Structure- and isoform-specific glucuronidation of six curcumin analogs

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Abstract
1. In the present study, we aimed to characterize the glucuronidation of six curcumin analogs (i.e. RAO-3, RAO-8, RAO-9, RAO-18, RAO-19, and RAO-23) derived from galangal using human liver microsomes (HLM) and twelve expressed UGT enzymes.
2. Formation of glucuronide was confirmed using high-resolution mass spectrometry. Single glucuronide metabolite was generated from each of six curcumin analogs. The fragmentation patterns were analyzed and were found to differ significantly between alcoholic and phenolic glucuronides.
3. All six curcumin analogs except one (RAO-23) underwent significant glucuronidation in HLM and expressed UGT enzymes. In general, the methoxy group (close to the phenolic hydroxyl group) enhanced the glucuronidation liability of the curcumin analogs.
4. UGT1A9 and UGT2B7 were primarily responsible for the glucuronidation of two alcoholic analogs (RAO-3 and RAO-18). By contrast, UGT1A9 and four UGT2Bs (UGT2B4, 2B7, 2B15 and 2B17) played important roles in conjugating three phenolic analogs (RAO-8, RAO-9, and RAO-19). Interestingly, the conjugated double bonds system (in the aliphatic chain) was crucial to the substrate selectivity of gastrointestinal UGTs (i.e. UGT1A7, 1A8 and 1A10).
5. In conclusion, glucuronidation of six curcumin analogs from galangal were structure- and isoform-specific. The knowledge should be useful in identifying a curcumin analog with improved metabolic property.

Keywords
Curcumin analogs, diarylheptanoids, galangal, glucuronidation

Introduction
Diarlyheptanoids are a class of natural products that share the 1,7-diphenylheptane skeleton. They are mainly distributed in the roots and rhizomes of Alpinia, Curcuma and Zingiber species (Lv & She, 2010). Curcumin from turmeric (Curcuma longa) is the one of the most famous diarylheptanoids for putative anti-inflammatory, anti-carcinogenic and anti-Alzheimer effects (Epstein et al., 2010). Galangal, the rhizome of Alpinia officinarum Hance (Zingiberae), is a spice widely used in Europe and China for over 1000 years. Phytochemical studies of this medicinal plant have led to the isolation of many diarylheptanoids (curcumin analogs) that possess diverse pharmacological effects such as antiemetic (Shin et al., 2002; Yang et al., 2002), anti-inflammatory (Li et al., 2011; Matsuda et al., 2006), anti-tumor (Chen et al., 2015; Lee et al., 2012; Matsuda et al., 2009), and anti-oxidant activities (Kose et al., 2015; Riethmuller et al., 2015).

Curcumin is poorly bioavailable after oral administration (Anand et al., 2007). Extensive metabolism is found to be one of the causes of the low oral bioavailability (Anand et al., 2007; Metzler et al., 2013). The reduction, glucuronidation and sulfation reactions are three major metabolic pathways for curcumin and its analogs (Anand et al., 2007; Metzler et al., 2013). Alcohol dehydrogenase is the enzyme responsible for reduction of curcuminoids (e.g. curcumin, demethoxy-curcumin and diemethoxy-curcumin), whereas UDP-glucuronosyltransferase (UGT) and sulfotransferase (SULT) enzymes are responsible for the conjugative reactions (glucuronidation and sulfation) of curcuminoids and their reductive products. The phase II metabolites (glucuronides and sulfates) represent the majorities of circulating curcuminoids in human and animals (Asai & Miyazawa, 2000; Liu et al., 2012; Vareed et al., 2008). It has been demonstrated that several UGTs (1A1, 1A8, 1A9, 1A10 and 2B7) and SULTs (1A3, 1B1, 1C4 and 1E1) are highly active in conjugating curcuminoids and hexahydro-curcuminoids (Hoehle et al., 2007; Lu et al., 2015; Pfeiffer et al., 2007).

