Research Article

Triple Recycling Processes Impact Systemic and Local Bioavailability of Orally Administered Flavonoids

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Abstract. Triple recycling (*i.e.*, enterohepatic, enteric and local recycling) plays a central role in governing the disposition of phenolics such as flavonoids, resulting in low systemic bioavailability but higher gut bioavailability and longer than expected apparent half-life. The present study aims to investigate the coexistence of these recycling schemes using model bioactive flavonoid tilianin and a four-site perfused rat intestinal model in the presence or absence of a lactase phlorizin hydrolase (LPH) inhibitor gluconolactone and/or a glucuronidase inhibitor saccharolactone. The result showed that tilianin could be metabolized into tilianin glucuronide, acacetin, and acacetin glucuronide, which are excreted into the bile and luminal perfusate (highest in the duodenum and lowest in the colon). Gluconolactone (20 mM) significantly reduced the absorption of tilianin and the enteric and biliary excretion of acacetin glucuronide. Saccharolactone (0.1 mM) alone or in combination of gluconolactone also remarkably reduced the biliary and intestinal excretion of acacetin glucuronide. Acacetin glucuronides from bile or perfusate were rapidly hydrolyzed by bacterial β -glucuronidases to acacetin, enabling enterohepatic and enteric recycling. Moreover, saccharolactone-sensitive tilianin disposition and glucuronide deconjugation, which was more active in the small intestine than the colon, points to the small intestinal origin of the deconjugation enzyme and supports the presence of local recycling scheme. In conclusion, our studies have demonstrated triple recycling of a bioactive phenolic (*i.e.*, a model flavonoid), and this recycling may have an impact on the site and duration of polyphenols pharmacokinetics *in vivo*.

KEY WORDS: enteric recycling; enterohepatic recycling; flavonoid; local recycling; phase II metabolism.

INTRODUCTION

Recycling schemes have been demonstrated to be involved in the disposition of phenolics including polyphenols such as flavonoids (1,2). Enterohepatic recycling, enteric recycling, and local recycling are three important recycling

schemes for flavonoids disposition, which are enabled by phase II conjugating enzymes (*e.g.*, glucuronides) and efflux transporters in intestine and liver. Through these recyclings, excreted flavonoid conjugates can be deconjugated into their respective aglycone forms, followed by their intestinal reabsorption (3,4). Enterohepatic recycling, the process demonstrated more than half a century ago (5), involves the hepatic excretion of the glucuronides into the intestine *via* bile. The flavonoid glucuronides can be hydrolyzed by bacteria-derived glucuronidase and reabsorbed into colon, thereby completing the enterohepatic recycling. Different from enterohepatic recycling, the intestinal excretion of glucuronides, instead of liver, is required for enteric and local recycling. Enteric recycling has been discovered for a decade (6), which needs bacterial β -glucuronidases in the colon to release aglycones from enterocyte-excreted conjugates (7). Remarkably, local recycling, a novel recycling mechanism proposed more recently, only requires enterocyte-derived β -glucuronidases to deconjugate the glucuronides in the upper small intestine, thereby completing the subsequent reabsorption and recycling loop without bacterial enzymes (2).

Flavonoids are a class of natural polyphenolic compound, which can be commonly found in human diet including fruits, vegetables, seeds, and medicinal herbs. Significant research interests in flavonoids exist because they are considered to be

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non-toxic and possess biological activities ranging from anti-oxidation to anti-tumor (8,9). The pharmacological effects of flavonoids, however, are critically limited by its low bioavailability *in vivo*. Since most flavone compounds are sufficiently absorbed (10), the main reason for their poor oral bioavailabilities is the extensive phase II metabolism mediated by UDP-glucuronosyltransferases (UGT) and sulfotransferases (SULT) (1,11). Because phase II metabolites are extensively secreted into the intestine *via* bile or direct route, metabolic recycling processes mentioned above (*i.e.*, enterohepatic recycling, enteric recycling and local recycling (1,2,7,12)) increase their local (*i.e.*, gut) bioavailability and prolong their apparent plasma half-life.

Similar to human, rat intestines also functionally express metabolizing enzymes (*e.g.*, UGT, SULT) and efflux transporters including multidrug resistance associated protein 2 (MRP2), breast cancer resistance protein (BCRP) and P-glycoprotein (P-gp) (2,7), which can be employed to mimic human intestinal transport. A perfused rat intestinal model is recognized by FDA and has been widely applied for evaluating drug absorption and metabolism mechanisms *in vivo*. Previous studies (7,12) have used the perfused rat intestinal model to propose the repeated shuffling of flavonoids through a duo recycling scheme involving both enteric and enterohepatic recycling is crucial for *in vivo* disposition of flavonoids. However, local recycling could prolong the residence time of flavonoids in the gut, which allows them to have good exposure locally even though they may have poor systemic bioavailabilities (2). Many publications have investigated “single” recycling scheme of phenolics (including flavonoids). For instance, disposition of biochanin A, glycitein, and formononetin (13–15) has been shown to undergo enterohepatic recycling. Apigenin (7), prunetin, daidzin, and genistein (10) were used as the model compounds to demonstrate the presence of enteric recycling. More recently, a flavonoid pair wogonin (aglycone) and wogonoside (glucoside) was used as a novel example to prove the presence of the local recycling (2). Although enterohepatic and enteric recycling have been known for a long period of time (5,7), the local recycling was only recently demonstrated (2). Nevertheless, none of the published reports was able to simultaneously investigate three recycling schemes.

