

# Characterization of Metabolites of the Chemopreventive Agent Curcumin in Human and Rat Hepatocytes and in the Rat *in Vivo*, and Evaluation of Their Ability to Inhibit Phorbol Ester-induced Prostaglandin E<sub>2</sub> Production<sup>1</sup>

Christopher Ireson, Samantha Orr, Don J. L. Jones, Richard Verschoyle, Chang-Kee Lim, Jin-Li Luo, Lynne Howells, Simon Plummer, Rebekah Jukes, Marion Williams, William P. Steward, and Andreas Gescher<sup>2</sup>

Medical Research Council Toxicology Unit [C. I., D. J. L. J., R. V., C-K. L., J-L. L., L. H., S. P., R. J., A. G.] and Department of Oncology [M. W., W. P. S.], University of Leicester, Leicester LE1 9HN, and School of Pharmacy and Pharmaceutical Sciences, De-Montfort University, Leicester LE1 9BH [S. O.], United Kingdom

## ABSTRACT

Curcumin, the yellow pigment in turmeric, has been shown to prevent malignancies in a variety of tissues in rodents, especially in the intestinal tract. Pharmacological activities of curcumin in cells *in situ* germane to chemoprevention, such as inhibition of expression of cyclooxygenase-2 (COX-2), require drug concentrations in the 10<sup>-5</sup>–10<sup>-4</sup> M range. The systemic bioavailability of curcumin is low, so that its pharmacological activity may be mediated, in part, by curcumin metabolites. To investigate this possibility, we compared curcumin metabolism in human and rat hepatocytes in suspension with that in rats *in vivo*. Analysis by high-performance liquid chromatography with detection at 420 and 280 nm permitted characterization of metabolites with both intact diferoylmethane structure and increased saturation of the heptatrienone chain. Chromatographic inferences were corroborated by mass spectrometry. The major metabolites in suspensions of human or rat hepatocytes were identified as hexahydrocurcumin and hexahydrocurcuminol. In rats, *in vivo*, curcumin administered i.v. (40 mg/kg) disappeared from the plasma within 1 h of dosing. After p.o. administration (500 mg/kg), parent drug was present in plasma at levels near the detection limit. The major products of curcumin biotransformation identified in rat plasma were curcumin glucuronide and curcumin sulfate whereas hexahydrocurcumin, hexahydrocurcuminol, and hexahydrocurcumin glucuronide were present in small amounts. To test the hypothesis that curcumin metabolites resemble their progenitor in that they can inhibit COX-2 expression, curcumin and four of its metabolites at a concentration of 20 μM were compared in terms of their ability to inhibit phorbol ester-induced prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) production in human colonic epithelial cells. Curcumin reduced PGE<sub>2</sub> levels to preinduction levels, whereas tetrahydrocurcumin, previously shown to be a murine metabolite of curcumin, hexahydrocurcumin, and curcumin sulfate, had only weak PGE<sub>2</sub> inhibitory activity, and hexahydrocurcuminol was inactive. The results suggest that (a) the major products of curcumin biotransformation by hepatocytes occur only at low abundance in rat plasma after curcumin administration; and (b) metabolism of curcumin by reduction or conjugation generates species with reduced ability to inhibit COX-2 expression. Because the gastrointestinal tract seems to be exposed more prominently to unmetabolized curcumin than any other tissue, the results support the clinical evaluation of curcumin as a colorectal cancer chemopreventive agent.

## INTRODUCTION

Curcumin [1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione] is the major yellow pigment extracted from turmeric, the powdered rhizome of the herb *Curcuma longa*. Turmeric is a spice used extensively in curries and mustards as a coloring and flavoring agent. Consumption of turmeric and curcumin has been associated

with a plethora of beneficial effects on human health; prominent among them are anti-inflammatory and cancer chemopreventive activities (1). Curcumin has been shown to inhibit tumor formation in the skin, forestomach, duodenum, and colon of mice and in the tongue, colon, mammary glands, and sebaceous glands of rats (for review see Ref. 2). Especially noteworthy are results of a number of recent studies of curcumin in colon cancer chemoprevention models in rodents. Curcumin (0.2% w/v) in the diet inhibited the development of azoxymethane-induced colonic adenocarcinomas in rats irrespective of whether the compound was administered during the initiation/postinitiation (3) or the promotion/progression stages of the disease (4). At dietary levels of 0.1%, curcumin caused a 64% reduction in adenoma formation in the intestine of Min mice, which harbor the defect in the adenomatous polyposis coli gene underlying familial adenomatous polyposis in humans (5). Curcumin has shown a variety of biological activities that might explain its chemopreventive action. These activities include antioxidation (6, 7), suppression of c-Jun/AP-1 activation (8), inhibition of prostaglandin biosynthesis (9), and inhibition of the activity and expression of the enzyme COX<sup>3</sup> (10). We have recently reported that curcumin interferes with the expression of the COX isoenzyme COX-2 and that this interference is probably linked to its ability to block activation of the transcription factor nuclear factor κB at the level of the NIK/IKKα/β signaling complex (11). In cell incubations *in vitro* these effects of curcumin were observed in the 10<sup>-5</sup>–10<sup>-4</sup> M concentration range. The bioavailability of curcumin in rodents has been shown to be low (12, 13). In a recent study, an oral dose of 1 g/kg administered to mice yielded a peak plasma level of only 0.5 μM (13). There is preliminary evidence derived from a clinical pilot study that suggests that the systemic availability of curcumin is also poor in humans, because oral doses of 4–8 g generated peak plasma levels of as little as 0.41–1.75 μM (14). These findings cast doubt on the assumption that consumption of curcumin as a drug or food constituent furnishes levels of compound in blood and tissues sufficient to elicit biological effects associated with chemoprevention, and they render rational selection of a potentially chemopreventive dose difficult. It is conceivable that curcumin is biotransformed to species that are responsible for, or contribute to, its chemopreventive efficacy. The metabolism of curcumin in humans is poorly understood. In rodents its major metabolic pathway involves successive reduction via dihydrocurcumin and tetrahydrocurcumin to hexahydrocurcumin (see Fig. 1) and conjugation of mainly tetrahydrocurcumin and hexahydrocurcumin with glucuronic acid (13, 15). The liver is the primary organ that generates metabolites from drugs and other xenobiotics. Early studies suggest that curcumin undergoes extensive metabolism in rat hepatocytes *in vitro*, although the metabolic products were not identified (16). The rat has served extensively as an experimental model in the evaluation of the ability of curcumin to prevent carcinogen-induced cancer (3, 4).