Curcumin and its analogs are a type of agents with diverse therapeutic potential. Thus, it is of great value to establish the structure-metabolism relationships in an attempt to identify a structure with favorable pharmacokinetics. In the present study, we aimed to determine the glucuronidation potential of six curcumin analogs using human liver microsomes (HLM) and twelve expressed UGT enzymes. Glucuronidation rate was measured by incubation assays of the analogs with...
UDPGA-supplemented enzyme preparations. Formation of glucuronide metabolite was confirmed using high-resolution mass spectrometry. Kinetic parameters were derived by fitting an appropriate model to the data of metabolic rates versus a wide range of substrate concentrations. Based on the metabolic rates and kinetic parameters, we analyzed the structure- and isoform-specific glucuronidation of curcumin analogs.

Materials and methods

Materials

Expressed human UGT enzymes (supersomes) including eight UGT1A members (1A1, 1A3, 1A4, 1A6, 1A7, 1A8, 1A9 and 1A10) and four UGT2B members (2B4, 2B7, 2B15 and 2B17), and pooled human liver microsomes (HLM, \( n = 50 \)) were purchased from BD Biosciences (Woburn, MA). Uridine diphosphoglucuronic acid (UDPGA), alamethicin, and D-saccharic-1,4-lactone monohydrate were purchased from Sigma-Aldrich (St Louis, MO). LC-grade acetonitrile and formic acid were purchased from Millipore (Billerica, MA). Deionized water was obtained using a Milli-Q water purification system (Millipore, Billerica, MA). Six curcumin analogs (Figure 1) used in this study were extracted and isolated from the rhizomes of *Alpinia officinarum* Hance (galangal) as described in our previous report (Tang et al., 2015). Their chemical structures were characterized and confirmed by 1-D and 2-D NMR. The purity of each compound was determined to be >98% by HPLC analysis.

Glucuronidation assay

A modified method was used to determine the glucuronidation activity of the tested enzymes toward six curcumin analogs as described previously (Lu et al., 2016). In brief, the incubation medium contained liver microsomes or expressed UGT enzyme (25 \( \mu \)g/ml), MgCl\(_2\) (0.88 mM), saccharolactone (4.4 mM), alamethicin (20 \( \mu \)g/ml), and curcumin analog (dissolved in DMSO) in a total volume of 0.2 mL of 50 mM potassium phosphate (pH 7.4). After pre-incubation at 37°C for 5 min, the reaction was initiated by the addition of UDPGA (2 mM) and subsequently incubated at 37°C for 60 min. Later 100 \( \mu \)L ice-cold acetonitrile was added to stop the reaction, followed by vortex and centrifugation (10 min; 15 000g), and 10-\( \mu \)L aliquot of the supernatant was subjected to UPLC-QTOF/MS analysis. The organic solvent (DMSO) concentration in the incubation mixture was kept at 1%. All experiments were performed in triplicate. Preliminary experiments showed that the glucuronide formation rate was linear with incubation time (up to 180 min) and protein concentration (up to 0.2 mg/ml).

Structural characterization of metabolites by UPLC-QTOF/MS

To identify and characterize UGT metabolites, samples containing curcumin analogs and their metabolites were injected to an UPLC-QTOF/MS system (Waters Corporation, Milford, MA). Chromatographic separation was performed using the Waters ACQUITY UPLC system (Waters Corporation, Milford, MA) equipped with a Kinetex C18 column (2.1 × 50 mm, 2.6 \( \mu \)m, Phenomenex, Torrance, CA). The mobile phase was formic acid (0.1%) in water (mobile phase A) and formic acid (0.1%) in acetonitrile (mobile phase B). The flow rate was set at 0.35 ml/min. The gradient elution program was 5% B at 0 to 1 min, 5 to 90% B at 1 to 3.3 min, and 90 to 5% B at 3.3 to 4 min. Mass spectrometry analysis was performed on the Xevo G2 Q-TOF/MS (Waters Corporation, Milford, MA) using the electrospray ionization source (under negative ion mode). The capillary, sampling cone, and extraction cone voltages were 2500, 25 and 4 V, respectively. The source and desolvation temperature were 110 and 350, respectively. The cone gas and desolvation gas was set to 30 and 600 l/h, respectively. To obtain informative fragment ions, collision energies ranging from 10 to 30 eV were used for MS/MS scanning. The spectra were acquired and processed using MassLynx software (Waters Corporation, Milford, MA).