Hence, in this paper, we have demonstrated the coexistence of enterohepatic, enteric and local recycling for model flavonoids tilianin and its aglycone acacetin, which are extensively glucuronidated in the gut and the liver. Tilianin is chosen because it is a flavonoid compound that has attracted much attention for its anti-atherogenic, antihypertensive, and anticonvulsive properties. It was originally isolated from *Dracocephalum moldavica* L. (DML) (16) and *Agastache rugosa* (17). It can be used for the treatment of hypertension (18). Additionally, tilianin has been found to reduce atherosclerotic lesion formation through the inhibition of cytokine-induced I κ B kinase activation (17). Acacetin, the flavone aglycone of tilianin, can be found in many more plants including propolis (19, 20), *Robinia pseudoacacia* (21), and DML (16). Acacetin is also selected because previous study have shown that acacetin exhibits peroxidative (22), antiangiogenic and anti-cancer properties, and is highly active against liver, prostate, lung, stomach, and breast cancer cells, most likely *via* apoptosis induction (22).

MATERIALS AND METHODS

Materials

Tilianin ($\geq 95\%$, HPLC grade, confirmed by LC–MS/MS) were kindly provided from by Dr. Xinchun Wang (First Affiliated Hospital of the Medical College, Shihezi University, Xin Jiang, China). Acacetin ($\geq 95\%$, HPLC grade, confirmed by LC–MS/MS) were purchased from Shanghai Winherb Medical Technology Co., Ltd (Shanghai, China). Saccharolactone, gluconolactone, glucose, NaHCO₃, and Hanks balanced salt solution (HBSS, powder form), were purchased from Sigma-Aldrich (St. Louis, MO, USA). All other reagents were typically analytical grade and used as received.

Animals

Male Sprague–Dawley rats (80–110 days old) weighing from 250 to 300 g were obtained from the Laboratory Animal Center of Guangzhou University of Chinese Medicine. The rats were housed four per cage in a unidirectional airflow room under relative humidity (40% to 70%), controlled temperature (20°C to 24°C) and 12 h light/dark cycle. No flavonoids were detected in pH 6.5 HBSS buffer that had been perfused through a segment of the rat upper small intestine, indicating that dietary tilianin or acacetin was not found in the rat gut. The rats were fasted overnight with free access to water before the day of the experiment.

Animal Surgery

The animal protocol used in the present study was approved by the Guangzhou University of Chinese Medicine's Ethics Committee. We perfused four segments (duodenum, jejunum, ileum, and colon) of the intestine simultaneously (four-site intestinal perfusion model) with a bile duct cannulation, which is approximately the same as those described previously (2). In brief, after anesthesia by an i.p. injection of 1.2 g/kg urethane (50%, *w/v*), we cannulated various segments of the intestine and bile duct using the method described previously (2). First, two cannula (one for inlet and one for outlet) at ≈ 10 cm apart were inserted into the duodenum, located adjacent to the stomach. Next, two cannula at ≈ 10 cm apart were inserted into two ends of the jejunum and ileum, respectively. Last, the colon inlet cannula was inserted into top part of colon (near cecum), and the outlet cannula was inserted through the anus. After the commencement of perfusion, HBSS buffer with the analyte (*i.e.*, tilianin) was perfused from the inlet cannula, while the outlet perfusate in the cannula was driven into a receiving tube with 100% acetonitrile (2 mL) for the purpose of denaturing the enzyme capable of the hydrolysis, except when blank perfusate (no flavonoid) was collected (no acetonitrile).

Perfused Rat Intestinal Model for Regional Transport and Metabolism Experiments

Four segments of the intestine (duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously with perfusate containing tilianin using an infusion pump (model PHD2000; Harvard Apparatus, Cambridge,

MA) at a flow rate of 0.168 mL/min. Two concentrations (40 and 5 μ M) of tilianin were perfused. After a 30-min washout period, which is considered to achieve steady-state absorption, perfusate and bile samples were collected from the outlet cannula every 30 min during the 2.5 h perfusion period.

Hydrolysis of Tilianin by Lactase Phlorizin Hydrolase

To determine the significant role of lactase phlorizin hydrolase (LPH) in the hydrolysis of tilianin into acacetin, 40 μ M tilianin was perfused alone or co-perfused with 20, 40, 80 mM LPH inhibitor (gluconolactone). Furthermore, blood samples from jugular vein were also collected every 30 min during the 2.5 h perfusion period. At the end of the experiment, four segments of intestine were removed, flushed with 0.9% NaCl at 4°C to remove intraluminal contents, cut longitudinally, and mucosa was obtained by scraping with a glass slide (notice: these steps took place on ice to minimize compound loss).

Hydrolysis Experiment by β -glucuronidase

To investigate whether conjugates could be hydrolyzed to their aglycone forms by bacteria-derived β -glucuronidase, a portion of intestinal perfusate and bile samples were incubated with β -glucuronidase at 37°C for 5 h to convert conjugated flavonoids to their respective aglycone forms. On the other hand, to consider the necessity of bacteria on glucuronide hydrolysis, acacetin glucuronide was incubated with blank perfusate, which was filtrated and centrifuged to remove intestinal bacteria.

