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Research Article

Metabolism of curcumin and induction of mitotic catastrophe in human cancer cells

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In cultured cells, curcumin (CUR) causes cell death by interfering with mitosis and leading to fragmented nuclei and disrupted microtubules, a process named mitotic catastrophe. In order to clarify the role of the known CUR metabolites hexahydro-CUR (HHC) and CUR-glucuronide (CUR-gluc) in mitotic catastrophe, the effects of CUR were studied in three human cancer cell lines with different metabolism of CUR. In Ishikawa and HepG2 cells, CUR was metabolized to HHC and small amounts of octahydro-CUR (OHC), whereas the only metabolism in HT29 cells was the formation of CUR-gluc. Despite their different metabolism, all three cell systems responded to CUR with arrest in G2/M phase and mitotic catastrophe. Fractionation of the cells showed that concentrations of CUR were higher in the ER and cytosol than in the incubation medium by a factor of up to about 150 and 8, respectively. In contrast to CUR, the metabolite HHC and the products of spontaneous degradation did not elicit any effects in Ishikawa cells. These results imply that the causative agent of mitotic catastrophe is the parent CUR molecule, whereas reductive metabolism and chemical degradation render CUR inactive.

Keywords: Curcumin / HepG2 / HT29 / Metabolism / Mitotic catastrophe

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1 Introduction

Curcumin (CUR, Fig. 1) is the major yellow-orange pigment of turmeric, which is obtained from the rhizomes of the Asian plant *Curcuma longa* and used extensively in traditional Indian cooking and Ayurvedic medicine [1, 2]. CUR represents the pungent principle of curry and is used as a food color in various countries [2]. It exhibits an extremely broad spectrum of biological activities [3, 4], which comprise anti-inflammatory [5], antioxidant [6], chemosensitizing [7], radiosensitizing [8], antiangiogenic [9], neuroprotective [10], antimicrobial [11], antiviral [12], antifungal [13], and wound-healing [14] properties, among others.

Of particular interest are the anticarcinogenic and cancer chemopreventive effects of CUR [15, 16]. Studies from several laboratories have shown that CUR causes cell death in various cancer cells *in vitro* by inhibiting cell proliferation, reducing cell viability and inducing symptoms of apoptosis [17 – 22]. However, cells with apoptosis-like morphology are observed rather late and thought to result from an arrest in G2/M phase and subsequent cellular and nuclear changes, a process named "mitotic catastrophe" and defined as cell death due to disrupted mitosis [23 – 25].

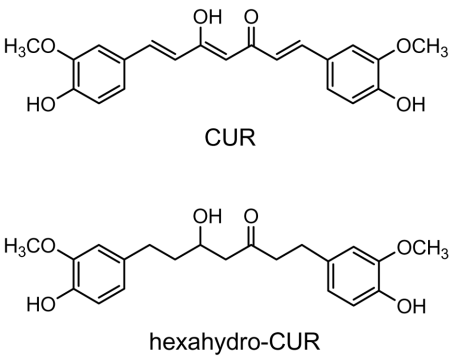

Figure 1. Chemical structures of CUR and HHC.

Studies on the absorption and metabolism of CUR in various animal species and in humans have shown low bioavailability and two major routes of biotransformation, *i.e.*, reduction to hexahydro-CUR (HHC) and octahydro-CUR (OHC), and conjugation of CUR and its reductive metabolites with glucuronic acid [26, 27]. Our laboratory has

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Abbreviations: CUR, curcumin; **CUR-gluc,** CUR-glucuronide; **DP,** degradation product; **HHC,** hexahydro-CUR; **OHC,** octahydro-CUR

recently reported that the major glucuronide of CUR formed in humans carries the glucuronic acid moiety at one of the phenolic hydroxyl groups, but small amounts of an alcoholic CUR glucuronide are also formed; in contrast, HHC is exclusively glucuronidated at the phenolic positions [28].