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<sup>2</sup> To whom requests for reprints should be addressed, at Medical Research Council Toxicology Unit, University of Leicester, Hodgkin Building, P.O. Box 138, Leicester LE1 9HN, United Kingdom. Phone: 44-116-252-5618; Fax: 44-116-252-5616; E-mail: ag15@le.ac.uk.

<sup>3</sup> The abbreviations used are: COX, cyclooxygenase; HCEC, human colonic epithelial cell; HPLC, high-performance liquid chromatography; PGE<sub>2</sub>, prostaglandin E<sub>2</sub>; PMA, phorbol-12-myristate-13-acetate.

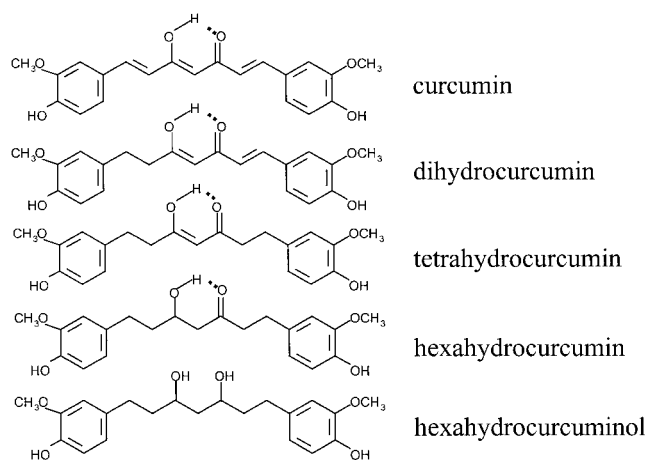


Fig. 1. Structures of curcumin and products of its metabolic reduction.

In view of the paucity of the existing data on curcumin metabolism, we tested the hypothesis that curcumin is biotransformed similarly by human and rat liver. To that end, hepatocytes obtained from humans and rats were incubated with curcumin, and their metabolites were identified. Curcumin was also administered to rats via the i.v. and p.o. routes, and its plasma metabolites were compared with those found in suspensions of liver cells. Finally, to investigate whether the identified metabolites possess pharmacological properties germane to chemoprevention, we compared their ability with that of curcumin to inhibit phorbol ester-induced COX-2 expression in human colon cells as reflected by PGE<sub>2</sub> levels.

## MATERIALS AND METHODS

**Chemicals and Reagents.** The following chemicals and reagents were purchased from the suppliers listed: curcumin, collagenase, uridine 5'-diphosphoglucuronic acid, uridine 5'-diphospho-*N*-acetyl glucosamine, magnesium chloride, uridine 5'-diphosphoglucuronosyl transferase, bacterial  $\beta$ -glucuronidase (type VII-A from *Escherichia coli*), sulfatase (type VIII from *Abalone* entrails), EGTA, glycerol formal, 1,4-dioxane (anhydrous), heparin, sodium borohydride, PMA, human serum albumin, and arachidonic acid (Sigma-Aldrich Comp. Ltd., Poole, Dorset, United Kingdom); HPLC-grade acetonitrile (Fisher Laboratory Supply Ltd., Loughborough, United Kingdom); HBSS concentrate without calcium, magnesium, sodium bicarbonate and phenol red, DMEM with high glucose and glutamax, media for liver digestion, liver perfusion, and hepatocyte suspension (Life Technologies, Inc., Paisley, United Kingdom); pentobarbitone (Sagatal; Rhone Merieux Ltd., Harlow, Essex, United Kingdom); sulfur-trioxide-*N*-triethylamine complex (Fluka Chemicals, Gillingham, Dorset, United Kingdom); DMSO, ammonium acetate (Merck Ltd., Poole, Dorset, United Kingdom); halothane (Zeneca, Macclesfield, Cheshire, United Kingdom); Baxters Soltran kidney perfusion solution (Baxters Healthcare, Berkshire, United Kingdom); PGE<sub>2</sub> immunoassay kit (Cayman Chemical Co., Ann Arbor, MI). Tetrahydrocurcumin and hexahydrocurcumin were synthesized as described (17) and provided by Dr. W. Wang (Phytopharm plc, Cambridge, United Kingdom).

**Cells and Animals.** Nonmalignant HCECs (18) were obtained from Dr. A. Pfeifer (Nestlé Research Institute, Lausanne, Switzerland). These cells were passaged in B50 medium (Biofluids Inc., Rockville, MD) containing BSA, bovine pituitary extracts, retinoic acid, vitamin C, and dexamethasone. Male (180–200 g) or female (160–180 g) F344 rats were purchased from Charles River UK Ltd. (Margate, Kent, United Kingdom) or Harlan UK Ltd. (Bicester, Oxon, United Kingdom). Rats that were maintained in a purpose-built animal house in negative pressure isolators (19–23°C) under a 12-h light/dark cycle received RM1 rodent maintenance diet (SDS, Kent, United Kingdom) and water *ad libitum*. Experiments using animals were conducted as stipulated by Project License 80/1250 granted to the Medical Research Council Toxicology Unit by the United Kingdom Home Office, and the experimental design was

vetted and approved by the Leicester University Ethical Committee for Animal Experimentation.

