

Curcuminoids Form Reactive Glucuronides In Vitro

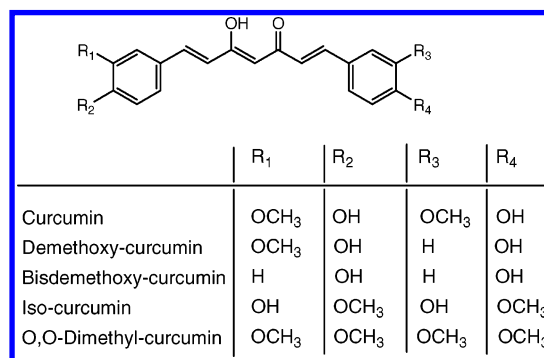
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Curcumin is of current interest because of its putative anti-inflammatory, anticarcinogenic, and anti-Alzheimer's activity, but its pharmacokinetic and metabolic fate is poorly understood. The present in vitro study has therefore been conducted on the glucuronidation of curcumin and its major phase I metabolite, hexahydro-curcumin, as well as of various natural and artificial analogs. The predominant glucuronide generated by rat and human liver microsomes from curcumin, hexahydro-curcumin, and other analogs with a phenolic hydroxyl group was a phenolic glucuronide according to LC-MS/MS analysis. However, a second glucuronide carrying the glucuronic acid moiety at the alcoholic hydroxyl group was formed from the same curcuminoids, but not hexahydro-curcuminoids, by human microsomes. Curcuminoids without a phenolic hydroxyl group gave rise to the aliphatic glucuronide only. The phenolic glucuronides of curcuminoids, but not of hexahydro-curcuminoids, were rather lipophilic and, in part, unstable in aqueous solution, their stability depending strongly on the type of aromatic substitution. The phenolic glucuronide of curcumin and of its natural congeners, but not the parent compounds, clearly inhibited the assembly of microtubule proteins under cell-free conditions, implying chemical reactivity of the glucuronides. These novel properties of the major phase II metabolites of curcuminoids deserve further investigation.

KEYWORDS: Curcumin; glucuronide; microsomes; microtubule proteins

INTRODUCTION

Curcuminoids are the yellow-orange pigments of turmeric, which is obtained from the rhizomes of the east Indian plant *Curcuma longa* and used extensively in traditional Indian cooking and Ayurvedic herbal remedies (1–3). Turmeric contains curcumin as the major component, together with smaller amounts of demethoxy-curcumin and bisdemethoxy-curcumin (4). The chemical structure of these curcuminoids is that of a diarylheptanoid with differently substituted aryl rings and a conjugated aliphatic structure (Figure 1). Turmeric has long been recognized for its broad spectrum of biological activities, in particular, its anti-inflammatory, antioxidant, and anticarcinogenic effects (2, 5, 6). More recent evidence suggests that curcumin may prevent or even cure Alzheimer's disease because it has been shown to decrease insoluble and soluble β -amyloid and plaque burden in the brain of an Alzheimer transgenic mouse model (7, 8). Accordingly, experimental and clinical studies on the beneficial effects of curcuminoids are increasing, and the number of nutritional supplements containing

**Figure 1.** Chemical structures of curcuminoids.

curcuminoids is rising. However, information on the metabolism of curcuminoids and their pharmacokinetic fate is still scant. Curcumin is known to be readily converted to several reductive metabolites, mostly hexahydro-curcumin, and conjugates of both the parent compound and its phase I metabolites with glucuronic acid and sulfate have been identified in studies with rats and mice in vivo (9–14).

In a recent study using rat liver slices and subcellular fractions, we have shown that the three natural curcuminoids differ in their chemical stability, whereas their hexahydro metabolites are stable (15). In the present study, we have investigated the glucuronidation of curcuminoids in more detail.

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To facilitate the elucidation of the chemical structures and the reactivities of the glucuronides, several non-natural analogs of curcumin and their hexahydro derivatives were synthesized and included in this study (**Figure 1**). Iso-curcumin is a positional isomer of curcumin in which the hydroxyl and methoxyl group at each aromatic ring have switched positions, whereas both phenolic hydroxyl groups of curcumin are replaced by methoxyl groups in *O,O*-dimethyl-curcumin. We have found that the phenolic hydroxyl group is the preferred site of glucuronidation for all curcuminoids. However, several of the phenolic glucuronides, including that of curcumin, exhibited unexpected reactivity and lipophilicity, which was strongly dependent on the exact position of the phenolic groups and the presence of olefinic double bonds in the aliphatic chain.

