glucuronides could be attributed to their low cellular uptake compared to quercetin-3′-*O*-sulfate (Figure 3). Of note, the human liver express an array of uptake transporters that are repressed in the HepG2 cells lines, which may contribute to an enhanced accumulation of flavonoid conjugates in liver [23]. Hence, the inhibitory activities of these quercetin conjugates could be greater in the liver *in vivo.* The elimination half lives of quercetin metabolites in human studies were in the range of 10-20 hours. The relatively long half-lives of the quercetin conjugates imply that consumption of quercetin-rich foods can potentially inhibit hydroxycinnamic acid metabolism *in vivo* over a significant period of time (~1 day) [24].

This study highlights the potential of flavonoids in modulating the metabolism of hydroxycinnamic acids via the inhibition of SULTs (Figure 5). In humans, hydroxycinnamic

acids are highly sulfated by SULTs, resulting in limited bioavailability of free acids in the plasma. Sulfation may also impact antioxidant activity. Through potent inhibition of SULTs, flavonoids and their *in vivo* conjugates have the potential to enhance the bioavailability and bioefficacy of hydroxycinnamic acids. Further *in vivo* studies will be needed to demonstrate the metabolic interaction between flavonoids and hydroxycinnamic acids in humans.

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References

[1] Poquet, L., Clifford, M. N., Williamson, G. (2008) Transport and metabolism of ferulic acid through the colonic epithelium. *Drug Metab. Dispos.* **36**, 190-197.

[2] Poquet, L., Clifford, M. N., Williamson, G. (2008) Investigation of the metabolic fate of dihydrocaffeic acid. *Biochem. Pharmacol.* **75**, 1218-1229.

[3] Stalmach, A., Mullen, W., Barron, D., Uchida, K., Yokota, T., Cavin, C., Steiling, H., Williamson, G., Crozier, A. (2009) Metabolite profiling of hydroxycinnamate derivatives in plasma and urine after the ingestion of coffee by humans: identification of biomarkers of coffee consumption. *Drug Metab. Dispos.* **37**, 1749-1758.

[4] Wong, C. C., Meinl, W., Glatt, H. R., Barron, D., Stalmach, A., Steiling, H., Crozier, A., Williamson, G. (2009) In vitro and in vivo conjugation of dietary hydroxycinnamic acids by UDP-glucuronosyltransferases and sulfotransferases in humans. *J. Nutr. Biochem.* **21**, 1060-1068.

[5] Menozzi-Smarrito, C., Wong, C. C., Meinl, W., Glatt, H., Fumeaux, R., Munari, C., Robert, F., Williamson, G., Barron, D. (2011) First chemical synthesis and in vitro characterization of the potential human metabolites 5-o-feruloylquinic acid 4'-sulfate and 4'-O-glucuronide. *J. Agric. Food Chem.* **59**, 5671-5676.

[6] Wong, C. C., Barron, D., Orfila, C., Dionisi, F., Krajcsi, P., Williamson, G. (2011) Interaction of hydroxycinnamic acids and their conjugates with organic anion transporters and ATP-binding cassette transporters. *Mol. Nutr. Food Res.* **55**, 979-988.

[7] Piazzon, A., Vrhovsek, U., Masuero, D., Mattivi, F., Mandoj, F., Nardini, M. (2012). Antioxidant activity of phenolic acids and their metabolites: synthesis and antioxidant properties of the sulfate derivatives of ferulic and caffeic acids and of the acyl glucuronide of ferulic acid. *J. Agric. Food Chem.* **60**, 12312-12323.

[8] Eaton, E. A., Walle, U. K., Lewis, A. J., Hudson, T., Wilson, A. A., Walle, T. (1996) Flavonoids, potent inhibitors of the human P-form phenolsulfotransferase. Potential role in drug metabolism and chemoprevention. *Drug Metab. Dispos.* **24**, 232-237.

[9] De Santi, C., Pietrabissa, A., Mosca, F., Rane, A., Pacifici, G. M. (2002) Inhibition of phenol sulfotransferase (SULT1A1) by quercetin in human adult and foetal livers. *Xenobiotica.* **32**, 363-368.

[10] Vietri, M., Vaglini, F., Cantini, R., Pacifici, G. M. (2003) Quercetin inhibits the sulfation of r(-)-apomorphine in human brain. *Int. J. Clin. Pharmacol. Ther.* **41**, 30-35.

[11] Manach, C., Williamson, G., Morand, C., Scalbert, A., Remesy, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **81**, 230S-242S.