**Isolation of Human and Rat Hepatocytes.** Isolation of hepatocytes from humans or rats was performed by the collagenase perfusion method of Berry and Friend (19) according to the protocol described by Seglen (20). Healthy liver tissue resected from four Caucasian patients with secondary hepatic tumors (two females, 38 and 61 years old; two males, 51 and 53 years old) were obtained from the United Kingdom Human Tissue Bank (Leicester, United Kingdom). Patients had not received medication known to interfere with liver metabolic activity. Following removal of the liver from the body, cannulas were immediately inserted into four to five large blood vessels of the lobe, which was immediately perfused in theater with kidney perfusion medium (500 ml) and transported in this fluid on ice. On arrival in the laboratory, the liver was transferred to a custom-built stainless steel tank and perfused for 20–30 min with liver perfusion medium maintained at 37°C to remove blood. The liver was then perfused with liver digestion media for approximately 45 min. The digested liver lobe was transferred to a tray containing liver suspension medium (DMEM supplemented with human serum albumin 2%), and the tissue was gently disrupted to release cells. Undigested tissue was removed by passing the cell suspension through a series of sieves (successive mesh size: 1 mm, 0.5 mm, and 100  $\mu$ m). For the isolation of rat liver cells, male F344 rats (180–220 g) were anesthetized with pentobarbitone, and the liver was perfused (5 min; rate, 50 ml/min) via the inferior portal vein with HBSS (containing 1 mM EGTA), which had been presaturated with carbogen (oxygen/CO<sub>2</sub> 5%). The liver was digested using collagenase (100 mg/liter) and calcium chloride (332 mg/liter) in HBSS. Tissue was gently disrupted and washed through a sieve (100- $\mu$ m mesh size) with liver suspension medium. Human or rat cells, thus, obtained were washed three times and centrifuged (3 min, 50  $\times$  g, 4°C). Cells were counted using a hemocytometer immediately following isolation. Hepatocyte viability determined by the trypan blue exclusion assay was routinely 80% or above. Hepatocytes in suspension were maintained on ice for a maximum of 30 min before use.

**Incubations with Hepatocytes.** Freshly isolated hepatocytes ( $2 \times 10^6$  cells per ml) were suspended in liver suspension medium (2 ml) and incubated in a slowly shaking incubator (37°C). Curcumin dissolved in DMSO was added to furnish a final concentration of 100  $\mu$ M. The concentration of DMSO (maximally 0.1% v/v) in the incubate did not interfere with cell viability. Control incubates included curcumin with heat-inactivated hepatocytes or hepatocytes incubated with the vehicle only. Incubations were terminated after 5, 30, 60, and 120 min by placing vials on dry ice. During the longest incubation period (2 h) cell viability decreased to between 60% and 40% of initial values (trypan blue exclusion test). Before HPLC analysis, suspensions were rapidly defrosted, immediately extracted twice with ethyl acetate (twice volume of sample), and mixtures were centrifuged (2800  $\times$  g, 4°C, 15 min). The organic layers were removed, combined, and evaporated to dryness under nitrogen. Samples were reconstituted in acetonitrile and immediately analyzed by HPLC. In control experiments, the ability of hepatocytes to conjugate the model substrate umbelliferone was assessed and found to be intact (21).

**Metabolism Studies *in Vivo*.** Female F344 rats received curcumin either p.o. (gavage, 500 mg/kg; vehicle, DMSO; dosage volume, 2.0 ml/kg) or i.v. (40 mg/kg; vehicle, glycerol formal; dosage volume, 1.0 ml/kg). The p.o. dose level was chosen because it is approximately equivalent to a daily dose of curcumin when ingested with the diet at 1%, a concentration that has been frequently used in intervention studies. The i.v. dose chosen was the highest feasible dose formulated in a solvent suitable for injection. Animals were subjected to terminal anesthesia (halothane/nitrous oxide), and blood was removed by cardiac puncture 30 min and 1, 2, 6, 12, and 24 h (p.o. administration) or 5 and 30 min and 1 and 6 h (i.v. administration) after dosing. Blood was also obtained from animals that had received vehicle only. Blood was transferred into heparinized centrifuge tubes, and plasma was obtained by centrifugation (1100  $\times$  g, 4°C, 25 min). Aliquots of plasma were extracted with twice the volume of ethyl acetate, or mixed with four times the volume of a mixture of DMSO:methanol (1:4). The mixtures were centrifuged (1000  $\times$  g, 15 min), and the supernatant was removed. In the case of the ethyl acetate extract, the organic layer was evaporated under nitrogen. Extraction efficiencies from plasma using the ethyl acetate extraction method for curcumin, hexahydrocurcumin, and curcumin sulfate were determined by HPLC (see below) as  $95 \pm 4\%$ ,  $70 \pm 5\%$ , and  $49 \pm 9\%$ , respectively. Recovery of curcumin in the case of treatment with DMSO:methanol was  $70 \pm 5\%$

(mean  $\pm$  SD,  $n = 3-6$  in all these studies). Extraction efficiencies from hepatocyte suspensions were identical to those determined for plasma.

**HPLC Analysis.** A reversed-phase HPLC method was used to determine the quantity of curcumin and its putative metabolites that is similar but not identical to that described before (13). A Varian Prostar (230 model) solvent delivery system coupled to a UV-visible detector (310 model) and autosampler (model 410) and a Symmetry Shield RP 18 column (150  $\times$  3.9 mm; Waters) were used. Detection of curcumin, curcumin sulfate, and curcumin glucuronide was achieved at 420 nm, whereas tetrahydrocurcumin, hexahydrocurcumin, and hexahydrocurcuminol were analyzed at 280 nm. Tetra-(*m*-hydroxyphenyl)-chlorin was used as an internal standard. Samples were reconstituted in acetonitrile:water (1:1), and the injection volume was 50  $\mu$ l. A linear gradient of 5–45% acetonitrile in 0.01% ammonium acetate (pH 4.5) was used for 30 min, followed by an increase over 20 min to 95% acetonitrile (flow rate, 1 ml/min). The retention times quoted in the results and in Table 1 were obtained using these conditions. The limits of detection of curcumin, tetrahydrocurcumin, and hexahydrocurcumin in plasma and hepatocyte suspensions were between 5 and 10 nM. In the case of curcumin, the quantitative method was validated using a 2.7- $\mu$ M solution yielding intra- and interday coefficients of variation of 5.1% and 9.8%, respectively ( $n = 4$ ), and a limit of quantitation of  $\sim$ 20 nM. Curcumin calibration curves spanned the concentration range of 20 nM to 40  $\mu$ M.