MATERIALS AND METHODS

Chemicals, Animals, and Cell Fractions. Demethoxy-curcumin was isolated from turmeric by extraction, column chromatography, and crystallization at the Arizona Center for Phytomedicine Research (Tucson, AZ). It was >99% pure according to HPLC analysis. Symmetrically substituted curcuminoids, i.e., curcumin, bisdemethoxy-curcumin, iso-curcumin, and *O,O*-dimethyl-curcumin, were chemically synthesized as described earlier (15), using the method of Pabon (16). Briefly, acetylacetone was reacted with the appropriate substituted benzaldehyde and boric acid anhydride as a catalyst. The final products obtained after crystallization from methanol were >99% pure according to HPLC analysis and exhibited the correct molecular ions in LC-MS analysis. Iso-curcumin and *O,O*-dimethyl-curcumin are novel compounds. Iso-curcumin had a melting point of 186 °C and a λ_{max} at 419 nm with $\epsilon = 59200$ in ethanol. The mass of its molecular ion as determined by high-resolution electron impact mass spectrometry (HREIMS) was 368.1257 (calculated for $\text{C}_{21}\text{H}_{20}\text{O}_6$: 368.1260). The ^1H and ^{13}C NMR spectral data of iso-curcumin were as follows: ^1H NMR (500 MHz, acetone- d_6): δ 3.92 (s, 6H), 6.05 (s, 1H), 6.71 (d, 2H, $^3J = 15.8$ Hz), 7.03 (d, 2H, $^3J = 8.2$ Hz), 7.17 (ddd, 2H, $^4J = 0.6$ Hz, $^4J = 2.0$ Hz, $^3J = 8.2$ Hz), 7.23 (d, 2H, $^4J = 2.0$ Hz), 7.60 (d, 2H, $^3J = 15.8$ Hz), 7.85 (bs, 1H). ^{13}C NMR (125 MHz, acetone- d_6): δ 55.4 (q, 2C), 101.1 (d, 2C), 111.5 (d, 2C), 113.5 (d, 2C), 121.6 (d, 2C), 122.0 (d, 2C), 128.4 (s, 2C), 140.3 (d, 2C), 147.0 (s, 2C), 149.7 (s, 2C), 183.6 (s, 2C). A distinct difference between curcumin and iso-curcumin was noted when hydroxide ions were added to a neutral aqueous solution of the curcuminoid: whereas the curcumin solution turned red, the solution of iso-curcumin remained yellow-orange due to the meta positions of the hydroxyl groups which prevent their participation in the conjugated π system. *O,O*-Dimethyl-curcumin had a melting point of 128–130 °C and a λ_{max} at 418 nm with $\epsilon = 56200$ in ethanol. The mass of its molecular ion was 396.1571 (calculated for $\text{C}_{23}\text{H}_{24}\text{O}_6$: 396.1573). ^1H NMR (400 MHz, CDCl_3): δ 1.70 (bs, 1H), 3.92 (s, 6H), 3.94 (s, 6H), 5.82 (s, 1H), 6.50 (d, 2H, $^3J = 15.8$ Hz), 6.88 (d, 2H, $^3J = 8.4$ Hz), 7.08 (d, 2H, $^4J = 2.0$ Hz), 7.14 (ddd, 2H, $^4J = 0.6$ Hz, $^4J = 2.0$ Hz, $^3J = 8.2$ Hz), 7.61 (d, 2H, $^3J = 15.8$ Hz). ^{13}C NMR (100 MHz, CDCl_3): δ 55.9 (q, 2C), 56.0 (q, 2C), 101.3 (d, 2C), 109.7 (d, 2C), 111.1 (d, 2C), 122.0 (d, 2C), 122.6 (d, 2C), 128.1 (s, 2C), 140.4 (d, 2C), 149.2 (s, 2C), 151.0 (s, 2C), 183.3 (s, 2C). Like iso-curcumin, *O,O*-dimethyl-curcumin did not change its λ_{max} when the pH of the solution was raised.

From each curcuminoid, the respective hexahydro derivative was obtained by hydrogenation in methanol with a Pd on charcoal catalyst as previously described in detail (15). The chemical structures of the synthetic hexahydro-curcuminoids, which had a purity of >95% according to HPLC analysis, were confirmed by UV spectroscopy, NMR spectroscopy, HREIMS analysis, LC-MS analysis, and GC-MS analysis after trimethylsilylation (15). The novel compound hexahydro-iso-curcumin had a λ_{max} at 282 nm ($\epsilon = 7400$ in methanol) and the molecular ion had a mass of 374.1725 (calculated for $\text{C}_{21}\text{H}_{26}\text{O}_6$: 374.1729). ^{13}C NMR (100 MHz, CDCl_3): δ 28.9 (t), 31.1 (t), 38.0 (t), 45.1 (t), 49.3 (t), 56.0 (q, 2C), 66.9 (d), 110.7 (d), 110.8 (d), 114.5 (d), 114.7 (d), 119.6 (d), 119.8 (d), 133.9 (s), 135.1 (s), 144.8 (s), 145.1 (s), 145.5 (s), 145.6 (s), 211.4 (s). The novel compound hexahydro-

O,O-dimethyl-curcumin had a λ_{max} at 280 nm ($\epsilon = 5100$ in methanol) and a molecular ion at m/z 402.2039 (calculated for $\text{C}_{23}\text{H}_{30}\text{O}_6$: 402.2042). ^1H NMR (500 MHz, CDCl_3): δ 1.60–1.71 (m, 1H), 1.78–1.85 (m, 1H), 2.57–2.61 (m, 2H), 2.61–2.68 (m, 1H), 2.75–2.77 (m, 2H), 2.73–2.80 (m, 1H), 2.86–2.89 (m, 2H), 3.10 (bs, 1H), 3.876 (s, 3H), 3.880 (s, 3H), 3.887 (s, 3H), 3.894 (s, 3H), 4.05–4.10 (m, 1H), 6.72–6.82 (m, 6H). ^{13}C NMR (125 MHz, CDCl_3): δ 29.2 (t), 31.4 (t), 38.3 (t), 45.3 (t), 49.4 (t), 55.86 (q, 2C), 55.94 (q, 2C), 66.9 (d), 111.3 (d), 111.3 (d), 111.7 (d), 111.8 (d), 120.1 (d), 120.2 (d), 133.3 (s), 134.4 (s), 147.2 (s), 147.5 (s), 148.88 (s), 148.94 (s), 211.3 (s).