Quantification of glucuronides

Glucuronides were quantified with the abovementioned Waters UPLC-QTOF/MS system using home-made reference standards (Waters Corporation, Milford, MA). The procedures for preparing glucuronide standards have been described elsewhere (Lu et al., 2016; Sun et al., 2015). In brief, the target

Figure 1. Structures, chemical names and coding names (in bracket) of six curcumin analogs from galangal. The arrows refer to the potential conjugating sites for UGTs.
glucuronides were synthesized according to the glucuronidation assay protocol. Samples containing the glucuronides were subjected to the UPLC system equipped with a photo-diode array detector (Waters Corporation, Milford, MA). The elution fractions containing the metabolites were collected in vials and then dried using Eppendorf Concentrator Plus (Hamburg, Germany). The residue was reconstituted in phosphate buffer (pH 7.4) to make a stock solution. The concentration of stock solution was determined through complete conversion of glucuronide to parent molecule (by β-glucuronidase, 25 U) and quantitation of the parent molecule using its reference standard (measured at 280 nm). A series of working standards (0.005–10 μM) of glucuronides were prepared by serial dilution of the stock solution. These working solutions were verified to be stable during the analytical period. Quantification of glucuronides was performed using the exacted ion chromatograms of their respective precursor ions with a mass window of ±0.05 Da (Table 1).

**Absolute quantification of UGT protein concentrations in BD supersomes**

The absolute amount of UGT isoform in BD supersomes was determined in order to make an accurate comparison of UGT activities. The enzymes of supersomes were trypsin-digested under the optimized condition and quantified using their activities. The enzymes of supersomes were trypsin-digested to make an accurate comparison of UGT activities. The absolute amount of UGT isoform in BD supersomes was determined in order to make an accurate comparison of UGT activities. The enzymes of supersomes were trypsin-digested under the optimized condition and quantified using their activities. The enzymes of supersomes were trypsin-digested under the optimized condition and quantified using their activities.

**Kinetic evaluation**

The formation rates of glucuronides were determined for six curcumin analogs according to the glucuronidation assay protocol. All substrates were evaluated at nine concentrations ranging from 0.39 to 75 μM (except for RAO-8). For RAO-8, substrate concentrations were 0.04–12.5 μM for UGT1A9, 0.04–25 μM for UGT2B4, and 0.2–75 μM for UGT2B15, respectively. The kinetic model Michaelis–Menten equation (Equation (1)) or substrate inhibition equation (Equation (2)) or Hill equation (Equation (3)) was fitted to the data of formation rates versus substrate concentrations using the Graphpad Prism V5 software (GraphPad Software, Inc., La Jolla, CA). Model selection was based on visual inspection of the characteristic Eadie–Hofstee plots (Hutzler & Tracy, 2002).

**Table 1. Chromatographic and mass spectrometric characteristics of six curcumin analogs and their glucuronides in human liver microsomes.**

| Analyte  | R<sub>t</sub> (min) precursor ion | Product ions | R<sub>t</sub> (min) precursor ion | Product ions | type          |
|----------|----------------------------------|--------------|----------------------------------|--------------|---------------|
| RAO-3    | N.A.<sup>a</sup>                | N.D.<sup>c</sup> | N.D.                             |              |               |
| RAO-8    | 2.83                             | 309.44 [M – H] | 309.45, 173.43                   |              |               |
| RAO-9    | 2.79                             | 279.40 [M – H] | 279.41, 173.43                   |              |               |
| RAO-18   | N.D.                             | N.D.         | N.D.                             |              |               |
| RAO-19   | 2.91                             | 355.49 [M – H] | 355.50, 309.49, 173.43           |              |               |
| RAO-23   | N.D.                             | N.D.         | N.D.                             |              |               |

<sup>a</sup>R<sub>t</sub>, retention time.
<sup>b</sup>N.A., not applicable.
<sup>c</sup>N.D., not detected.

where

\[
V = \frac{V_{\text{max}} \cdot [S]}{K_m + [S]} \tag{1}
\]

where \(V\) is the formation rate of product, \(V_{\text{max}}\) is the maximal velocity, \(K_m\) is the Michaelis constant and \([S]\) is the substrate concentration.