**Synthesis of Curcumin Sulfate and Curcumin Glucuronide.** For the synthesis of curcumin sulfate, curcumin (1.36 mmol), dissolved in anhydrous 1,4-dioxane, was incubated with sulfur trioxide *N*-triethylamine complex (6.8 mmol) and maintained at 37°C for 2 h. The precipitate was washed (ethyl acetate) to remove unreacted curcumin. For the synthesis of curcumin glucuronide according to a published method (22), curcumin (1 mM), uridine diphosphoglucuronic acid (4 mM), uridine diphospho-*N*-acetyl glucosamine (2 mM), HEPES buffer (25 mM, pH 7.4), magnesium chloride (10 mM), and uridine diphosphoglucuronosyl transferase (150 units/liter) were incubated (3 ml, 37°C, 3 h). The incubation medium was extracted twice with ethyl acetate, and the combined organic extracts were evaporated under nitrogen. The residues of either reaction were reconstituted in acetonitrile:water (1:1). Products were separated by preparative HPLC using a Hypersil column (250  $\times$  21.2 mm, 5  $\mu$ , detection at 420 nm). Eluent corresponding to the peaks that were tentatively assigned to curcumin sulfate or curcumin glucuronide was collected, and the solvent removed from the collected fractions by freeze-drying. The isolated materials were reanalyzed by HPLC (detection at 420 nm). The extinction coefficients of curcumin sulfate and curcumin glucuronide were approximately equivalent to that of curcumin, as established by a standard curve (see below). Consequently, their quantitation in the plasma was based on calibration curves established with curcumin. The structural identity of the products as curcumin sulfate and curcumin glucuronide was confirmed by mass spectrometry (see “Results” and Table 1).

**Synthesis of Hexahydrocurcuminol.** An equimolar amount of sodium borohydride was added to hexahydrocurcumin (3 mM) dissolved in methanol. HPLC analysis (detection at 280 nm) showed that after 2 h at ambient temperature all of the hexahydrocurcumin had reacted. Methanol was removed by evaporation under nitrogen, and the residue was reconstituted in water (2 ml) and adjusted to pH 4.5. The product was extracted with ethyl acetate, and the solvent was evaporated under nitrogen. The structural identity of the product as hexahydrocurcuminol was confirmed by mass spectrometry (see “Results” and Table 1).

Table 1 Characterization of major curcumin metabolites by HPLC and mass spectrometry

Compound	HPLC retention time (min)	Mass spectrometric product ions (m/z) <sup>a</sup>
Hexahydrocurcumin	24.4	179 (100); 165 (11); 193 (12); 373 (10) <sup>b</sup>
Hexahydrocurcuminol	22.5	135 (100); 375 (50) <sup>b</sup> ; 109 (32); 179 (22)
Curcumin glucuronide	25	177 (100); 369 (72); 545 (15) <sup>b</sup>
Curcumin sulfate	31	367 (100); 217 (85); 149 (61); 447 (50) <sup>b</sup>
Hepatocyte metabolite 1	24.4	179 (100); 165 (16); 193 (17); 373 (10)
Hepatocyte metabolite 2	22.5	135 (100); 375 (67); 179 (23); 109 (22)
Plasma metabolite 1	25	545 (100); 369 (45); 177 (30)
Plasma metabolite 2	31	447 (100); 367 (25); 217 (24); 149 (22)

<sup>a</sup> m/z values of the most prominent product ions in the mass spectrum; the percentage abundance is in parentheses. For details of chromatography and spectrometry see “Materials and Methods.”

<sup>b</sup> Deprotonated molecular ion.

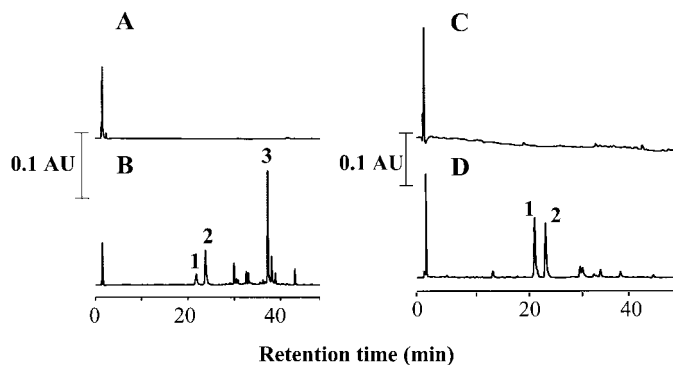


Fig. 2. High-performance liquid chromatograms with detection at 280 nm of extracts of suspensions of hepatocytes from humans (A and B) or rats (C and D) incubated for 2 h without (A and C) or with (B and D) curcumin (100  $\mu$ M). Chromatograms are representative of incubations with six (rat) and four (human) separate hepatocyte preparations. Peaks were identified by cochromatography and mass spectrometry (see text) as curcumin (3), hexahydrocurcumin (2), and hexahydrocurcuminol (1). AU, absorbance units. Note that commercially available curcumin contains 15% desmethoxycurcumin and 5% bis-desmethoxycurcumin, which furnished the two small peaks just beyond curcumin. For details of hepatocyte isolation, incubation conditions, and HPLC analysis see “Materials and Methods.”