β -Glucuronidase (type B-1 from bovine liver) and all other chemicals and reagents were obtained from Sigma-Aldrich Chemical Co. (Steinheim, Germany). HPLC grade acetonitrile was from Carl Roth Co. (Karlsruhe, Germany).

Male Sprague–Dawley rats were purchased from Harlan Winkelmann GmbH (Borchen, Germany). Animals were kept under a 12-h light/dark cycle and received water and commercial lab chow ad libitum. The livers of untreated male rats with 200–300 g weight and the liver of a 63-year-old male white (kindly provided by Dr. J. Weymann, former Knoll AG, Ludwigshafen, Germany) were used for the preparation of hepatic microsomes as described previously by Lake (17). Human intestinal microsomes and supersomes, i.e., microsomes from insect Sf-9 cells infected with a baculovirus strain containing the DNA of human UGT1A1, 1A3, 1A6, 1A7, 1A8, 1A9, 1A10, or 2B7, were from Gentest (Woburn, MA). Protein concentrations were measured according to Bradford (18) with bovine serum albumin as standard. The concentration of active cytochrome P450 was determined by the method of Omura and Sato (19).

Microsomal Incubations. In vitro glucuronidation of curcuminoids was carried out in a total volume of 0.2 mL of 0.1 M phosphate buffer pH 7.4 containing 0.2 mg of hepatic microsomal protein. In a typical incubation, the enzyme protein was first mixed with 5 μg of alamethicin in about 50 μL of buffer and placed on ice for 15 min. Subsequently, magnesium chloride (final concentration 10 mM), the β -glucuronidase inhibitor saccharolactone (10 mM), UDPGA (20 mM), and finally the curcuminoid (100 μM) dissolved in DMSO (final concentration 2%) were added and the mixture was incubated at 37 °C for up to 60 min. The workup procedure for HPLC analysis was either centrifugation of the protein for 3 min at 1000g or addition of 0.2 mL of 0.7 M glycine/chloride buffer pH 1.2 to yield pH 1.8 of the mixture, which was subsequently extracted three times with 0.4 mL of ethyl acetate. The extract was evaporated to dryness under reduced pressure and dissolved in methanol. Control incubations were conducted without UDPGA. In some experiments, saccharolactone was omitted, and an aliquot of the incubation mixture was mixed with the same volume of 0.15 M acetate buffer pH 5.0 containing 5000 Fishman units of β -glucuronidase per milliliter and incubated for 2 h at 37 °C prior to extraction with ethyl acetate and HPLC analysis.

For studies on the effects of curcuminoid glucuronides on microtubule proteins, larger amounts of the glucuronides were required. As attempts to scale up the 0.2 mL incubations failed, multiple parallel 0.2 mL incubations were conducted using protein concentrations of up to 6 mg/mL and incubation times of 90 min. Ethyl acetate extracts were combined and evaporated to dryness under reduced pressure, and glucuronides were dissolved in 25 μL of DMSO. Their concentration was in the low millimolar range as determined by HPLC analysis of an aliquot diluted with methanol.

For studies on the enzymatic activity of hepatic and intestinal microsomes and of supersomes expressing human UGTs, incubations were conducted in 50 mM Tris buffer pH 7.5, containing 0.05–1.0 mg of protein/mL and 20 μM curcumin or 4-(trifluoromethyl)-umbelliferone (TFMU) for up to 2 h with linear product formation. After being cooled to 0 °C, aliquots were directly analyzed by HPLC. Glucuronides were quantified through their absorbance, assuming that they have the same molar extinction coefficients as their aglycones.

NMR and HREIMS Analysis. ^1H and ^{13}C NMR spectra were recorded in acetone- d_6 or CDCl_3 on a Bruker AM-400 and Bruker DRX-500 spectrometer (Karlsruhe, Germany) at 400 or 500 MHz (^1H) and 100 or 125 MHz (^{13}C), using the residual solvent signal as internal standard. Multiplicity determinations (DEPT 90, DEPT 135) were acquired using standard Bruker pulse programs. HREIMS were obtained

by direct injection using a Finnigan MAT 95 instrument (Thermo Finnigan, Austin, TX) with EI ionization at 70 eV.