CUR is chemically stable in organic solvents and acidic aqueous phase, but decomposes spontaneously in aqueous solution at pH above 6.5; for example, the half-life of CUR in 0.1 M phosphate buffer pH 7.4 is only about 10 min [29, 30]. The chemical structures of the degradation products (DPs) have not been completely elucidated. Degradation is slower in the presence of proteins [30], and CUR has a high propensity for protein binding, probably through hydrophobic interactions [31].

The aim of the present study was to clarify the role of CUR metabolites and DPs in the induction of mitotic catastrophe in three human cancer cell lines *in vitro*. To this end, the kinetics of CUR uptake, intracellular distribution, and metabolite formation, as well as the effects of CUR, HHC and DPs on cell cycle and cell morphology were determined in Ishikawa, HepG2, and HT29 cells, which are derived from carcinoma of the endometrium, liver, and colon, respectively.

2 Materials and methods

2.1 Chemicals and cell lines

CUR was synthesized according to the method of Pabon [32], and HHC was obtained by hydrogenation of synthesized CUR as described earlier [27]. The purity of both compounds was >98% according to HPLC. 3,5,3′,5′-Tetramethyl-bisphenol A (TMBPA), penicillin, streptomycin, trypsin, β-glucuronidase type B-1 from bovine liver, and all other chemicals and reagents were of the highest quality available and were purchased from Sigma/Aldrich/Fluka (Taufkirchen, Germany). DMEM and DMEM/Ham's F12 (DMEM/F12) were also from Sigma/Aldrich/Fluka, and fetal bovine serum (FBS) was obtained from Invitrogen (Karlsruhe, Germany). HPLC grade ACN was from Carl Roth (Karlsruhe, Germany).

A mixture of CUR DPs was obtained by keeping CUR at a concentration of 15 or 30 μM in 1 mL DMEM/F12 at 37°C for 24 h, followed by extraction with 2 mL ethyl acetate. The extract was evaporated under reduced pressure and the residue dissolved in 50 μL methanol.

Ishikawa cells, HepG2 cells and HT29 cells were obtained from the German Collection of Microorganism and Cell Cultures (Braunschweig, Germany).

2.2 Cell culture conditions

Cells were cultured at 37°C in a humidified incubator with 5% carbon dioxide in air atmosphere, using DMEM/F12 for Ishikawa and HepG2 cells, and DMEM for HT29 cells. All cell culture media contained 10% FBS, 100 U/mL penicillin, and 0.1 mg/mL streptomycin.

2.3 Cell fractionation

A total of 3×10^6 cells in 10 mL cell culture medium were seeded in 10 cm dishes and allowed to attach for 24 h. CUR or HHC (300 nmol) dissolved in 50 μL DMSO was added to the culture medium to achieve a concentration of 30 μM. After incubation for 0.2, 1, 3, 6, 9, 24, and 30 h, the incubation media were removed and the dishes rinsed with cold PBS pH 7.4. Adherent cells were trypsinized with 1% trypsin/0.02% EDTA for 5 min and centrifuged at $2000 \times g$ for 5 min. All centrifugations were carried out in a TL-100 table centrifuge with a TLA-45 fixed angle rotor (Beckman Instruments). The pellets were suspended in 200 μL PBS and stored at −80°C until analysis.

For fractionation, 2.5×10^6 cells were homogenized by sonification for 15 s and centrifuged at 4°C and $9000 \times g$ for 15 min. The supernatant was further centrifuged at 4°C and $100\,000 \times g$ for 1 h. The resulting supernatant contained the cytosol, and the microsomal pellet consisted of fragments of the ER. Microsomes were resuspended in 250 μL PBS and homogenized by sonification for 15 s. The fractions of cytosol and microsomes were extracted with Folch's reagent (chloroform/methanol 2:1 v/v), the extracts evaporated to dryness under reduced pressure, and the residues dissolved in 50 μL methanol and a 20 μL aliquot analyzed by HPLC as described below.