Substrate inhibition equation:

\[
V = \frac{V_{\text{max}} \cdot [S]}{K_m + [S] \times \left(1 + \frac{[S]}{K_{i}}\right)} \tag{2}
\]

where \(K_{i}\) is the substrate inhibition constant.

Hill equation:

\[
V = \frac{V_{\text{max}} \cdot [S]^n}{S_{50}^n + [S]^n} \tag{3}
\]

where \(S_{50}\) is the substrate concentration resulting in 50% of \(V_{\text{max}}\), and \(n\) is the Hill coefficient. For the Michaelis–Menten
and substrate inhibition models, the intrinsic clearance (CL_{int}) was derived by \( V_{\text{max}}/K_m \); for the Hill model, the maximal clearance (CL_{max}) was obtained using Equation (4).

\[
CL_{\text{max}} = \frac{V_{\text{max}}}{S_{50}} \times \frac{n - 1}{n(n - 1)^{1/n}}
\]

### Statistical analysis

Data are presented as mean ± SD. Statistical differences were analyzed by one-way analysis of variance or Student's t test as appropriate, and the level of significance was set at \( p < 0.05 \).

### Results and discussion

#### Structural characterization of six curcumin analogs and their glucuronides by UPLC-QTOF/MS

The six curcumin analogs were introduced into the QTOF mass spectrometer. The analogs containing a phenolic hydroxyl group (i.e. RAO-8, RAO-9, and RAO-19) were detected, whereas those containing alcoholic hydroxyl (i.e. RAO-3, RAO-18, and RAO-23) were not (Table 1). This indicated that ESI source may not be a good choice when analyzing RAO-3, RAO-18, and RAO-23. RAO-8, RAO-9, and RAO-19 formed deprotonated molecules \([M - H]^-\) at m/z 309.44, 279.40 and 355.49, respectively (Table 1). They produced an identical product ion at m/z 173.43 (Table 1), suggestive of a similar fragmentation pattern. RAO-19 reduced the ethanol group to the chemical skeleton shared by RAO-8 and RAO-9 (Figure 2). Further, the bond between C6 and C7 was cleaved, and the typical fragment ion at m/z 173.43 was generated upon a chemical rearrangement (Figure 2).

HLM generated a single metabolite from each of the six curcumin analogs. The metabolites have an increase of 176 Da in mass compared with their respective parent compounds, indicating that monoglucuronides were generated (Table 1). This was corroborated by the fact that the metabolites cannot be generated in the absence of UDPGA, the glucuronide acid donor. Interestingly, the alcoholic and phenolic glucuronides differed in the fragmentation pattern (Table 1 and Figure 3). The alcoholic glucuronides (of RAO-3, RAO-18 and RAO-23) generated a fragment ion at m/z 193 Da, whereas the phenolic glucuronides (of RAO-8, RAO-9 and RAO-19) formed a fragment ion at m/z 175 Da (Figure 3). A difference of 16 Da in the molecular weight corresponded exactly to the addition/lack of an oxygen atom, revealing a distinct fragmentation between the alcoholic and phenolic glucuronides (Figure 3).

The product ion of m/z 193 Da was not observed in the alcoholic glucuronides such as the glucuronides of estradiol (17-glucuronide) and 3’-azido-3’-deoxythymidine (Barbier et al., 2000; Diaz-Cruz et al., 2003). However, this product ion was observed in the alcoholic glucuronide of nabumetone (Noblis et al., 2004), as well as the acyl glucuronide of phenylacetic acids (Karlsson et al., 2010). Moreover, the curcumin analog hexahydro-O,O-dimethyl-curcumin also produced such a characteristic ion (Pfeiffer et al., 2007). The results from previous and current study suggested this ion might be a characteristic fragment for alcoholic glucuronides of curcumin analogs (in which the conjugated double bond system in the aliphatic chain of curcumin is reduced). Therefore, our findings provided a useful MS/MS approach for quick differentiation of alcoholic and phenolic glucuronides of curcumin analogs.