HPLC Analysis. A HP 1100 system equipped with a binary pump, a photodiode array detector, and HP Chemstation software for data collection and analysis (Agilent Technologies, Waldbronn, Germany) was used and separation was carried out on a 250 × 4.6 mm i.d., 5 μ m, reversed-phase Prodigy 5ODS(2) column (Phenomenex, Torrance, CA). Samples were dissolved in methanol and the injection volume was 10–20 μ L. Four different linear gradients of acetonitrile (solvent B) in deionized water (solvent A, adjusted to pH 3.0 with formic acid) were used for different curcuminoids. Gradient I: 0–35 min 30–70% B; gradient II: 0–5 min 35% B, 5–30 min 35–55% B; gradient III: 0–6 min 15% B, 7–17 min 30–35% B, 17–32 min 35–45% B; gradient IV: 0–35 min 30–80% B. The flow rate was 1 mL/min. The curcuminoids were recorded at 420 nm and the hexahydro-curcuminoids at 280 nm.

LC-MS/MS Analysis. The HPLC system was the same as described above for HPLC analysis but with a 250 × 4.6 mm i.d., 4 μ m, reversed-phase Synergy Hydro column (Phenomenex). The eluate of the HPLC column was split, one part being analyzed by the photodiode array detector and the other part by an Agilent MSD-Trap-SL ion trap mass spectrometer operated in the negative electrospray ionization (ESI) mode. The flow rate of the nitrogen drying gas was 10 L/min, the nebulizer pressure was set to 60 psi, the drying temperature was maintained at 200 °C, the capillary voltage was 4500 V, and the capillary current was 18.3 nA. MS/MS experiments were performed in AutoMS(n) mode. The first scan was a full MS scan, and then one or two precursor ions were selected, isolated, and selectively fragmented in the ion trap.

Hydrogenation of Glucuronides. The glucuronides of various curcuminoids extracted from the microsomal incubations with ethyl acetate were dissolved in methanol and stirred with a small amount of PtO₂ under a H₂ atmosphere at 20 °C for 1 h to yield the glucuronides of the respective hexahydro-curcuminoids. The reaction mixture was centrifuged (5 min at 1000g) and the supernatant analyzed by LC-MS/MS.

Chemical Hydrolysis of Glucuronides. The stability of the glucuronides and aglycones of various curcuminoids and of ketoprofen was determined in 0.1 M potassium phosphate buffer pH 7.4 at 37 °C, using 30 μ M concentrations of the test compounds. Thirty microliters of a 0.5 mM stock solution of the aglycone or the corresponding volume of the concentrated glucuronide solutions in DMSO were mixed with the prewarmed buffer to obtain a total volume of 500 μ L, and 20 μ L aliquots were analyzed by HPLC at 0, 1, 2, 3, and 24 h after mixing.

Interaction with Microtubule Proteins. Microtubule proteins (MTP) were prepared from fresh bovine brain according to Shelanski et al. (20) and the inhibition of microtubule polymerization was determined in a total volume of 500 μ L of reassembly buffer containing 10 μ M MTP as previously described (21). The aglycones and glucuronides of the test compounds were added as DMSO solutions. The final concentrations were 2% (v/v) for DMSO, 40–100 μ M for the free curcuminoids and ketoprofen, 9–45 μ M for the glucuronides, and 2 μ M for colchicine. The test compounds were first incubated with the MTP solution for 20 min at 20 °C, followed by the addition of guanosine triphosphate (GTP) dissolved in reassembly buffer (final concentration 0.5 mM) and warming to 35 °C to start the assembly of MTP, which was measured by the increase in absorbance (due to turbidity) at 350 nm for 30 min in 5 min intervals. Subsequently, the assembled MTP was depolymerized by cooling to 4 °C for 30 min, followed by a second and third cycle of assembly and disassembly. Control incubations contained DMSO but no test compound. The absorbance of the curcuminoids with a fully conjugated double bond system was compensated by measuring against an incubation mixture containing the curcuminoid but no GTP. Colchicine and ketoprofen glucuronide, which are known inhibitors of MTP assembly, were used as positive controls.

RESULTS AND DISCUSSION

Microsomal Glucuronides of Curcuminoids. When curcumin was incubated with rat liver microsomes in the presence

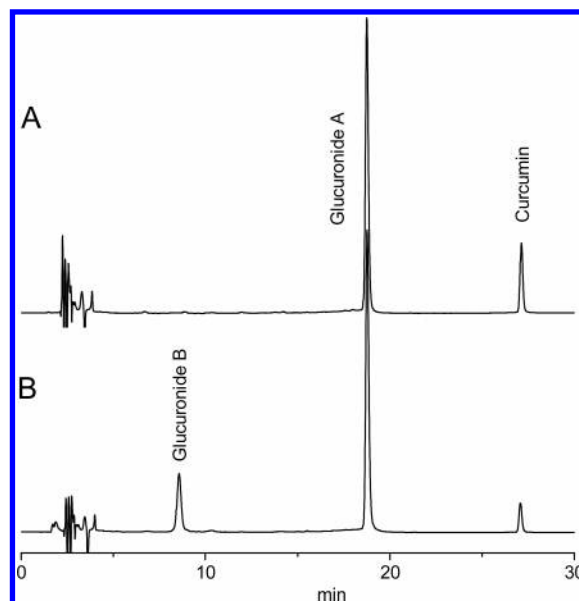


Figure 2. HPLC profiles of the incubations of curcumin with UDPGA and hepatic microsomes from (A) rats and (B) humans.

of UDPGA and the incubation mixture analyzed by HPLC, a product eluting earlier than curcumin from the reversed-phase column was observed (**Figure 2A**). This product was absent when the incubation mixture was treated with β -glucuronidase prior to HPLC and it was therefore concluded to represent a glucuronide of curcumin. The same glucuronide A was formed upon incubation with human hepatic microsomes; however, a smaller peak of an even more polar product eluted from the HPLC column (**Figure 2B**), which could also be cleaved by β -glucuronidase.