Cell culture media removed after incubation with the compounds were also stored at −20°C. For workup, 10 μL of a 5 mM solution of TMBPA in DMSO was added *per* mL medium as internal standard. For the determination of unconjugated compounds, a 1.5 mL aliquot of the medium was extracted with 6 mL ethyl acetate. The extract was evaporated to dryness under reduced pressure, the residue dissolved in methanol and analyzed by HPLC. For measuring the sum of unconjugated and conjugated material, another 1.5 mL aliquot of medium was mixed with 1.5 mL of 0.15 M acetate buffer pH 5.0, incubated with 5000 U of β-glucuronidase for 3 h at 37°C, extracted and analyzed as described above. The amount of glucuronides was calculated as the difference between the values obtained with and without hydrolysis.

2.4 HPLC analysis

An 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. Separation was carried out on a 250×4.6 mm^2 id, 5 μm RP Prodigy 5ODS(2) column (Phenomenex, Torrance, CA, USA) with a flow rate of 1 mL/min. Solvent A was deionized water adjusted to pH 3.0 with

formic acid, and solvent B was ACN. A linear solvent gradient was used, changing from 30 to 70% B in 35 min. The retention times of CUR, HHC, OHC, and CUR-glucuronide (CUR-gluc) were 25.8, 12.1, 9.4, and 17.3 min, respectively. CUR and CUR-gluc were recorded and quantified at 420 nm and compounds containing reduced metabolites and DP at 280 nm, as previously reported [27, 28, 33].

2.5 Determination of cell diameter, cell number, and cell cycle distribution

A total of 10^5 cells were mixed with 10 mL isotonic salt solution and measured with an electronic cell counter (CASY, Schaerfe Systeme, Reutlingen, Germany), taking the peak of the Gauss distribution as the average cell diameter as described by the manufacturer. The mean values of three independent experiments were 14.3 ± 0.3 μm for Ishikawa, 15.0 ± 0.4 μm for HepG2, and 14.4 ± 0.7 μm for HT29 cells.

For studying the effects on cell number and cell cycle, 5×10^4 Ishikawa, HepG2, or HT29 cells in 1 mL medium *per* well were seeded on 24-well plates (Nunc, Wiesbaden, Germany) and allowed to attach and resume proliferation for 24 h. Cells were then incubated for 24 h with various concentrations of the test compounds, dissolved in DMSO and added to the cell culture medium. The final DMSO concentration did not exceed 0.5%. After treatment, the media were removed and cells were lysed with 150 μL lysis buffer *per* well and stained with 750 μL CyStain DNA 2step staining solution according to the manufacturer's instructions (Partec, Münster, Germany). For each concentration of the test compound, cells from three wells were analyzed in parallel by flow cytometry for cell number and relative DNA content with a Ploidy Analyzer®-II flow cytometer (Partec).

2.6 Studies on cell morphology

Cells were plated on microscope glass slides in a quadriperm vial. A total of 1×10^5 cells *per* slide in 5 mL medium were allowed to attach for 24 h, then incubated with various concentrations of the test compounds for 24 h as described above, followed by a postincubation period of 24 h in compound-free medium. Subsequently, cells were fixed in ice-cold methanol. After blocking of nonspecific binding by incubation with goat serum for 1 h at 37°C, slides were incubated with a 1% solution of BSA in PBS containing monoclonal mouse anti-α-tubulin antibody for 1 h at 37°C, followed by incubation with a solution of BSA in PBS containing the secondary goat antimouse antibody (CY3-conjugated, Jackson Immune Research Laboratories, West Grove, USA). Slides were finally stained in antifade solution containing 1 μg/mL 4,4′-diamidino-2-phenylindole (DAPI) and analyzed by fluorescence microscopy for mitotic cells, fragmented nuclei, and disrupted cytoplasmic microtubules.

2.7 Statistical analysis

Data are expressed as mean ± SD of at least three individual experiments, and the two-tailed independent Student's *t*-test of significant differences was conducted using the Origin program of Microcal software.