Excretion of Acacetin Glucuronide Affected by a Glucuronidase Inhibitor

How glucuronidase would impact the excretion of acacetin glucuronide was determined by 40 μ M tilianin co-perfused with or without 0.1 mM glucuronidase inhibitor (saccharolactone).

Excretion of Acacetin Glucuronide Affected by Combination of a Glucuronidase Inhibitor and a LPH Inhibitor

To determine how glucuronidase and LPH inhibitors when used together would impact the excretion of acacetin glucuronide, 40 μ M tilianin was perfused with or without 0.1 mM glucuronidase inhibitor (saccharolactone) in combination with 20 mM LPH inhibitor (gluconolactone).

Identification of Triple Recycling (i.e., Enterohepatic recycling, Enteric Recycling, and Local Recycling) in the Perfusion Model and Hydrolysis Experiments

To demonstrate enterohepatic recycling, *in vitro* studies incubated with bacteria-derived β -glucuronidase were required to prove the hydrolysis of glucuronide in metabolic recycling processes. Additionally, biliary and enteric excretion of tilianin and its metabolites in the perfusion experiment were also examined. To prove enteric recycling, in addition to the hydrolysis of glucuronide by bacteria-derived β -glucuronidase, the enteric excretion of acacetin glucuronide in colon

was also studied in the presence of a glucuronidase inhibitor. Lastly, to demonstrate local recycling, the importance of enteric β -glucuronidase should be further emphasized. Therefore, glucuronide was incubated with blank perfusate, which was filtrated and centrifuged to remove intestinal bacteria. The enteric excretion of acacetin glucuronide in upper small intestine was also examined in the presence of a glucuronidase inhibitor.

Preparation of Biosamples

To detect tilianin and its metabolites in the perfusate, 20 ng/mL propiophenone (IS) was added into the mixture of the perfusate and acetonitrile or the bile sample (1:2). The supernatant after centrifugation at 13,000 rpm for 30 min was injected into HPLC for analysis. With respect to rat plasma and mucosa samples, 20 μ L of plasma or mucosa was mixed with 100 μ L methanol containing 200 nM testosterone (IS). The mixture was centrifuged at 13,000 rpm for 30 min and 100 μ L of the supernatant was transferred to a disposable tube and evaporated to dryness under a stream of nitrogen at room temperature. The residue was reconstituted with 100 μ L of methanol–water ($v/v=1:1$) and then injected into the UPLC-MS/MS for analysis.

LC/MSⁿ and HPLC Analysis of Tilianin and its Metabolites

Agilent 6540 Accurate-Mass Q-TOF MS System and Agilent HPLC were used for the qualification and quantification of tilianin and its metabolites. The conditions were as follows: column, ZORBAX SB-C18, 5 μ m, 4.6 $\times$ 150 mm; mobile phase A, 100% acetonitrile, mobile phase B, 100% aqueous buffer (0.1%, v/v formic acid, pH 2.5); flow rate, 1 mL/min; gradient, 0 to 15.0 min, 5–35% A, 15.0 to 15.5 min, 35–50% A, 15.5 to 16.0 min, 50–55% A, 16.0 to 17.5 min, 55–70% A, 17.5 to 18.5 min, 70–10% A, 18.5 to 19 min, 10–5% A, 19.0 to 20 min, 5–5% A; wavelength, 330 nm for tilianin and its metabolites and IS; and injection volume, 20 μ L. Ionization was achieved using electrospray ionization in the positive mode.

The main mass working parameters for the mass spectrometers were set as follows: capillary voltage, 3500 V; fragmentor 175 V; cone voltage, 35 V; skimmer, 65 V; OCT 1 RF V_{pp} , 750 V; pressure of nebulizer, 35 psi; drying gas temperature, 300°C; sheath gas temperature, 300°C. Nitrogen was used as sheath and drying gas at a flow rate of 8.0 and 3.0 L/min, respectively. Data acquisition and analysis were performed using Agilent Mass hunter software.

Data Analysis

Permeability of tilianin was represented by P^*_{eff} , which was obtained as described previously (6,23). Briefly,

$$P^*_{eff} = \frac{1 - C_m/C_o}{4G_z} \quad (1)$$

P^*_{eff} represents the apparent permeability of a compound, which is a measurement for the amount loss in the perfusate. Where C_o and C_m are inlet and outlet

concentrations corrected for water flux using the sample weight, respectively; while G_z , or Graetz number ($G_z = \pi DL/2Q$), is a scaling factor incorporating flow rate (Q) and intestinal length (L), and diffusion coefficients (D) to make the permeability dimensionless.

Amounts of tilianin absorbed (M_{ab}) and amounts of corresponding acacetin glucuronide excreted into the intestinal lumen (M_{met}) were calculated as described previously (7,23). Generally, M_{ab} was expressed as

$$M_{ab} = Q\tau(C_0 - C_m) \quad (2)$$

where τ is the sampling interval (30 min), and other parameters were identical with those defined in Eq. 1. Amounts of metabolites (M_{met}) were formulated as

$$M_{met} = Q\tau C_{met} \quad (3)$$

where C_{met} is the outlet concentration (nmol/mL) of metabolites corrected for water flux.

$$M_{bile} = VCM_{bile} \quad (4)$$

where CM_{bile} is the bile concentrations (nmol/mL) of metabolites, and V is the volume of bile collected over a 30 min time period.