#### Reaction kinetics for glucuronidation of curcumin analogs by HLM

Kinetic profiling revealed that formation of RAO-3 glucuronide in HLM followed the classical Michaelis–Menten kinetics (Figure 4). The glucuronidation profiles of RAO-8, RAO-9, RAO-18, and RAO-19 were well modeled by the substrate inhibition equation (Figure 4). In contrast, the glucuronide formation of RAO-23 in HLM was too slow (<2 pmol/min/mg protein), disallowing a full kinetic characterization. The \( V_{\text{max}}, K_m \), and CL_{int} values ranged from 230 to 2320 pmol/min/mg protein, from 3.04 to 12.0 μM, and from 30.5 to 650 μl/min/mg protein, respectively (Table 2). Three phenolic substrates (RAO-8, RAO-9 and RAO-19) had significantly higher CL_{int} values than those of RAO-3 and RAO-18 (Table 2). In addition, the CL_{int} values of RAO-8 and RAO-19 were more than 2-fold higher than that of RAO-9 (Table 2).

#### Absolute enzyme levels in BD supersomes

Signature peptides of twelve UGT isoforms were synthesized and their structures were confirmed by MS/MS spectra (Figures S1 & S2). The absolute levels of various UGT enzymes were determined by UPLC-QTOF/MS using their respect signature peptides. The UGT level in their respect BD supersomes was as follow: 1371 (UGT1A1), 722 (UGT1A3), 644 (UGT1A4), 523 (UGT1A6), 1004 (UGT1A7), 456 (UGT1A8), 1135 (UGT1A9), 1770 (UGT1A10), 1347 (UGT2B4), 354 (UGT2B7), 689 (UGT2B15), and 1549 (UGT2B17) pmol/mg. There was a large difference between UGT concentrations with a maximal difference of 5.0-fold. Consistently, Fallon et al. (2013a) revealed a large variability (up to 6.9-fold) in enzyme levels of recombinant UGTs. The results suggested that glucuronidation rates per mg total protein in vitro will not accurately reflect the true specific activity of UGT enzymes for a particular compound (Fallon et al., 2013a). Absolute quantification of UGT levels in recombinant materials herein allowed determination of glucuronidation as per nmol of UGT isoform, facilitating precise evaluation of enzyme selectivity toward a compound.

#### Reaction phenotyping with UGT enzymes

To identify the enzymes involving in the glucuronidation of curcumin analogs, twelve UGT isoforms were screened for
their catalysis activities (expressed as pmol/min/nmol) at the substrate concentrations of 5 and 50 μM. The metabolic profiles were similar at two test substrate concentrations (Figure 5). UGT1A9 and UGT2B7 were the two enzymes capable of glucuronidating all six curcumin analogs (Figure 5). UGT1A6, 1A8, 1A10 and 2B15 did not show any activities...
toward three alcoholic substrates (RAO-3, RAO-18 and RAO-23) (Figure 5). RAO-3 and RAO-18 were primarily metabolized by UGT1A9 and UGT2B7, whereas three phenolic substrates (RAO-8, RAO-9 and RAO-19) were extensively glucuronidated by multiple UGT1As and UGT2Bs (Figure 5). In general, UGT2B7 showed the highest glucuronidation activities, whereas UGT1A3, 1A4 and 1A7 showed negligible activities. Compared with other five curcumin analogs, RAO-23 was a very-poor substrate for all UGT isoforms. This was consistent with its exceedingly low glucuronidation activity in HLM.

**Glucuronidation kinetics by recombinant UGT enzymes**

Based on the results of reaction phenotyping, we further performed kinetic analyses for active UGTs using a series of substrate concentrations (Figures S3–S5 for kinetic profiles, Tables 3–5 for derived kinetic parameters). The $V_{max}$, $K_m$, and $CL_{int}$ values varied from 19.1 to 9681 pmol/min/mg, from 1.08 to 5797 μM, and from 1.08 to 5797 μL/min/mg, respectively. UGT1A9 and UGT2B7 were the main enzymes contributing to the glucuronidation of RAO-3 and RAO-18, whereas multiple enzyme (UGT1A9 and four UGT2Bs) were actively involved in glucuronidation of RAO-8, RAO-9 and RAO-19 (Figure 6). Compared with alcoholic hydroxyl substrates, all UGT enzymes except UGT1A9 showed high affinity (with lower $K_m$ values) and high $CL_{int}/CL_{max}$ values toward the phenolic substrates (Tables 3–5 and Figure 6).