3 Results and discussion

3.1 Cellular uptake, distribution and metabolism of CUR and HHC

The time course of the concentrations of CUR, its reductive metabolites and its glucuronide in the ER and in the cytosol of Ishikawa, HepG2, and HT29 cells, as well as in the incubation medium was followed over a time period of 30 h. For each time point and cell line, 3 million cells were incubated with 30 μM CUR. Cells were then harvested, lysed, and fractioned by centrifugation into ER (microsomes) and cytosol. Cell fractions and aliquots of the incubation media were directly extracted and extracts analyzed by HPLC. In other aliquots of the incubation media, an enzymatic hydrolysis of glucuronides was conducted prior to extraction. The results are shown in Fig. 2.

CUR is rapidly taken up by all three cell types, and is clearly detectable in the ER and cytosol after only 12 min of incubation. The peak concentrations of CUR in ER and cytosol were reached after 1 h in HepG2 and HT29 cells, and at 3 h in Ishikawa cells. No reductive metabolites of CUR were detectable in the ER fractions of the three cell systems; in contrast, the cytosol of Ishikawa and HepG2, but not of HT29 cells, contained significant amounts of the reductive metabolite HHC (Fig. 2). Likewise, HHC and small amounts of OHC were found in the incubation media of Ishikawa and HepG2 cells at later time points. In the culture medium of HT29 cells, no reductive metabolites but large amounts of the phenolic glucuronide of CUR were measurable (Fig. 2). The identification as phenolic glucuronide is based on its HPLC retention time, which differs from that of the alcoholic CUR glucuronide [28]. Thus, HT29 cells efficiently glucuronidate CUR but are unable to form reductive metabolites, whereas Ishikawa and HepG2 cells are devoid of CUR glucuronidation but capable of CUR reduction.

It is obvious from Fig. 2, that the total amount of CUR is two to three times higher in the ER than in the cytosol. In order to estimate the concentrations of CUR and its reductive metabolites in these subcellular compartments of the three cell types, the diameters of the cells were measured and the corresponding cell volumes calculated. With the assumption that the ER takes 10% and the cytosol 50% of the cell volume, peak concentrations of 4400 μM CUR were calculated in the ER and 250 μM in the cytosol of HepG2 cells after 1 h incubation, whereas the respective concentrations in Ishikawa cells were somewhat lower but