**Mass Spectrometry.** Mass spectrometry was performed on a Quattro Bio-Q tandem quadrupole mass spectrometer upgraded to Quattro MK II specifications (Micromass, Altrincham, Cheshire, United Kingdom) with a pneumatically assisted electrospray interface. Samples were analyzed in negative ion mode. The temperature was maintained at 120°C, the operating voltage of the electrospray capillary was 3.88 kV and the cone voltage 32 V. Tandem mass spectrometric experiments were conducted using argon as the collision gas and a collision energy of 25 eV. Samples were dissolved in water:methanol (1:1) and introduced into the mass spectrometer via flow injection using a Varian 9012 Solvent delivery system (Varian, Walton-on-Thames, United Kingdom) and a Rheodyne 7125 injector (Cotatai, CA). HPLC conditions were as described under HPLC analysis above, except that the linear gradient program was: acetonitrile (5–45%) in 0.01% ammonium acetate (pH 4.5) for 60 min (rather than 30 min), followed by an increase for 20 min to 95% acetonitrile (flow rate, 1 ml/min). These are the conditions of the chromatogram shown in Fig. 4, and they differ from those shown in Fig. 2. The flow was split so that only 115  $\mu$ l was introduced into the mass spectrometer. In some experiments, the solution was introduced by continuous infusion using a Harvard Apparatus model 22 syringe pump (Harvard Apparatus, South Natick, MA) pumped at a flow rate of 10  $\mu$ l/min.

**Immunoassay for PGE<sub>2</sub> in HCECs.** HCECs were seeded onto collagen/fibronectin precoated dishes (9 cm) containing 10 ml of DMEM (with glutamax-1, 4500 g/liter glucose, and pyridoxine, but excluding sodium pyruvate) and 10% FCS and left for 48 h. Subsequently the cells were serum-starved for 24 h and then treated for 30 min with curcuminoids (20  $\mu$ M) dissolved in DMSO, after which PMA (final concentration, 75 nM) was added. The mixture was incubated for 6 h. Subsequently arachidonic acid (10  $\mu$ M) was added to cellular incubations for a further 30 min. Aliquots (1 ml) were removed for determination of PGE<sub>2</sub> by immunoassay (23), which was carried out using a PGE<sub>2</sub> immunoassay kit (Cayman Chemical Co.). Quantitation was performed using a Labsystems iEMS plate reader.

## RESULTS

**Metabolism of Curcumin by Hepatocytes.** Hepatocytes from humans or rats were incubated with curcumin at a concentration of 100  $\mu$ M, and extracts of the incubates were analyzed by HPLC. Curcumin and products of its biotransformation were detected at 420 nm, indicating the presence of molecules containing the intact yellow-colored diferoylmethane structure. Analysis at 280 nm also allowed the characterization of molecules generated from curcumin by reduction or cleavage of the chromophoric diarylheptatrienone chain. Chromatographic analysis at 420 nm of extracts of hepatocyte suspensions, incubated for up to 2 h, yielded traces containing only one prominent

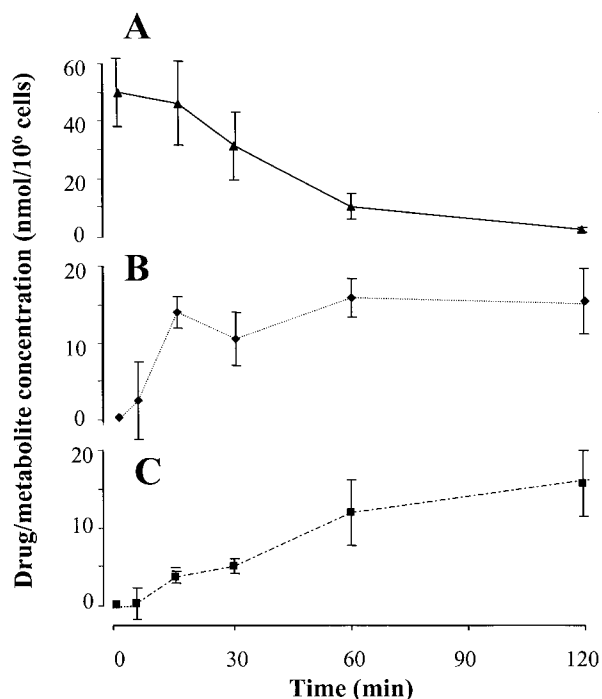


Fig. 3. Time course of disappearance of curcumin (A) and generation of metabolites hexahydrocurcumin (B) and hexahydrocurcuminol (C) in suspensions of rat hepatocytes. HPLC detection was by UV at 280 nm. The results are the mean  $\pm$  SD of three incubations with separate hepatocyte preparations. For details of hepatocyte isolation, incubation, and HPLC analysis see "Materials and Methods."

peak that coeluted with curcumin (data not shown). Although 35% of the initial amount of curcumin was still present in suspensions of human hepatocytes after incubation for 2 h, curcumin concentrations were reduced to near the detection limit when incubated with rat hepatocytes for that time period. In addition to curcumin, there were two small peaks in extracts from both types of hepatocytes, characterized by retention times of  $\sim$ 25 and 31 min, consistent with curcumin sulfate and glucuronide, respectively (see below). HPLC analysis using detection at 280 nm yielded at least four metabolite peaks; the two major ones were characterized by retention times of 22.5 and 24.4 min (Fig. 2). Both species were found in hepatocytes from humans (Fig. 2A) and rats (Fig. 2B), and they were absent from chromatograms of hepatocyte incubations from which curcumin had been omitted. The peak characterized by the retention time of 24.4 min coeluted with authentic hexahydrocurcumin, and mass spectrometric analysis of a dried residue of the eluent was collected at the pertinent retention time confirmed its identity (Table 1). The other major metabolite with a retention time of 22.5 min afforded a molecular ion of  $m/z$  375 on mass spectrometric analysis (Table 1), thus containing two mass units more than hexahydrocurcumin. In a separate experiment, authentic hexahydrocurcumin was incubated with rat hepatocytes and rapidly metabolized to the species characterized by a retention time of 22.5 min and the molecular ion  $m/z$  375. These findings are consistent with the possibility that the metabolite was generated from curcumin via hexahydrocurcumin. Furthermore, the same molecule was generated chemically on treatment of hexahydrocurcumin with the reducing agent sodium borohydride. We infer from these results that the second major hepatocytic metabolite of curcumin is hexahydrocurcuminol (Fig. 1). Fig. 3 shows the time course of disappearance of curcumin and concurrent generation of its two major metabolites in suspensions of rat hepatocytes. The ratio of integrated peak areas of hexahydrocurcumin over hexahydrocurcuminol after incubation of rat or human hepatocytes with curcumin for 2 h fur-

nished values of  $1.0 \pm 0.1$  in the case of rat hepatocytes as compared with  $3.2 \pm 0.6$  (mean  $\pm$  SD,  $n = 3$  for each) for human hepatocytes. Taken together, the results obtained in experiments with hepatocytes demonstrate, first, that metabolic reduction of curcumin to hexahydrocurcumin is rapid, followed by the reduction of the carbonyl moiety to hexahydrocurcuminol and, second, that overall reduction of curcumin to hexahydrocurcuminol, the ultimate reduction product of curcumin, occurs more extensively in rat than in human hepatocytes.