The kinetics profiles of RAO-3 and RAO-18 by UGT1A9 (and UGT2B7) were well described by the Michaelis–Menten and substrate inhibition equation, respectively (Figures S3 & S5). These were in line with their glucuronidation profiles in HLM (Figure 4), suggesting that UGT1A9 and UGT2B7 were indeed two crucial enzymes responsible for hepatic glucuronidation of RAO-3 and RAO-18. UGT1A9 and four UGT2Bs were primary contributors to the glucuronidation of RAO-8 in HLM, as evidenced by the consistent substrate inhibition kinetics (Figures 4, S3 & S5). HLM-mediated glucuronidation of RAO-9 and RAO-19 followed the substrate inhibition kinetics (Figure 4), whereas glucuronidation of the two compounds by UGT2Bs (the main contributors) did not always follow the same kinetics (Figure S5). RAO-23 was resistant to the metabolism by all UGTs, suggesting that a keto-enol tautomerism or a tight intramolecular hydrogen bond may preclude binding of the molecule to the UGT enzymes. Taken together, UGT1A9 and UGT2B7 played the most important roles in the glucuronidation of curcumin analogs.

Our finding that UGT1A9 and UGT2B7 made significant contributions to metabolism of alcoholic curcumin analogs (RAO-3 and RAO-18) were consistent with previous studies in which the two enzymes show marked activities toward hexahydro-cumurinoids and alcoholic gingerols (a class of curcumin structurally related compounds) (Hoehle et al., 2007; Wu et al., 2015). In fact, UGT1A9 and UGT2B7 showed glucuronidation activities toward a broad range of compounds containing an alcoholic hydroxyl group. These compounds include 1’-hydroxyestragole (Iyer et al., 2003), 3’-azido-3’-deoxyxymidine (Barbier et al., 2000), 4-(methyl-nitro-samin)-1-(3-pyridyl)-1-butanol (Ren et al., 2000), 4-hydroxyretinoic acid (Samokyszyn et al., 2000), almokalant (Gaiser et al., 2003), bicalutamide (Grosse et al., 2013), dihydroartemisinin (Ilett et al., 2002), ethanol (Al Saabi et al., 2013; Schwab & Skopp, 2014), ornidazole (Du et al., 2013), propafenone (Xie and Zeng, 2010), propranolol (Yu et al., 2013; Schwab & Skopp, 2014), ornidazole (Du et al., 2013), propafenone (Xie and Zeng, 2010), propranolol (Yu et al., 2010) and R-oxazepam (Court et al., 2002). It should be noted that both hepatic and renal UGTs would contribute to the metabolism of these curcumin analogs, because UGT1A9 and UGT2B7 were abundantly expressed in human liver and kidney (Fallon et al., 2013a; Sato et al., 2014).

RAO-8 and RAO-19, but not RAO-9, possess a methoxy group at the ortho-position of the phenolic hydroxyl group. RAO-8 and RAO-19 were much more efficiently glucuronidated compared with RAO-9. This clearly indicated that the addition of a methoxy group increased the metabolic susceptibility. It was interesting to note that the glucuronidation activities of three curcuminoids by human liver, intestine, and recombinant UGTs were in the order of curcumin > demethoxy-curcumin > bisdemethoxy-curcumin (Hoehle et al., 2007). Similar results were observed when hexahydro-cumurinoids were used as the substrates (Hoehle et al., 2007). Therefore, it may be a general rule that the methoxy group (close to the phenolic hydroxyl group) enhances the glucuronidation liability of the curcumin analogs.