LC-MS/MS analysis of curcumin glucuronides A and B showed that both products were monoglucuronides (**Table 1**). Because curcumin is a symmetrical molecule, it was assumed that one glucuronide carries the glucuronic acid at a phenolic and the other one at the alcoholic hydroxyl group. As the daughter ion spectra obtained by MS/MS analysis from the molecular ions of curcumin glucuronide A and B were virtually identical and dominated by the loss of glucuronic acid (**Table 1**), it was not possible to assign the proposed structures to the two glucuronides.

Demethoxy-curcumin, bisdemethoxy-curcumin, and iso-curcumin behaved like curcumin upon microsomal glucuronidation, yielding two glucuronides with human hepatic microsomes and one with rat hepatic microsomes. The MS and MS/MS data of the glucuronides A and B of bisdemethoxy-curcumin and iso-curcumin are listed in **Table 1**. Again, the daughter ion spectra of the glucuronides A and B were very similar. As expected, only one glucuronide was obtained when *O,O*-dimethyl-curcumin was glucuronidated. In this case, the glucuronic acid must be attached to the alcoholic hydroxyl group because this is the only site available for glucuronidation. The MS/MS data of this glucuronide were consistent with those of the others, exhibiting the preferential loss of glucuronic acid (**Table 1**).

When hexahydro-curcumin was glucuronidated with human or rat hepatic microsomes, only one glucuronide was formed. In this case, LC-MS/MS analysis gave rise to a daughter ion spectrum which was dominated by the loss of water from the molecular ion (**Table 1**), strongly suggesting the presence of a free alcoholic hydroxyl group and therefore a phenolic monoglucuronide. In contrast, the alcoholic glucuronide obtained from hexahydro-*O,O*-dimethyl-curcumin did not release water upon

Table 1. Chromatographic and Mass Spectrometric Characteristics of Glucuronides of Various Curcuminoids Generated with Rat and Human Hepatic Microsomes

glucuronide ^a	microsomes ^b	retention time (min) in HPLC ^c	negative ESI (<i>m/z</i> ; % relative intensity)		type of glucuronide	yield of extraction ^d
			MS	MS/MS		
curcumin A	RH, HH	18.8 (gradient I)	543 [M-H] ⁻	451 (12), 393 (25), 367 (100), 353 (13), 217 (39), 175 (80)	phenolic	98
curcumin B	HH	8.8 (gradient I)	543 [M-H] ⁻	367 (100), 217 (86)	alcoholic	n.d. ^e
bisdemethoxy-curcumin A	RH, HH	16.4 (gradient I)	483 [M-H] ⁻	363 (68), 307 (100), 257 (7), 175 (64)	phenolic	98
bisdemethoxy-curcumin B	HH	8.3 (gradient I)	483 [M-H] ⁻	363 (33), 307 (77), 215 (21), 175 (100)	alcoholic	n.d.
iso-curcumin A	RH, HH	18.9 (gradient I)	543 [M-H] ⁻	543 (100), 525 (12), 367 (83), 175 (11)	phenolic	73
iso-curcumin B	HH	13.0 (gradient I)	543 [M-H] ⁻	543 (100), 367 (43)	alcoholic	n.d.
<i>O,O</i> -dimethyl-curcumin	RH, HH	15.3 (gradient II)	571 [M-H] ⁻	571 (7), 395 (100), 175 (6)	alcoholic	99
hexahydro-curcumin	RH, HH	18.0 (gradient III)	549 [M-H] ⁻	549 (19), 531 (100), 513 (12), 373 (13), 359 (23), 175 (29)	phenolic	36
hexahydro-bisdemethoxy-curcumin	RH, HH	18.5 (gradient III)	489 [M-H] ⁻	489 (39), 471 (100), 313 (64), 175 (95)	phenolic	n.d.
hexahydro-iso-curcumin	RH, HH	18.2 (gradient III)	549 [M-H] ⁻	549 (26), 531 (100), 373 (18)	phenolic	45
hexahydro- <i>O,O</i> -dimethyl-curcumin	RH, HH	11.3 (gradient IV)	577 [M-H] ⁻	392 (5), 193 (100), 175 (6)	alcoholic	n.d.

^a A and B denote type of glucuronide. ^b RH, rat hepatic; HH, human hepatic. ^c Gradients I–IV, see Materials and Methods. ^d Percent of curcuminoid glucuronide extracted with ethyl acetate from the microsomal glucuronidation mixture after incubation for 60 min and addition of glycine/chloride buffer to adjust to pH 1.8. ^e n.d., not determined.