[12] Fumeaux, R., Menozzi-Smarrito, C., Stalmach, A., Munari, C., Kraehenbuehl, K., Steiling, H., Crozier, A., Williamson, G., Barron, D. (2010) First synthesis, characterization, and evidence for the presence of hydroxycinnamic acid sulfate and glucuronide conjugates in human biological fluids as a result of coffee consumption. *Org. Biomol. Chem.* **8**, 5199-5211.

[13] Day, A. J., Bao, Y. P., Morgan, M. R. A., Williamson, G. (2000) Conjugation position of quercetin glucuronides and effect on biological activity. *Free Radical Bio. Med.* **29**, 1234-1243.

[14] Wong, C. C., Botting, N. P., Orfila, C., Al-Maharik, N., Williamson, G. (2011) Flavonoid conjugates interact with organic anion transporters (OATs) and attenuate cytotoxicity of adefovir mediated by organic anion transporter 1 (OAT1/SLC22A6). *Biochem. Pharmacol.* **81**, 942-949.

[15] Day, A. J., Mellon, F., Barron, D., Sarrazin, G., Morgan, M. R. A., Williamson, G. (2001) Human metabolism of dietary flavonoids: Identification of plasma metabolites of quercetin. *Free Radical Res.* **35**, 941-952.

[16] Wong, C. C., Akiyama, Y., Abe, T., Lippiat, J. D., Orfila, C., Williamson, G. (2012) Carrier-mediated transport of quercetin conjugates: involvement of organic anion transporters and organic anion transporting polypeptides. *Biochem. Pharmacol.* **84**, 564-570.

[17] Harris, R. M., Wood, D. M., Bottomley, L., Blagg, S., Owen, K., Hughes, P. J., Waring, R. H., Kirk, C. J. (2004) Phytoestrogens are potent inhibitors of estrogen sulfation: implications for breast cancer risk and treatment. *J. Clin. Endocrinol. Metab.* **89**, 1779-1787.

[18] Petri, N., Tannergren, C., Holst, B., Mellon, F. A., Bao, Y., Plumb, G. W., Bacon, J., O'Leary, K. A., Kroon, P. A., Knutson, L., Forsell, P., Eriksson, T., Lennernas, H., Williamson, G. (2003) Absorption/metabolism of sulforaphane and quercetin, and regulation of phase II enzymes, in human jejunum in vivo. *Drug Metab. Dispos.* **31**, 805-813.

[19] Walsh, K. R., Haak, S. J., Bohn, T., Tian, Q., Schwartz, S. J., Failla, M. L. (2007) Isoflavonoid glucosides are deconjugated and absorbed in the small intestine of human subjects with ileostomies. *Am. J. Clin. Nutr.* **85**, 1050-1056.

[20] Auger, C., Mullen, W., Hara, Y., Crozier, A. (2008) Bioavailability of polyphenon E flavan-3-ols in humans with an ileostomy. *J. Nutr.* **138**, 1535S-1542S.

[21] Scalbert, A., Williamson, G. (2000) Dietary intake and bioavailability of polyphenols. *J. Nutr.* **130**, 2073S-2085S.

[22] O'Leary, K. A., Day, A. J., Needs, P. W., Mellon, F. A., O'Brien, N. M., Williamson, G. (2003) Metabolism of quercetin-7-and quercetin-3-glucuronides by an in vitro hepatic model: the role of human beta-glucuronidase, sulfotransferase, catechol-O-methyltransferase and multi-resistant protein 2 (MRP2) in flavonoid metabolism. *Biochem. Pharmacol.* **65**, 479-491.

[23] Hilgendorf, C., Ahlin, G., Seithel, A., Artursson, P., Ungell, A. L., Karlsson, J. (2007) Expression of thirty-six drug transporter genes in human intestine, liver, kidney, and organotypic cell lines. *Drug Metab. Dispos.* **35**, 1333-1340.

[24] Manach, C., Williamson, G., Morand, C., Scalbert, A., Rémésy, C. (2005) Bioavailability and bioefficacy of polyphenols in humans. I. Review of 97 bioavailability studies. *Am. J. Clin. Nutr.* **81**, 2305-2425.