Statistical Analysis

One-way ANOVA with or without Tukey–Kramer multiple comparison and Student's t test were used to evaluate between control and treatment. Differences were considered significant at $p < 0.05$ or $p < 0.01$.

RESULTS

Identification of Tilianin and its Metabolites by LC–MS/MS

The HPLC method developed for tilianin and its metabolites had a run time of 20 min (Fig. 1). The tested linear response range was 0.3125–80 μ M (for a total of 9 concentrations) with the lower limit of quantification (LLOQ) of 0.2 μ M.

In bile samples, metabolites eluted at 11.94 min (M1: tilianin glucuronide) and 16.31 min (M2: acacetin glucuronide) (Fig. 1a). In outlet perfusate samples, metabolites eluted at 16.31 min (M2) (Fig. 1b). A high-resolution mass spectrometry (HRMS) scan running at a positive ion mode was used to determine the MS spectrum of the metabolite. M1 showed a pseudo-molecule ion $[M+H]^+$ of m/z 623.1617 in full-scan mass spectra (Fig. 1c) and fragment ions at m/z 447 and 285 (Fig. 1d), indicating that they had the molecular formula of $C_{28}H_{30}O_{16}$ (or tilianin-7-glucuronide). Tilianin-7-glucuronide was a new metabolite that had never been reported previously. M2 showed a pseudo-molecule ion $[M+H]^+$ of m/z 461.1069 in full-scan mass spectra (Fig. 1e) and fragment ions at m/z 285 (Fig. 1f), indicating that they had the molecular formula of $C_{22}H_{20}O_{11}$ (or acacetin-7-glucuronide). HPLC retention times and parameters of the Q-TOF tandem mass spectrometer were summarized in Table I.

Regional Transport and Metabolism of Tilianin in a Perfused Rat Intestinal Model

After perfusion with 5 or 40 μ M tilianin, its absorption was fast in duodenum, jejunum, and ileum, all of which were higher than those in colon, at both concentrations. Moreover, there was no significant difference in P^*_{eff} values in duodenum, jejunum, and ileum between 5 and 40 μ M concentrations (Fig. 2a). Tilianin was found to be converted to acacetin and acacetin glucuronide in the rat intestine (Figs. 1 and 2). Large amounts of acacetin glucuronide and acacetin were also found in perfusate with the highest excretion in duodenum and lowest excretion in colon (Fig. 2c). Surprisingly, in addition to tilianin, acacetin, and acacetin glucuronide, a small amount of tilianin-7-glucuronide (M1) was also discovered in the bile (Fig. 2d–g). A small amount of acacetin sulfate was also found in the perfusate and bile but their concentrations were below LLOQ of the HPLC analysis method (0.2 μ M). At the same time, tilianin sulfate could not be detected in either perfusate or bile.

Stability of Tilianin, Acacetin, and Their Glucuronides

To ensure that the biotransformation of the flavonoids was not due to chemical instability, stability testing of the tilianin and acacetin and their glucuronides in HBSS solution, bile, and plasma was conducted. The results demonstrated that the stabilities in HBSS solution and bile displayed 90–110% recoveries after storage at 1, 2, 3, and 4 h incubation at 37°C (Table S1 and S2 in the Electronic Supplementary Material (ESM)). In addition, the stabilities in rat plasma also displayed no less than 85% recoveries after storage at 25°C for 4 h, –80°C for 7 days, going through three freeze–thaw cycles (–80°C and 25°C) and 10°C for 12 h (Table S3 in the ESM). These results could eliminate the possibility that the flavonoids undergo hydrolysis in the HBSS buffer, bile, or plasma.

Hydrolysis of Tilianin and Acacetin Glucuronide by β -glucuronidase

A variety of *in vitro* studies were conducted to investigate whether glucuronides undergo hydrolysis in the perfused buffer. The results showed that acacetin glucuronide was unstable in the blank perfusate (generated by perfusing HBSS buffer through four segments of the intestine) (Fig. 3). It was possibly caused by the β -glucuronidase, because 0.1 mM saccharolactone (a glucuronidase inhibitor) significantly reduced the hydrolysis of acacetin glucuronide, while 4.4 mM saccharolactone almost completely abolished the hydrolysis (Fig. 3). The importance of β -glucuronidase was further confirmed by the fact that after incubation with β -glucuronidase from *Escherichia coli*, the peak area of tilianin glucuronide and acacetin glucuronide was significantly reduced while that of tilianin and acacetin was proportionately increased (see Fig. S1 in the ESM).

Notably, bacteria might not be the major source of this enzyme because removal of bacteria *via* centrifugation and aseptic filtration did not significantly improve stability of

Fig. 1. HPLC chromatograms as well as HRMS and MS2 scan profiles along with structures of tilianin, acacetin and their respective glucuronide. HPLC was used to separate and quantify tilianin and its metabolites in the experimental samples. Panels **a** and **b** represent the HPLC chromatograms of bile and perfusate showing the retention time and/or structures of tilianin, acacetin, their glucuronide and internal standard (IS), respectively. HRMS was used to identify the metabolites as glucuronide of tilianin and acacetin, and panels **c** and **e** represent HRMS scans for the glucuronide of tilianin and acacetin, respectively. MS2 scan was used to confirm their identities, and panels **d** and **f** represent MS2 scan for the glucuronide of tilianin and acacetin, respectively.

acacetin glucuronide in the blank perfusate. In addition, the hydrolysis of acacetin glucuronide was faster in the blank perfusate collected from the upper small intestine than those collected from the colon (Fig. 3), since it has been commonly known that colon has higher bacterial count than the upper small intestine.