LC-MS/MS analysis (Table 1). Thus, only the phenolic and alcoholic glucuronides of the hexahydro-curcuminoids, but not of the curcuminoids, differ in their daughter ion spectra.

To elucidate the exact structures of the two monoglucuronides of curcumin, glucuronide A was prepared using rat liver microsomes, extracted from the incubation mixture with ethyl acetate and chemically hydrogenated in methanol using a platinum catalyst. The product of this reaction had the same HPLC retention time and daughter ion spectrum as the microsomal glucuronide of hexahydro-curcumin, clearly showing that curcumin glucuronide A is a phenolic glucuronide. Curcumin glucuronide B must then be the alcoholic glucuronide. The small amount of this minor glucuronide prevented hydrogenation and mass spectrometric confirmation of the structure.

The similar pattern of the microsomal glucuronides of curcumin, bisdemethoxy-curcumin, and iso-curcumin, i.e., predominant and rather unpolar glucuronides A and minor but more polar glucuronides B (Table 1), implies that the glucuronides A of bisdemethoxy-curcumin and iso-curcumin are also phenolic glucuronides. This was confirmed by hydrogenation of these glucuronides A and comparison of the hydrogenation products with the phenolic glucuronides of the respective hexahydro-curcuminoids by HPLC and LC-MS/MS analysis (Table 1).

During the experiments aiming to obtain larger amounts of the glucuronides for chemical derivatization, several unusual features of the glucuronides of the curcuminoids were noted. First, the glucuronides of the curcuminoids appeared to be more readily extractable from the aqueous incubation mixture than the glucuronides of the hexahydro-curcuminoids. As shown in Table 1, virtually complete extraction of the glucuronides A of curcumin and some of its analogs, but not of hexahydro-curcuminoids, was achieved with ethyl acetate when the pH of the aqueous phase was adjusted to 1.8. Second, several of the curcuminoid glucuronides but not the hexahydro-curcuminoid glucuronides appeared to be unstable. The stability and chemical reactivity of the glucuronides was therefore studied in more detail.

Stability and Reactivity of Curcumin Glucuronide. Curcumin itself has long been known to degrade in aqueous solution at pH values above 7, and some but not all of the degradation products have been identified (15). Instability of curcumin glucuronide has not been reported before. We have therefore studied the stability of various curcuminoids and their major

glucuronides obtained with rat liver microsomes. The nonsteroidal anti-inflammatory drug ketoprofen and its acyl glucuronide, which is known to be unstable (22), were included for comparison. The compounds were kept in 0.1 M phosphate buffer pH 7.4 at 37 °C and analyzed by HPLC after various time periods. The results are depicted in Figure 3. As expected, ketoprofen was quite stable for up to 24 h, whereas its glucuronide decomposed with a half-life of about 1 h. With curcumin, a rapid disappearance from the aqueous solution was observed for both the parent compound and its phenolic glucuronide; after 1 h, more than 90% of the initial amount had degraded, and the glucuronide appeared to be even more unstable than the unconjugated curcumin. For bisdemethoxy-curcumin, there was a clear difference between the unconjugated and the glucuronidated form, the glucuronide decomposing much faster than the aglycone. In contrast, no difference in stability was observed between the glucuronides and parent compounds of iso-curcumin and hexahydro-curcumin. However, only hexahydro-curcumin and its glucuronide were completely stable, whereas iso-curcumin and its glucuronide decomposed slowly. Thus, pronounced differences in stability were obvious for curcuminoids and their phenolic glucuronides as a result of small alterations of the substituents at the aromatic rings. One of the obvious questions is the nature of the degradation products of the glucuronides. According to HPLC analysis, the aqueous solutions of the glucuronides of ketoprofen, curcumin, and bisdemethoxy-curcumin after 1 and 2 h did not contain the parent compounds, and none of the known degradation products of curcumin were detected in the solution of curcumin glucuronide. Therefore, the decomposition of the curcuminoid glucuronides is probably not a simple hydrolysis of the glycosidic bond, and further studies are needed to clarify the mechanism of degradation.

To find out whether the unstable glucuronides are reactive toward proteins, the glucuronides of various curcuminoids were probed for their effects on microtubule proteins (MTP). MTP consist predominantly of heterodimers of α - and β -tubulin as well as smaller amounts of microtubule-associated proteins. Under cell-free conditions in the presence of GTP and Mg^{2+} , MTP assemble at 35 °C to form microtubules, which disassemble again to MTP at 4 °C. It is known that chemical modification of MTP, e.g., by reaction with the acyl glucuronides of nonsteroidal anti-inflammatory drugs, causes MTP to lose their ability to form microtubules (23). With native