**Metabolism of Curcumin *in Vivo*.** Metabolites were characterized in rat plasma *in vivo* after administration of curcumin via the i.v. (40 mg/kg) or p.o. (0.5 g/kg) routes. Plasma samples were analyzed by HPLC with detection either by UV absorption at 420 nm or by ion-selected monitoring in mass spectrometry mode. Spectrophotometric analysis of plasma at 280 nm did not allow useful inferences to be made because specific curcumin metabolite peaks were indistinguishable from a host of peaks attributable to endogenous constituents. Plasma from rats that had received curcumin p.o. afforded a peak that coeluted with curcumin, but at levels below the limit of quantitation (data not shown). In addition, there were three metabolites harboring the intact diferoylmethane structure with retention times of approximately 20, 25, and 31 min in the plasma of rats after both routes of administration. Incubation of plasma extracts with  $\beta$ -glucuronidase led to a reduction of the height of the peak with a retention time of 25 min with a concurrent increase in the peak height of parent curcumin (data not shown). Similarly, incubation of the plasma ex-

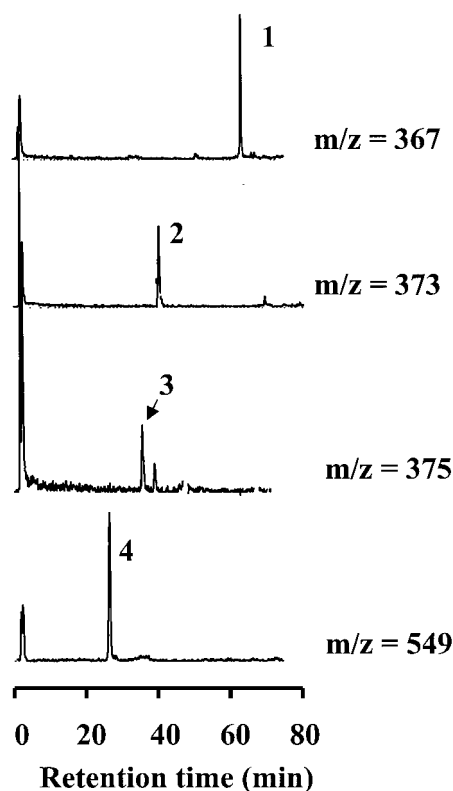


Fig. 4. Mass spectrometric analysis by selected ion monitoring at the indicated  $m/z$  values of extracts of rat plasma 30 min after administration of curcumin (40 mg/kg, i.v.). Peaks can be assigned to curcumin (1;  $m/z = 367$ ), hexahydrocurcumin (2;  $m/z = 373$ ), hexahydrocurcuminol (3;  $m/z = 375$ ), and hexahydrocurcumin glucuronide (4;  $m/z = 549$ ). Note that retention times are longer than those shown in Fig. 2 and Table 1 because the chromatographic conditions were slightly different; the retention times shown here are 67.1 min for curcumin, 39.5 min for hexahydrocurcumin, 38 min for hexahydrocurcuminol, and 24.9 min for hexahydrocurcumin glucuronide. Selected ion chromatograms of plasma extracts from rats that had not received curcumin did not show any of the peaks seen here. The chromatograms are representative of three experiments. For details of curcumin administration and HPLC and mass spectral analyses see "Materials and Methods."

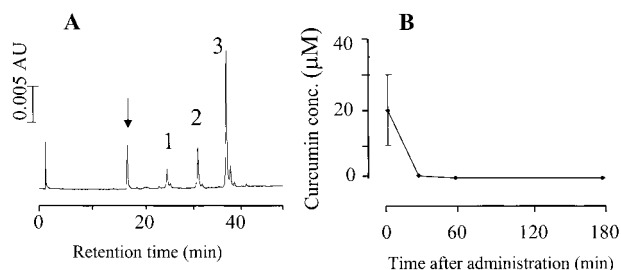


Fig. 5. High-performance liquid chromatogram with detection at 420 nm of an extract of rat plasma obtained 30 min after i.v. administration of curcumin (40 mg/kg; A), and time course of disappearance of curcumin after i.v. administration (B). The identity of the peaks in A was established by cochromatography and mass spectrometry as curcumin (3), curcumin sulfate (2), and curcumin glucuronide (1; see "Results"). Note that commercially available curcumin contains 15% desmethoxycurcumin and 5% bisdesmethoxycurcumin, which furnished the two small peaks just beyond curcumin. The arrow in A marks the position of a peak characterized by mass spectrometry as curcumin glucuronide sulfate (see "Results"). HPLC analysis of extracts of plasma from control rats did not furnish any detectable peaks. AU, absorbance units. The chromatogram is representative of three experiments, and the values in B are the mean  $\pm$  SD values of three separate animals. For details of curcumin administration and HPLC analysis see "Materials and Methods."