The gastrointestinal UGTs (i.e. UGT1A7, UGT1A8, and UGT1A10) showed negligible or weak activities toward the six curcumin analogs (Table 4 and Figure 6). In line with this finding, glucuronidation activities of the gastrointestinal UGTs were significantly reduced when curcuminoids were transformed to their hexahydro derivatives (Hoehle et al., 2007). In contrast, the gastrointestinal UGTs (e.g. UGT1A8, and UGT1A10) showed high metabolic activities for curcuminoids (Hoehle et al., 2007; Pfeiffer et al., 2007). Our results and previous one strongly indicated the
conjugated double bonds system in the aliphatic chain was important for the substrate selectivity of gastrointestinal UGTs toward the curcumin analogs. Our results overall suggested that glucuronidation may be a determining factor to the pharmacokinetics of curcumin analogs from galangal. The oral bioavailability of these compounds may be significantly influenced by first-pass glucuronidation in the intestine and liver, highly expressing UGT1As and UGT2Bs. It was noteworthy that other metabolic enzymes such as cytochrome P450 and sulfotransferases might also contribute to the metabolism and pharmacokinetics of these curcumin analogs. On the other hand, it was uncovered that the glucuronidation was highly dependent on the chemical structure (Figure 6). Therefore, it represented a promising approach to improve the pharmacokinetics of curcumin analogs through modification of their chemical structures. For example, an analog with a chemical structure of RAO-23 would be least glucuronidated thus is expected to have an improved oral bioavailability.

**Conclusion**

We have characterized the glucuronidation of six curcumin analogs by HLM and twelve expressed UGTs. It was found that the alcoholic and phenolic glucuronides differed in the generation of MS/MS product ions, allowing quick differentiation of these two types of glucuronides. Further,
### Table 3. Kinetics parameters of four hepatic UGT1As-catalyzed glucuronidation of curcumin analogs.

| Substrate | $V_{\text{max}}$ pmol/min/nmol | $K_{\text{m}}/S_{50}$ μM | $n$ | $K_i$ μM | $C_{\text{int}}/C_{\text{max}}$ μL/min/nmol | Model$^a$ |
|-----------|-------------------------------|--------------------------|-----|----------|------------------------------------------|---------|
| RAO-3     | 113 ± 7.06                    | 8.29 ± 1.21              | 1.57 ± 0.30 | N.A.$^b$ | 13.6 ± 2.16                               | Hill    |
| RAO-8     | 19.1 ± 0.90                   | 4.92 ± 0.89              | N.A. | N.A.     | 3.88 ± 0.73                               | MM      |
| RAO-19    | 298 ± 12.7                    | 7.85 ± 1.15              | N.A. | N.A.     | 38.0 ± 5.79                               | MM      |
| UGT1A3    | RAO-3                         | 168 ± 12.7               | N.A. | N.A.     | 5.92 ± 1.00                               | MM      |
|           | RAO-8                         | 78.4 ± 3.91              | N.A. | N.A.     | 26.05 ± 5.60                              | MM      |
|           | RAO-19                        | 97.2 ± 6.86              | N.A. | N.A.     | 42.4 ± 13.5                               | MM      |
|           | RAO-23                        | 60.6 ± 2.34              | N.A. | N.A.     | 2.34 ± 0.24                               | MM      |

$^a$MM, Michaelis–Menten model. SI, substrate inhibition. Hill, fitted to the Hill equation.

$^b$N.A., not applicable.

### Table 4. Kinetics parameters of three gastrointestinal UGT1As-catalyzed glucuronidation of curcumin analogs.

| Substrate | $V_{\text{max}}$ pmol/min/nmol | $K_{\text{m}}/S_{50}$ μM | $n$ | $K_i$ μM | $C_{\text{int}}/C_{\text{max}}$ μL/min/nmol | Model$^a$ |
|-----------|-------------------------------|--------------------------|-----|----------|------------------------------------------|---------|
| UGT1A7    | RAO-3                         | 379 ± 26.7               | 41.6 ± 6.03 | N.A. | 9.11 ± 1.47                               | MM      |
|           | RAO-8                         | 91 ± 18.7                | 0.80 ± 0.35 | N.A. | 114 ± 55                                  | SI      |
|           | RAO-18                        | 25.3 ± 0.6               | 2.33 ± 0.27 | 1.95 ± 0.24 | 10.8 ± 1.28                              | MM      |
| UGT1A8    | RAO-8                         | 202 ± 25.7               | 1.46 ± 0.49 | N.A. | 61.7 ± 23.4                               | MM      |
|           | RAO-9                         | 51.9 ± 3.67              | 48.3 ± 6.67 | N.A. | 1.08 ± 0.17                               | MM      |
|           | RAO-19                        | 794 ± 26.5               | 21.6 ± 1.86 | N.A. | 36.8 ± 3.39                               | MM      |
| UGT1A10   | RAO-8                         | 254 ± 9.60               | 1.35 ± 0.33 | 1.17 ± 0.13 | 217 ± 25.5                                | SI      |
|           | RAO-9                         | 38.7 ± 7.32              | 9.76 ± 2.01 | N.A. | 187 ± 36.9                                | SI      |
|           | RAO-19                        | 162 ± 23.3               | 13.2 ± 3.70 | N.A. | 178 ± 30.1                                | SI      |

$^a$MM, Michaelis–Menten model. SI, substrate inhibition. Hill, fitted to the Hill equation.