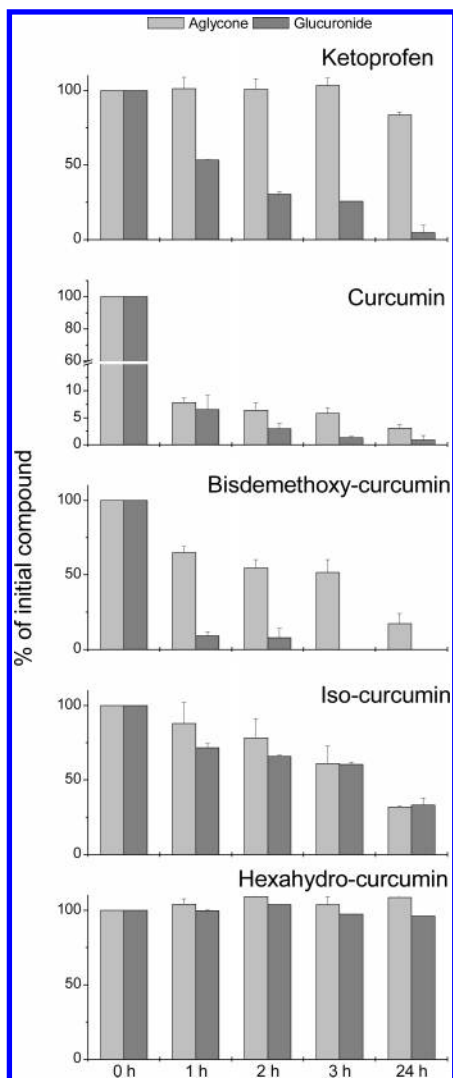


Figure 3. Stability of various curcuminoids and their phenolic glucuronides in aqueous phosphate buffer at pH 7.4. Ketoprofen and its acyl glucuronide were included for comparison. Data represent mean of two independent experiments.

MTP, a second and third cycle of assembly and disassembly can be conducted without adding new GTP by renewed warming and cooling. The assembly and disassembly causes changes in turbidity and can be measured by light scattering at 350 nm. Because curcuminoids with a conjugated double bond system and their glucuronides absorb light at 350 nm, the absorbance of the complete polymerization mixture was measured against a reference containing the same components but no GTP. A typical assembly and disassembly of MTP in the presence of curcumin and its phenolic glucuronide is depicted in **Figure 4**. Whereas unconjugated curcumin had no effect on MTP polymerization, about 50% inhibition of MTP assembly was observed with 40 μ M curcumin glucuronide. About the same inhibitory effect was obtained with the same concentration of ketoprofen glucuronide, whereas the strong microtubule inhibitor colchicine was about 10 times more effective (**Figure 5**). Among the tested curcuminoids, the phenolic glucuronide of bisdemethoxy-curcumin proved to be the strongest inhibitor and iso-curcumin glucuronide the weakest one, whereas the glucuronide of hexahydro-curcumin was devoid of any inhibitory effect (**Figure 5**). Thus, the inhibitory activities of the phenolic glucuronides of various curcuminoids for MTP assembly paralleled their instability in aqueous solution as shown in **Figure 3**, whereas

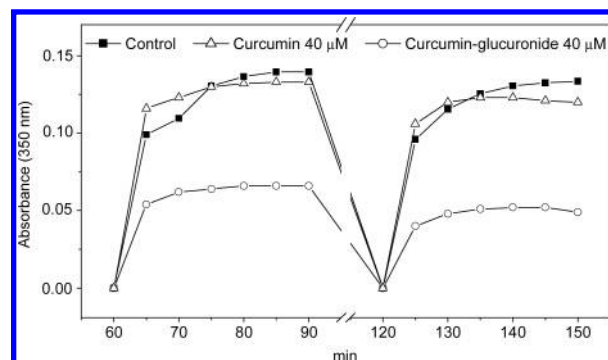


Figure 4. Effect of curcumin and its phenolic glucuronide on the polymerization of MTP under cell-free conditions. The second and third cycle of assembly and disassembly are depicted.

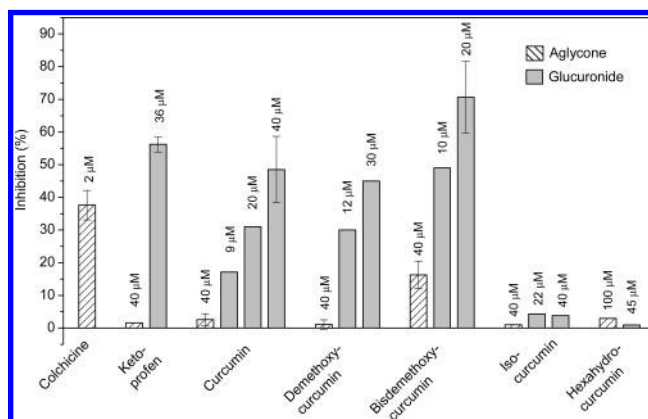


Figure 5. Inhibitory effect of various curcuminoids and their phenolic glucuronides on the polymerization of MTP under cell-free conditions. The known MTP inhibitors colchicine and ketoprofen glucuronide were used as positive controls. All experiments were independently repeated three times. Error bars calculated from experiments with identical concentrations represent standard deviations. Columns without error bars are from one experiment, but were supported by two other experiments with similar but not identical concentrations.

the unconjugated parent compounds did not affect MTP. This suggests that the unstable curcuminoid glucuronides are reactive toward proteins.