tracts with arylsulfatase resulted in the reduction of the height of the peak with a retention time of 31 min and a concurrent increase in the peak height of curcumin. These findings characterize these two peaks as being due to curcumin glucuronide and curcumin sulfate, respectively. This conclusion was corroborated by chromatographic analysis with ion-selected monitoring yielding molecular ions of  $m/z$  543 and 447, respectively. Furthermore, eluent corresponding to either peak was collected and analyzed by mass spectrometry. The spectra obtained were consistent with the postulated identity of the peaks as curcumin glucuronide and curcumin sulfate (Table 1). Both species persisted in the plasma after p.o. dosing for up to 6 h. The concentrations of conjugates measured at 30 min and 6 h after p.o. administration were between 1.7 and 1.5  $\mu\text{M}$  for curcumin glucuronide and between 0.21 and 0.35  $\mu\text{M}$  for curcumin sulfate (mean of three experiments in each case). At later time points, curcumin conjugates were detectable but at levels below the limit of quantitation. The third major metabolite in the extract of plasma decreased in size during incubation of plasma extracts with  $\beta$ -glucuronidase with concomitant increase of the curcumin sulfate peak. It was also decreased on incubation with arylsulfatase with concomitant elevation of the curcumin glucuronide peak (data not shown). Mass spectral analysis with ion-selected monitoring furnished a molecular ion of  $m/z$  622. These results characterize this metabolite as curcumin glucuronide sulfate. Further chromatographic analysis of the plasma extracts by ion-selected monitoring yielded peaks with  $m/z$  367, 373, 375, and 549, in addition to the curcumin conjugate peaks, consistent with the presence of curcumin, hexahydrocurcumin, hexahydrocurcuminol, and hexahydrocurcumin glucuronide, respectively (Fig. 4). Finally, for unambiguous identification, curcumin glucuronide and curcumin sulfate were synthesized using UDP glucuronic acid plus bacterial glucuronyltransferase and triethylamine sulfur trioxide, respectively. On HPLC analysis these molecules afforded peaks with retention times identical to those of the two curcumin conjugates identified in rat plasma after oral dosing (data not shown). They also furnished mass spectral fragmentation patterns compatible with those obtained from the conjugates isolated from the rat plasma (Table 1). These curcumin conjugates were also observed after i.v. administration of curcumin (Fig. 5A). Parent drug disappeared very rapidly, and, at 30 min after i.v. administration, curcumin levels were below the limit of quantitation (Fig. 5B). Curcumin conjugates were present at levels near the limit of detection at the 1 h time point, but they were not evident at 6 h.

**Effect of Curcumin Metabolites on Phorbol Ester-induced PGE<sub>2</sub> Production in HCECs.** One of the biological effects of curcumin considered to be potentially associated with chemoprevention is its ability to inhibit the expression of inducible COX-2 (11). We tested the hypothesis that the hepatic metabolites of curcumin share this pharmacological property with the parent drug. COX-2 expression was induced by PMA in HCECs, which had been exposed to curcumin or its metabolites (20  $\mu\text{M}$ ). COX-2 activity was inferred from PGE<sub>2</sub> levels. Fig. 6 shows that curcumin decreased PMA-inducible PGE<sub>2</sub> production down to almost preinduction levels. Tetrahydrocurcumin, hexahydrocurcumin, and curcumin sulfate reduced it by 31%, 37%, and 22%, respectively. Hexahydrocurcuminol was devoid of inhibitory activity. In a confirmatory Western analysis using a COX-2 monoclonal antibody, curcumin was shown to reduce PMA-induced COX-2 protein expression consistently by 60–70%. In contrast, the curcumin metabolites interfered with COX-2 protein induction only weakly or not at all (data not shown).

## DISCUSSION

The results outlined above allow the following four novel conclusions to be drawn concerning the metabolism of curcumin, which contribute to the understanding of its preclinical pharmacology and, thus, aid with the planning of its clinical evaluation: (a) human and rat liver reduces curcumin first to hexahydrocurcumin and then to hexahydrocurcuminol, whereas conjugation of curcumin is only a minor hepatic biotransformation route; (b) the biotransformation step curcumin  $\rightarrow$  hexahydrocurcumin is rapid, and the overall rate of curcumin reduction seems slower in human than in rat liver cells; (c) the predominant metabolites of curcumin in rat plasma *in vivo* are curcumin glucuronide and curcumin sulfate, whereas hexahydrocurcumin and hexahydrocurcuminol, the major metabolites of curcumin in hepatocyte suspensions, occur only in small amounts in rat plasma after curcumin administration; (d) curcumin metabolites are markedly less able to inhibit inducible COX-2 expression than their metabolic progenitor.

Whereas tetrahydrocurcumin, hexahydrocurcumin, and curcumin glucuronide have been described as products of the metabolic reduc-

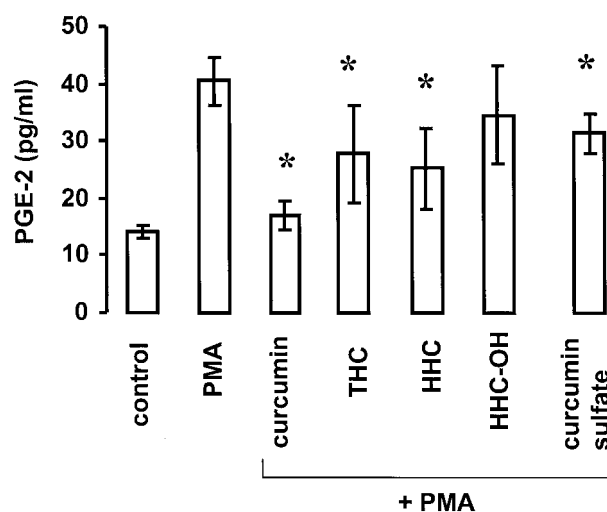


Fig. 6. Inhibition of PMA-induced PGE<sub>2</sub> generation by curcumin, tetrahydrocurcumin (THC), hexahydrocurcumin (HHC), hexahydrocurcuminol (HHC-OH) and curcumin sulfate, each at 20  $\mu\text{M}$ . The asterisks indicate that values are significantly different from those obtained with PMA alone ( $P > 0.05$ , balanced ANOVA). The results are representative of two experiments, each performed in triplicate. Curcumin or its metabolites alone did not affect PGE<sub>2</sub> production. For details of PGE<sub>2</sub> determination see "Materials and Methods."