Importance of LPH in Absorption of Tilianin

LPH inhibitor gluconolactone was used to inhibit the hydrolysis of tilianin to acacetin in order to determine how this would impact the absorption and metabolism of either compound. As expected, gluconolactone reduced absorption

Table I. Retention Time of Tilianin and its Metabolites in HPLC Chromatograms with the HRMS Data

	HPLC t_R (min)	$[M+H]^+$ (m/z)	Molecular formula
Tilianin glucuronide	11.9	623.1617	$C_{28}H_{30}O_{16}$
Tilianin	16.1	447.1282	$C_{22}H_{22}O_{10}$
Acacetin glucuronide	16.3	461.1069	$C_{22}H_{20}O_{11}$
Acacetin	19.2	285.0770	$C_{16}H_{12}O_5$

Fig. 2. Absorption and metabolism of tilianin in a four-site rat intestinal perfusion model. Four segments of the intestine (*i.e.*, duodenum, upper jejunum, terminal ileum, and colon) were perfused simultaneously at a flow rate of 0.168 mL/min using tilianin at the concentrations of 40 and 5 μ M. Effective permeabilities of tilianin (panel a), amounts of tilianin absorbed (panel b), and acacetin glucuronide excreted (panel c) in the perfusate were determined and normalized over a 10 cm intestinal length. The amounts of tilianin glucuronide (panel d), tilianin (panel e), acacetin glucuronide (panel f), and acacetin (panel g) excreted in the bile were also determined. Each column (in panels a–c) and each curve (in panels d–g) represents the average of four determinations, and the error bar is the S.D. ($n=4$). The “*” symbol means a statistically significant difference at $p<0.05$; and “**” means $p<0.01$.

of tilianin and excretion of acacetin glucuronide in the perfusate, and the effect was concentration-dependent

(Fig. 4a–c). The effect of gluconolactone on the excretion of acacetin glucuronide was more significant at the upper

Fig. 3. Biological stability of acacetin glucuronide (5 μ M) in blank perfusate of upper small intestine (panel **a**) or colon (panel **b**) ($n=3$). Acacetin glucuronide (AG') was added into the intestinal perfusate, amount of AG' was determined at 0, 60, 120, 180 min, and used as the control group. Additional studies were performed in the sterile filtered blank perfusate, or in the presence of 0.1 mM saccharolactone (SCL) or 4.4 mM SCL, respectively. The incubation experiments were performed at 37°C. Each data point is the average of the three determinations, and the error bar represents S.D. ($n=3$). The “*” symbol means a statistically significant difference at $p<0.05$; and “**” means $p<0.01$.

small intestine than that of colon, since LPH is present at much higher quantities in the small intestine. Compared to the control group, the excretion of tilianin glucuronide, tilianin, acacetin, and acacetin glucuronide in the bile were significantly reduced in the presence of gluconolactone in the perfusate, which was consistent with reduced absorption in the perfusate (Fig. 4d–g). Tilianin glucuronide, tilianin, acacetin glucuronide, and acacetin were also found in the plasma and the intestinal mucosa (Fig. 5). The mucosa (Fig. 5a, b) and plasma (Fig. 5e, f) concentrations of tilianin and tilianin glucuronide were enhanced after perfusion with gluconolactone. On the contrary, the concentrations of acacetin and acacetin glucuronide in the mucosa (Fig. 5c, d) were significantly reduced in the presence of gluconolactone. Concentrations of these two compounds were also moderately reduced in plasma (Fig. 5g, h) but the effects were not statistically significant.

Effects of Glucuronidase and/or LPH Inhibitor on the Excretion of Acacetin Glucuronide

Because acacetin glucuronide is the major metabolite undergoing excretion, much of the remaining study is focused on this conjugate. First, a glucuronidase inhibitor saccharolactone was used to inhibit the hydrolysis of acacetin glucuronide to acacetin in order to determine how this would impact the disposition of acacetin glucuronide. The excretion of acacetin glucuronide in both the upper small intestine (Fig. 6) and the colon (Fig. 7) was significantly reduced in the presence of saccharolactone. Then, a combination of glucuronidase inhibitor saccharolactone and LPH inhibitor gluconolactone was also able to significantly reduce excretion of acacetin glucuronide in the upper small intestine (Fig. 6) and colon (Fig. 7).