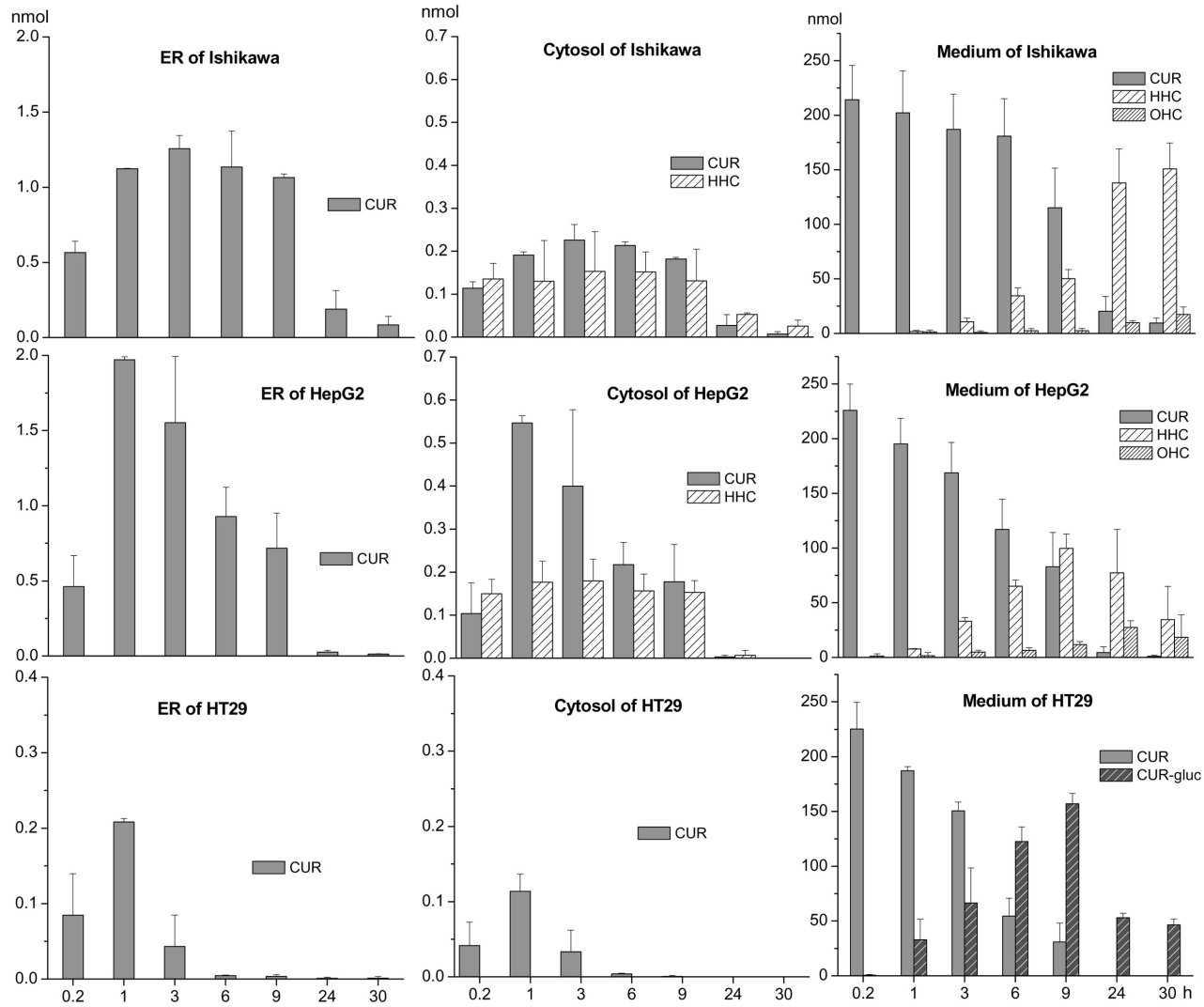

Figure 2. Kinetics of CUR and its metabolites HHC, OHC, and CUR-gluc in the ER and cytosol of cultured human cancer cells. Data represent the total amount of compound in the ER and cytosol from 2.5 million cells or from 10 mL incubation medium, and are the mean ± SD of three independent experiments.

Table 1. Estimated concentrations of CUR and HHC in the ER and cytosol of cells incubated with 30 μM CUR

Cell type	Ishikawa	HepG2	HT29
Volume *per* cell (L)	1.54×10^{-12}	1.77×10^{-12}	1.57×10^{-12}
Volume of 2.5×10^6 cells (L)	3.85×10^{-6}	4.42×10^{-6}	3.94×10^{-6}
Concentration of CUR (μM)			
In ER after 1 h	2900	4400	530
In ER after 9 h	2800	1600	9
Concentration of CUR/HHC (μM)			
In cytosol after 1 h	100/70	250/80	60/n.d.
In cytosol after 9 h	94/70	80/70	n.d.

The LOD was 2 μM for CUR and 15 μM for HHC.
HHC, hexahydro-CUR; n.d., not detectable.