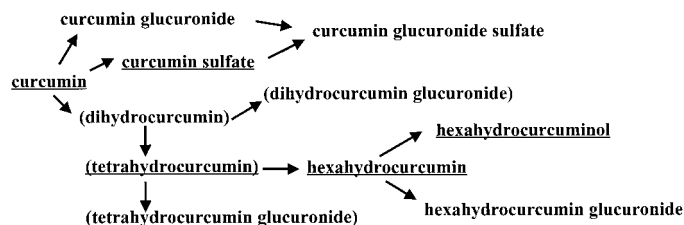


Fig. 7. Pathways of metabolism of curcumin described in this study. Underlined molecules have been characterized in terms of COX-2 inhibitory properties (see Fig. 6). *Parentheses* indicate generation of metabolites that have been previously identified in mice (13) but not observed here in human or rat hepatocytes or in rat plasma *in vivo*.

tion of curcumin in rodents before (13, 15), this is the first study that describes hexahydrocurcuminol and curcumin sulfate as curcumin metabolites. Hexahydrocurcuminol occurs naturally in the rhizomes of the ginger plant *Zingiber officinale* (24) and of *Curcuma xanthorrhiza* (17), the latter of which is, together with *Curcuma longa*, the major plant source of curcumin. Our results suggest that curcumin glucuronide and curcumin sulfate are generated only in small amounts in hepatocytes, whereas they are abundant in rat plasma after administration of curcumin. This discrepancy is consistent with the hypothesis that they are generated, at least in part, extra-hepatically, probably in the gastrointestinal tract (25). The metabolic conversions described here and their interrelationship are described in Fig. 7: the figure shows that curcumin undergoes metabolism to its sulfate and glucuronide and sulfate-glucuronide conjugates. The liver reduces curcumin to hexahydrocurcumin and hexahydrocurcuminol, probably via the intermediacy of dihydrocurcumin and tetrahydrocurcumin, two species that were identified in mice (13), but not in the present study in rat plasma or rat and human hepatocytes. Dihydrocurcumin, tetrahydrocurcumin, and hexahydrocurcumin generate glucuronides, all three of which were characterized in mice (13), and hexahydrocurcumin glucuronide was also found here in rats. Hexahydrocurcuminol constitutes the ultimate product of curcumin reduction, and it is conceivable that it is also a substrate of conjugating enzymes. However, identification of hexahydrocurcuminol glucuronide or hexahydrocurcuminol sulfate has thus far been elusive.

Curcumin and its metabolites have not been compared before in terms of pharmacological potency. Inhibition of prostaglandin biosynthesis by inhibition of COX-2 induction is arguably an important mechanism that contributes to the chemopreventive activity of curcumin (11). Here, we show that stepwise metabolic reduction of the diarylheptadienone chain was accompanied by a significant loss of ability to inhibit COX-2 expression in a bioassay as reflected by PGE<sub>2</sub> activity. Of the reduced species, tetrahydrocurcumin, found as glucuronide in the plasma of mice (13) but not in human or rat hepatocytes, and hexahydrocurcumin, a major curcumin metabolite in human and rat hepatocytes, were less capable than curcumin of interfering with the induction of PGE<sub>2</sub> production and COX-2 expression. Hexahydrocurcuminol, the other major metabolite of curcumin in the liver, lacked COX-2-suppressing activity, and curcumin sulfate interfered only weakly with COX-2 expression. It is pertinent to mention that the data presented above does not allow judgement as to whether the effect of curcumin and its metabolites on COX-2 expression is concentration dependent. The results render it unlikely that the major hepatic metabolites of curcumin are responsible for, or contribute in a major way to, its chemopreventive activity via the inhibition of COX-2 expression. It is, therefore, possible that the metabolic conversions of curcumin described here and shown in Fig. 7 are pharmacological deactivation pathways. Information as to the biological potency of curcumin metabolites is scarce. Only tetrahydrocurcumin has previously been subjected to comparative pharmacological studies. It was found to be more potent than curcumin in the carrageenin-

induced rat paw edema test for anti-inflammatory activity (26), and at least as potent an antioxidant as curcumin in rabbit erythrocyte membrane ghosts and rat liver microsomes *in vitro* (27, 28). In contrast, tetrahydrocurcumin was much less potent than curcumin as inducer of quinone reductase in cells *in vitro* (29) or as inhibitor of 12-*O*-tetradecanoylphorbol-13-acetate-induced tumor promotion in mouse skin (30). Our results intimate that the unsaturated nature of the diarylheptanoid chain and free phenolic moieties may be pivotal pharmacophoric features of molecules related to curcumin for optimal inhibition of COX-2 expression, the *in vitro* paradigm of chemopreventive activity chosen here.

The prolonged presence in rat plasma of curcumin glucuronide and curcumin sulfate after oral administration as described here may be the corollary of slow absorption of curcumin from the gastrointestinal tract and/or of its intrahepatic circulation. This contention is consistent with results of a recent drug distribution study in mice (13). It suggests low, probably subefficacious, curcumin levels in a variety of tissues, which amounted to between 1 nmol/g in brain and 72 nmol/g in liver 1 h after an i.p. dose of 100 mg/kg of the drug. The intestine was the exception in that it contained 300 nmol/g tissue. The results presented here, together with information published previously, suggest that curcumin taken p.o. might prevent cancer of the colon more effectively than malignancies in other tissues. This conclusion provides a rationale for trials of curcumin to be conducted with the aim of preventing human colorectal cancer.

In conclusion, the results described here shed new light on the role of the liver in the metabolic fate of curcumin because they suggest that hepatic metabolism of curcumin is a pharmacological deactivation step. Overall, the results buttress the rationale for clinical evaluation of curcumin in the chemoprevention of human colorectal cancer. The relevance of the findings discussed here for humans who consume curcumin will eventually be established in clinical studies of curcumin, in which pharmacokinetic and pharmacodynamic parameters will be correlated.

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