Although the formation of curcumin glucuronide *in vivo* and *in vitro* has been reported before (9, 11–14), these publications do not mention the chemical reactivity and unusual lipophilicity of the glucuronide. Moreover, our study has, for the first time, disclosed that two curcumin glucuronides are formed and unambiguously clarified their exact chemical structures, showing that the phenolic hydroxyl group is the preferred site of glucuronidation. Prior to the rigorous structure elucidation based on daughter ion mass spectrometry, we were misled by the chemical reactivity of the major glucuronide, which could be more readily explained by assuming the structure of an alcoholic glucuronide (24, 25). The alcoholic glucuronide of curcumin represents a vinylogous acyl glucuronide. Acyl glucuronides are formed from several nonsteroidal anti-inflammatory drugs, e.g., ketoprofen, ibuprofen, and diclofenac, and other carboxylic acid-bearing compounds, and have been amply demonstrated to be labile electrophiles (22).

With the chemical structure of curcumin glucuronide A established, the chemical instability of this phenolic glucuronide in aqueous solution and its reactivity with proteins remain to be explained. Comparison with the glucuronides of other curcuminoids used in this study clearly show that two prerequisites must be fulfilled for reactivity, i.e., a para position of

Table 2. Glucuronidation of Curcumin by Microsomes Expressing Human Recombinant UGT Isoforms and by Microsomes from Various Tissues

microsomes	enzymatic activity (pmol/min/mg protein)	
	for curcumin	for TFMU ^a
UGT1A1	1858 ± 77	426 ± 33
UGT1A3	540 ± 14	422 ± 49
UGT1A6	20 ± 2	3886 ± 227
UGT1A7	187 ± 4	8735 ± 306
UGT1A8	1537 ± 146	341 ± 24
UGT1A9	97 ± 15	5547 ± 278
UGT1A10	1535 ± 92	421 ± 35
UGT2B7	306 ± 38	1537 ± 108
human hepatic	4641 ± 126	24079 ± 1361
human intestinal	12687 ± 1138	10326 ± 499
rat hepatic	4589 ± 170	29654 ± 859
rat intestinal	3933 ± 104	13817 ± 640

^a TFMU represents a nonspecific substrate for various UGT isoforms and was used as reference.

the phenolic hydroxyl group to the aliphatic chain, and the presence of a conjugated double bond system in the aliphatic chain. The glucuronides of curcuminoids lacking one of these structural requirements, e.g., iso-curcumin with meta-positioned phenolic groups or hexahydro-curcumin with a saturated aliphatic chain, are markedly more stable and less reactive. In contrast, the phenolic glucuronides A of the natural congeners demethoxy- and bisdemethoxy-curcumin, which meet the requirements, exhibit the same or even higher reactivity as compared to that of curcumin glucuronide A. Recent high-level ab initio molecular orbital calculations have been conducted on curcumin and have shown that curcumin has numerous electrophilic regions over the entire molecule and a low molecular hardness, implying electrophilic reactivity (26). Possibly similar calculations for the glucuronide A of curcumin as well as for other curcuminoids and their glucuronides might help to better understand why some curcuminoids and/or their phenolic glucuronides are stable whereas others are not. Furthermore, the synthesis and study of other curcuminoids, e.g., a "mixed curcumin" carrying one curcumin-type and one iso-curcumin-type aromatic ring, and their glucuronides might prove useful.

UGT Isoforms Involved in the Glucuronidation of Curcumin. No information is available to date about the activity of different isoforms of UGTs for curcuminoids. We have therefore determined the enzymatic activity of eight human UGTs for the in vitro glucuronidation of curcumin (Table 2). All UGTs gave rise to the formation of the phenolic glucuronide A of curcumin, but with some isoforms, small amounts of glucuronide B were also obtained. For the calculation of the enzymatic activity, the amounts of glucuronide A and B were added. The nonspecific UGT substrate TFMU was included in this experiment for comparison. In addition to human UGT isoforms, hepatic and intestinal microsomes from humans and rats were assayed for their glucuronidation activity.

The human UGT isoforms with the highest activity for curcumin were UGT1A1, 1A8, and 1A10 (Table 2). High levels of all three UGTs are expressed in the human gastrointestinal tract (27). In fact, the activity for the glucuronidation of curcumin was 3-fold higher in human intestinal microsomes than in human hepatic microsomes, whereas no such difference was noted for the rat intestinal and hepatic microsomes (Table 2). Thus, curcumin may be efficiently glucuronidated during absorption, and the lipophilicity of the glucuronide may facilitate its distribution into various organs, possibly explaining, in part, the extremely low plasma levels of curcumin observed even

after very high doses of several grams per day (28). In conclusion, our study has demonstrated that the glucuronides of curcuminoids, which are major phase II metabolites, exhibit novel properties that should be further investigated.

ABBREVIATIONS

DMSO, dimethyl sulfoxide; ESI, electrospray ionization; GTP, guanosine triphosphate; HREIMS, high-resolution electron impact mass spectrometry; MTP, microtubule proteins; TFMU, 4-(trifluoromethyl)umbelliferone; UDPGA, uridine-5'-diphosphoglucuronic acid; UGT, uridine-5'-diphosphoglucuronosyl-transferase.

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