$^b$N.A., not applicable.

### Table 5. Kinetics parameters of hepatic UGT2Bs-catalyzed glucuronidation of curcumin analogs.

| Substrate | $V_{\text{max}}$ pmol/min/nmol | $K_{\text{m}}/S_{50}$ μM | $n$ | $K_i$ μM | $C_{\text{int}}/C_{\text{max}}$ μL/min/nmol | Model$^a$ |
|-----------|-------------------------------|--------------------------|-----|----------|------------------------------------------|---------|
| UGT2B4    | RAO-3                         | 197 ± 6.53               | 6.95 ± 1.32 | 1.47 ± 0.15 | 28.3 ± 5.46                               | MM      |
|           | RAO-8                         | 345 ± 61.3               | 0.35 ± 0.14 | N.A. | 5.73 ± 2.29                              | SI      |
|           | RAO-9                         | 211 ± 19.8               | 1.50 ± 0.76 | N.A. | 36.7 ± 7.07                              | SI      |
|           | RAO-19                        | 303 ± 53.7               | 1.44 ± 0.70 | 19.6 ± 3.66 | 210 ± 109                                 | SI      |
| UGT2B7    | RAO-3                         | 4073 ± 143               | 11.8 ± 1.28 | N.A. | 113 ± 4.61                                | MM      |
|           | RAO-8                         | 9681 ± 996               | 1.67 ± 0.44 | N.A. | 63.7 ± 19.4                              | SI      |
|           | RAO-9                         | 792 ± 33.0               | 2.29 ± 0.41 | N.A. | 352 ± 65.8                                | SI      |
|           | RAO-18                        | 635 ± 19.8               | 5.31 ± 0.62 | N.A. | 120 ± 14.5                                | MM      |
|           | RAO-19                        | 5724 ± 235               | 2.50 ± 0.28 | N.A. | 417 ± 156                                 | SI      |
| UGT2B15   | RAO-8                         | 1617 ± 203               | 0.44 ± 0.14 | N.A. | 5.08 ± 1.21                              | SI      |
|           | RAO-9                         | 1377 ± 73.6              | 4.07 ± 0.39 | N.A. | 25.3 ± 2.52                              | SI      |
|           | RAO-19                        | 1143 ± 333               | 4.20 ± 1.46 | N.A. | 41.0 ± 13.3                              | SI      |
| UGT2B17   | RAO-3                         | 82.86 ± 3.57             | 17.0 ± 2.03 | N.A. | 4.87 ± 0.62                               | MM      |
|           | RAO-8                         | 757 ± 42.6               | 1.42 ± 0.24 | N.A. | 189 ± 58.3                               | SI      |
|           | RAO-9                         | 39.0 ± 1.35              | 5.54 ± 0.71 | N.A. | 7.04 ± 0.93                               | SI      |
|           | RAO-19                        | 2698 ± 68.8              | 4.06 ± 0.41 | N.A. | 665 ± 69.2                                | MM      |

$^a$MM, Michaelis–Menten model. SI, substrate inhibition. Hill, fitted to the Hill equation.

$^b$N.A., not applicable.
glucuronidation was highly dependent on both the chemical structures and the isozymes. Compared with other analogs, RAO-23 was least glucuronidated by HLM and UGTs. The two alcoholic analogs (i.e. RAO-3 and RAO-18) were primarily glucuronidated by UGT1A9 and UGT2B7, whereas three phenolic analogs (i.e. RAO-8, RAO-9, and RAO-19) were mainly metabolized by UGT1A9 and four UGT2B enzymes. The knowledge should be useful in identifying a curcumin analog with improved metabolic property.

Declaration of interest

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