Demonstration for Triple Recycling (i.e., Enterohepatic Recycling, Enteric Recycling, and Local Recycling)

The model compound tilianin underwent enterohepatic, enteric, and local recycling, these claims are backed by a variety of *in situ* and *in vitro* studies. When tilianin was perfused *in situ*, large amounts of acacetin glucuronide and small amounts of tilianin, tilianin glucuronide and acacetin were demonstrated to be excreted into the bile *via* the liver (Fig. 2). In addition, *in vitro* incubation with bacteria-derived β -glucuronidase notably deconjugated glucuronides to aglycones (Fig. S1 in the ESM), thus suggesting the important role of enterohepatic recycling. After perfused with glucuronidase inhibitor, the enteric excretion of acacetin glucuronide in colon (Fig. 7) was significantly reduced, further indicating enteric recycling. Additional studies also proved local recycling because the excretion of acacetin glucuronide was reduced in upper small intestine after co-perfused with glucuronidase inhibitor (Fig. 6). The hypothesis was further supported by that fact that incubation with blank perfusate after bacteria removal did not influence the hydrolysis of acacetin, thus demonstrating the importance of enteric β -glucuronidase hydrolysis of glucuronides in local recycling (Fig. 3).

DISCUSSION

The results of this study showed for the first time that flavonoids, including the glucosides of flavone, could be subjected to a triple recycling scheme, where enterohepatic recycling, enteric recycling and local recycling occur simultaneously (see Fig. 8). This triple recycling process starts with hydrolysis of a model compound, tilianin, to its aglycone, acacetin, catalyzed by LPH. Once hydrolyzed, acacetin is rapidly absorbed into intestinal epithelial cells and eventually travel to hepatocytes. Acacetin appears to be rapidly metabolized into glucuronides in both enterocytes and hepatocytes by glucuronidation, and the glucuronides were then rapidly excreted into lumen or bile. Conjugates can be hydrolyzed back to its aglycone forms by bacterial β -glucuronidases in the colon or enteric β -glucuronidases in the upper small intestine. The released aglycone, acacetin could be subsequently reabsorbed in the colon (for enterohepatic and enteric recycling) or any enterocytes with

◀ **Fig. 4.** Effects of a LPH inhibitor gluconolactone on the absorption and metabolism of tilianin and its aglycone acacetin. Absorption and metabolism of tilianin were measured in a “four-site” perfused rat intestinal model using a flow rate of 0.168 mL/min. Tilianin was perfused alone or co-perfused with 20, 40, 80 mM gluconolactone (GCL). Effective permeabilities of tilianin (panel **a**), the amounts of tilianin absorbed (panel **b**) and amounts of acacetin glucuronide excreted (panel **c**) in the perfusate were determined and normalized over a 10 cm intestinal length. The amounts of tilianin glucuronide (panel **d**), tilianin (panel **e**), acacetin glucuronide (panel **f**) and acacetin (panel **g**) excreted in the bile were also determined. Each column (panel **a–c**) and each curve (panel **d–g**) represents the average of four determinations, and the error bar is the S.D. ($n=4$). The “*” symbol means a statistically significant difference at $p<0.05$; and “***” means $p<0.01$.

enteric β -glucuronidases (for local recycling), completing the recycling process. We termed this phenomenon triple recycling to signify the fact that one compound (acacetin in this case) can undergo these three recycling processes simultaneously.

To demonstrate whether our model compound tilianin underwent enterohepatic, enteric, and local recycling, a variety of *in situ* and *in vitro* experiments were conducted in our present study. We hypothesized that enterohepatic recycling participated in the disposition of tilianin and its metabolites, including the hydrolysate (*i.e.*, acacetin) and conjugates (*i.e.*, acacetin glucuronide). When perfused with tilianin in the intestine, large amounts of acacetin glucuronide and small amounts of acacetin rapidly appeared in the bile, indicating absorption from the gut (Fig. 2). When tilianin was continually perfused, the biliary excretion of acacetin glucuronide and acacetin amounted in 2 h to the maximum (Fig. 2). After incubation with β -glucuronidases from *E. coli*, acacetin glucuronide in the bile was deconjugated to form aglycone acacetin (Fig. S1 in the ESM), which suggested that glucuronides could be hydrolyzed back to its aglycones by bacterial β -glucuronidases in colon and reabsorbed into the enterocyte thereby completing the recycling processes (*i.e.*, enterohepatic recycling). Large amounts of flavonoids and their conjugates, such as biochanin A, glycitein, and formononetin (9,13) are demonstrated to be excreted *via* bile, and then reach the colon where they are reconverted to aglycones and reabsorbed into the blood, completing the enterohepatic recycling process.

Enteric recycling of acacetin and its glucuronides is also possible, since we found extensive enteric excretion of acacetin glucuronide (Fig. 2). Acacetin could be formed rapidly following its hydrolysis from its glucuronides by microflora-derived β -glucuronidase (Fig. S1 in the ESM). In the presence of glucuronidase inhibitor alone, the colonic excretion of acacetin glucuronide was significantly reduced by approximately 80% (Fig. 6). This is consistent with the previous literature that the glucuronidase released from the large amounts of bacteria in the colon could deconjugate glucuronides into aglycones, and enabled them to be reabsorbed in intestine (4, 10). Since recycling is a dynamic process, termination on any link would have a negative effect on the entire process, leading to a progressive decrease in the recycling. We extrapolated that the hydrolysis inhibition of acacetin glucuronide to acacetin, led by glucuronidase inhibitor, might decrease the amount of acacetin in

enterocytes, resulting in the less production and excretion of acacetin glucuronides into the lumen. Previous studies have shown that a large number of flavonoids including formononetin (4), apigenin (7,24), prunetin (23), biochanin A (3), daidzin, and genistein (7) also underwent enteric recycling.