Table 1. Inhibition of caffeic acid and ferulic acid sulfation by flavonoids in intestinal S9

	IC_{50} (μM, mean ± SD) for the inhibition of sulfation of	
Flavonoids	Caffeic acid	Ferulic acid
Apigenin	0.96 ± 0.01	2.30 ± 0.75
Luteolin	1.33 ± 0.59	2.95 ± 1.18
Kaempferol	2.37 ± 1.63	3.76 ± 0.45
Isorhamnetin	4.40 ± 0.16	4.66 ± 0.21
Quercetin	4.15 ± 0.34	5.10 ± 0.54
Hesperetin	3.51 ± 1.40	5.04 ± 0.49
Genistein	0.77 ± 0.02	1.50 ± 0.84
Daidzein	0.73 ± 0.11	2.26 ± 0.43
(+)-Catechin	5.80 ± 1.98	7.05 ± 0.64
(-)-Epicatechin	7.65 ± 1.09	11.6 ± 3.2
Phloretin	0.86 ± 0.27	2.17 ± 1.67

Table 2. Inhibition of caffeic acid and ferulic acid sulfation by flavonoids in human liver S9

	IC_{50} (μM, mean ± SD) for the inhibition of sulfation of	
Flavonoids	Caffeic acid	Ferulic acid
Apigenin	0.46 ± 0.02	0.90 ± 0.17
Luteolin	0.08 ± 0.01	0.53 ± 0.01
Kaempferol	0.84 ± 0.01	3.98 ± 1.17
Isorhamnetin	2.05 ± 1.49	3.78 ± 0.00
Quercetin	0.41 ± 0.02	0.64 ± 0.03
Hesperetin	4.31 ± 0.02	5.25 ± 0.24
Genistein	0.58 ± 0.14	1.02 ± 0.02
Daidzein	0.60 ± 0.06	0.61 ± 0.10
(+)-Catechin	4.16 ± 0.11	4.43 ± 0.03
(-)-Epicatechin	7.36 ± 0.10	7.28 ± 0.56
Phloretin	0.90 ± 0.05	1.03 ± 0.02

Table 3. Inhibition of caffeic acid and ferulic acid sulfation by quercetin conjugates in liver S9

	IC_{50} (μM, mean ± SD) for the inhibition of sulfation of	
Conjugate	Caffeic acid	Ferulic acid
Quercetin-3-*O*-glucuronide	11.5 ± 0.2	11.3 ± 0.6
Quercetin-7-*O*-glucuronide	9.56 ± 0.28	8.35 ± 0.59
Quercetin-3′-*O*-sulfate	6.56 ± 0.03	6.82 ± 0.28

Figure legends:

Figure 1. Inhibition of hydroxycinnamic acid sulfation by daidzein (A) and genistein (B) in human intestinal S9. 0.4 mg / mL intestinal S9 was pre-incubated for 15 min with daidzein, genistein or 0.2 % DMSO. Formation of conjugates was measured after incubation of hydroxycinnamic acids with intestinal S9 for 30 min.

Figure 2. Inhibition of hydroxycinnamic acid sulfation by luteolin (A) and quercetin (B) in human liver S9. 1 mg / mL liver S9 was pre-incubated for 15 min with luteolin, quercetin or 0.2 % DMSO. Conjugate formation was measured 30 min after the addition of hydroxycinnamic acids.

Figure 3. (A) Effect of β-glucuronidase on the inhibitory activity of quercetin glucuronides. 1 mg / mL liver S9 was pre-incubated with quercetin-3-*O*-glucuronide (Q3G) and quercetin-7-*O*-glucuronide (Q7G), alone or with saccharolactone, for 15 min. Caffeic acid was then added and sulfate conjugate formation was measured after 30 min. **(B)** Uptake of quercetin conjugates into HepG2 cells. Uptake was assessed incubating quercetin conjugates (50 μM) with HepG2 cells for 4 h. Quercetin-3′-*O*-sulfate: Q3′S.

Figure 4. Inhibitory effect of luteolin, quercetin, and quercetin conjugates on sulfation of caffeic acid (A) and ferulic acid (B) in HepG2 cells. Caffeic acid and ferulic acid (10 μM) were incubated in the presence of luteolin, quercetin or quercetin conjugates for 4 h. Quercetin-3-*O*-glucuronide: Q3G; quercetin-7-*O*-glucuronide: Q7G; quercetin-3′-*O*-sulfate: Q3′S.

Figure 5. The potential impact of flavonoids on hydroxycinnamic acid (HCA) metabolism. In the intestinal tract, flavonoid aglycones are potent inhibitors of hydroxycinnamic acid sulfation. In the liver, flavonoid conjugates are taken up and further inhibit the sulfation of hydroxycinnamic acid.