Additionally, we have showed that the β -glucuronidase activities in upper intestinal perfusate were mediated by enteric β -glucuronidase since the activities were higher in the upper small intestine than in colon (Fig. 3), which has the highest count of bacteria among the blank perfusate solutions collected from different regions of the intestine (2). Because less acacetin in the enterocyte might result in the less generation and excretion of acacetin glucuronide, when co-perfused with a glucuronidase inhibitor, the excretion of acacetin glucuronide in the upper small intestine was also remarkably reduced by approximately 50% (Fig. 7), which supports the hypothesis of the presence of local recycling. It has been previously coined by us that glucuronides can be rigorously hydrolyzed by enterocyte-derived β -glucuronidase (2), completing the local recycling. Wogonin and wogonoside (a wogonin glucuronide) (2) have been used to demonstrate the existence and function of the local recycling. Taken together, these important findings supports our assumption of triple recycling, which is novel because there is a lack of literature indicating a flavonoid that participated in enterohepatic, enteric, and local recycling simultaneously.

Generally, many flavonoids distributed in the plants are flavone glucosides; a deglycosylation process that releases aglycones appears to be a crucial first step in disposition, since it serves as the initiator of all subsequent disposition processes. The hydrolysis of tilianin by LPH is an initial enabling step for all three recycling processes. The main evidence in support of this claim is that LPH inhibitor reduced the absorption of tilianin and excretion of acacetin glucuronide in the bile and perfusate in a dose-dependent manner (Fig. 4). This is expected since deglycosylation by LPH is an important step in the absorption and metabolism of dietary phenolic glycosides, including monoglycosides (quercetin-3-glucoside (25), genistein-7-glucoside (26), and daidzein-7-glucoside (27,28)), diglycosides (icarrin (29), pyridoxine-5'-beta-D-glucoside (30, 31) and resveratrol-glucoside (32)) and triglycosides (epimedin B, epimedin C (29)). The substrates of LPH are proposed to be rapidly hydrolyzed to aglycones and absorbed into the enterocyte, then extensively glucuronidated in the gut. The excreted metabolites from bile or intestine could then be hydrolyzed back to aglycones and reabsorbed into the intestine, completing various recycling processes (*e.g.*, enterohepatic, enteric recycling, and/or local recycling). On the other hand, some phenolic glucosides, such as rutin and baohuoside I (29), which may not be the substrates for LPH, could only be hydrolyzed by bacterial glycosidases in the colon or absorbed by enterocytes directly. And for these glycosides, LPH is not a contributing factor and their disposition could be quite different.

In our present studies, the glucuronidase inhibitor and LPH inhibitor remarkably affect different enzymes that would lead to lower excretion of acacetin glucuronide. When both inhibitors were used simultaneously, however, the excreted amounts of acacetin glucuronide had no difference compared to the glucuronidase inhibitor alone (Fig. 6). We

Fig. 5. Effects of a LPH inhibitor GCL on the mucosa and plasma accumulation/concentration of tilianin and its metabolites were measured in a “four-site” perfused rat intestinal model using a flow rate of 0.168 mL/min. Tilianin was perfused alone or co-perfused with 80 mM GCL. The concentrations of tilianin glucuronide (panel **a**), tilianin (panel **b**), acacetin glucuronide (panel **c**) and acacetin (panel **d**) in the mucosa were determined. The concentrations of tilianin glucuronide (panel **e**), tilianin (panel **f**), acacetin glucuronide (**g**) and acacetin (**h**) in the plasma were also determined. Each column represents the average of four determinations, and the error bar is the S.D. ($n=4$). The “*” symbol means a statistically significant difference at $p<0.05$; and “**” means $p<0.01$.

speculated that although intracellular concentration of acacetin is reduced because of the inhibition of both LPH and glucuronidase, efflux transporter is still the rate-limiting

step. Reduced rate of glucuronidation still provided sufficient amounts of glucuronides for efflux. Hence, the two inhibitors used together did not result in further decrease in glucuronide

Fig. 6. Effects of a glucuronidase inhibitor saccharolactone alone or in combination with a LPH inhibitor on the excretion of acacetin glucuronide in colon. Excretion of acacetin glucuronide in the colon was measured in perfused intestinal model using a flow rate of 0.168 mL/min. Tilianin was perfused alone or in the presence of 0.1 mM SCL or 0.1 mM SCL+20 mM GCL. Rate of acacetin glucuronide excretion (expressed as % per 30 min, panel **a**) and amounts of acacetin glucuronide (panel **b**) excreted in the colon were determined and normalized over a 10 cm intestinal length. Each column represents the average of four determinations, and the error bar is the S.D. ($n=4$). The “*” symbol means a statistically significant difference at $p<0.05$; and “***” means $p<0.01$.

excretion when compared to using either compound alone (Fig. 6).

In addition, it was commonly assumed that inhibition of glucoside hydrolysis might increase the enteric accumulation and biliary excretion of flavone glucoside (*i.e.*, tilianin) (12). It was somewhat unexpected, however, to observe that LPH inhibitor significantly reduced the biliary excretion of tilianin and its glucuronide. To account for this unexpected difference, their accumulation in the intestinal mucosa and plasma were also determined and we were quite surprised that the accumulation of tilianin and tilianin glucuronide in the plasma and intestinal mucosa were enhanced in the presence of glucuronolactone (Fig. 5). The reasons of the phenomenon are not clear. Studies are currently ongoing to determine the mechanisms of hepatic uptake inhibition.

Enterohepatic recycling, enteric recycling, and local recycling of phenolics may exert profound influence on their

Fig. 7. Effects of a glucuronidase inhibitor alone or combination with a LPH inhibitor on the excretion of acacetin glucuronide in the upper small intestine (jejunum). Excretion of acacetin glucuronide in the upper small intestine was measured in perfused intestinal model using a flow rate of 0.168 mL/min. Tilianin was perfused alone or in the presence of 0.1 mM SCL or 0.1 mM SCL+20 mM GCL. Rate of acacetin glucuronide excretion (expressed as % per 30 min, panel **a**) and amounts of acacetin glucuronide (panel **b**) excreted in the upper small intestine were determined and normalized over a 10 cm intestinal length. Each column represents the average of four determinations, and the error bar is the S.D. ($n=4$). The “*” symbol means a statistically significant difference at $p<0.05$; and “***” means $p<0.01$.

disposition in the intestine and liver. Due to its propensity to undergo repeated recycling through the digestive system, phenolics including flavonoids often display apparent plasma half-life much longer than predicted from their low systemic bioavailability. Since glucuronidated flavonoids can be reconverted back to active aglycones by glucuronidase in the small intestine and colon, the long apparent half-life may contribute to the pharmacokinetics of flavonoids. Notably, each recycling is of relative importance. For instance, when an aglycone is administered at high concentration, enterohepatic recycling plays a crucial role in its disposition, since it is rapidly absorbed (saturating the metabolism in the gut) and predominantly metabolized in the liver, followed by the excretion of its glucuronide *via* bile. On the contrary, the aglycone at lower concentration might be extensively absorbed and metabolized in the gut, due to the much higher concentration of the aglycone in the enterocyte than that in

Fig. 8. Schematic representations of triple recycling of acacetin using tilianin as the starting compound and metabolite profile of tilianin [symbols: *T* tilianin, *TG'* tilianin glucuronide, *A* acacetin, *AG'* acacetin glucuronide, β -GUS β -glucuronidase, *UGT* UDP-glucuronosyltransferase, *LPH* lactase phlorizin hydrolase]. Deglycosylation pathway by LPH is marked with red arrow(s). Biliary or enteric excretion of compounds is marked with orange/black letter(s). The local or enteric recycling only needs the involvement of players enclosed in the “round” green or and “smaller” blue circle, whereas enterohepatic recycling needs the involvement of players enclosed in the “larger” purple circle

the portal vein. The excreted glucuronide could be hydrolyzed back to its aglycone, followed with enteric and local recycling without the involvement of enterohepatic recycling. For phenolics whose glucuronides are more bioactive (e.g., ezetimibe (33,34) and morphine (35)), the involvement of three recycling schemes results in longer duration of time in the circulation system and more potential pharmacological activities. For phenolics whose glucuronides are less bioactive (e.g., raloxifene (36–38)), the involvement of the triple

recycling schemes means longer half-life and perhaps long duration of action, since the conjugates could be converted to its aglycone *in vivo*.

Taken together, our data support the hypothesis that tilianin, a glycoside, could directly and indirectly participate in various recycling processes. However, since very little of excreted tilianin is likely to stay intact in the gut after it is excreted, we believe that tilianin and other phenolic glycosides mostly participate in recycling, indirectly, *via* their

corresponding aglycone (for tilianin, it is acacetin). We used tilianin instead of acacetin for the perfusion study because of its much better aqueous solubility (than acacetin). Better solubility allows us to do dose–response study at higher concentrations (5 and 40 μ M tilianin). Higher concentration 40 μ M was chosen because of the need to detect and measure tilianin glucuronide, which was difficult owing to slow glucuronidation rate of tilianin. Our kinetic study has also shown that 40 μ M was a proper concentration used for perfusion study. Detection and measurement of tilianin-7-glucuronide is very important here since it is a newly discovered compound, not yet reported in the literatures.

Based on previous observations that efflux transporters including MRP2 (39, 40) and BCRP (4,41,42), are responsible for the efflux of glucuronides, the involvement of efflux transporters in enterohepatic, enteric, and local recycling holds strong possibility. Further studies are underway to define the specific efflux transporters for the recycling process of tilianin and its metabolites.

In conclusion, our studies described the possible coexistence of enterohepatic, enteric, and local recycling schemes of an aglycone (*i.e.*, acacetin). The fact that a glycoside tilianin could be rapidly hydrolyzed by intestinal LPH to produce acacetin and then participate in triple recycling suggests that many naturally occurring phenolics might share this disposition mechanism. For phenolics (including drugs such as ezetimibe, morphine, and raloxifene), triple recycling processes would also play a vital role in prolonging their plasma half-lives, and increasing their intestinal bioavailability. The latter may be particularly important for drugs such as ezetimibe whose glucuronide is more active in the target organ (*i.e.*, the gut). Future studies that focus on the robustness and wide applicability of this triple recycling mechanism may help us determine how important it is for us to investigate if a compound will undergo triple recycling.

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