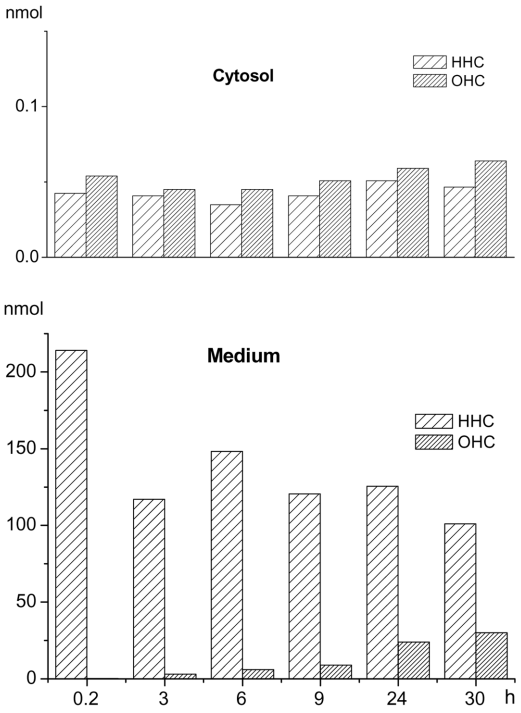

Figure 3. Kinetics of HHC and its reductive metabolite OHC in cultured Ishikawa cells. Data represent the total amount of compound in the cytosol from 2.5 million cells and from 10 mL incubation medium.

persisted over a longer time span (Table 1). Thus, peak concentrations of CUR in the ER were up to 150-fold and in the cytosol up to 8-fold higher than the initial CUR concentration in the incubation medium. The concentration of HHC remains at the level of about 70 μM from 1 to 9 h in both cell types. In HT29 cells, the peak concentration of CUR is much lower, probably due to its efficient glucuronidation. The rapid decline of free CUR in the incubation medium and the corresponding increase of CUR glucuronide provide indirect evidence that CUR is rapidly taken up by HT29 cells, glucuronidated, and the glucuronide excreted back into the medium. Because uridine-5′-diphosphoglucuronosyltransferases (UGTs) are know to be located at the luminal side of the ER, an efficient distribution of CUR into the ER must precede glucuronidation. No attempts were made to detect CUR glucuronide in the fractions of ER and cytosol of HT29 cells because of the small amounts of these cell fractions.

When the three cell systems were incubated with 30 μM HHC under the same conditions, peak concentrations of this compound reached about 20 μM in cytosol, and no HHC was detectable in the ER of any of the cells. In Ishikawa (Fig. 3) and in HepG2 cells, reduction of HHC to OHC was observed, which also showed up in the culture media of these cells at later time points (Fig. 3). In HT29 cells incubated with HHC, no reductive metabolite but considerable amounts of the glucuronide of HHC were detected (data not shown).

In summary, these metabolic studies have shown that CUR but not the structurally similar HHC have the propensity to transiently accumulate in the ER of three different types of cancer cells. These cells exhibit different metabolic activities: whereas HepG2 and, with somewhat lower efficiency, Ishikawa cells convert both compounds to reduced metabolites, HT29 cells lack reductive metabolism but efficiently generate glucuronides.

3.2 Effects of CUR, HHC, and DPs on cell cycle and cell morphology

Ishikawa, HepG2, and HT29 cells were incubated with various concentrations of CUR ranging from 0 to 30 μM for 24 h and subsequently analyzed by flow cytometry. A concentration-dependent decline of the total cell number and a shift of the cell cycle distribution was observed with each of the three cell systems (Fig. 4). At concentrations of 20–30 μM CUR, all cells exhibited a significantly reduced percentage of cells in G0/G1 phase and an increased proportion in G2/M phase. The three cell systems appeared to be of similar susceptibility, although the effects of CUR in the different cells could not be compared quantitatively due to differences in the cell culture medium and in the extent of their attachment to the culture dishes (see below).

In another experiment, cells were treated with CUR for 24 h and subsequently kept in fresh medium for 24 h prior to fluorescence staining of chromatin and microtubules. Analysis by fluorescence microscopy revealed a concentration-dependent decline of the number of mitotic cells and a concomitant increase of cells with fragmented nuclei and disrupted microtubules (Table 2). The fragmentation of nuclei in conjunction with disruption of the microtubule network is typical for the mitotic catastrophe induced by CUR [23, 25, 34]. Regarding this endpoint and the number of mitotic cells, HepG2 cells appeared to be somewhat more sensitive than Ishikawa and HT29 cells (Table 2). However, a quantitative comparison of the cell lines was precluded, because the lower number of HT29 cells with fragmented nuclei and disrupted microtubules may also be due to the smaller number of attached cells observed after treatment, *i.e.*, damaged HT29 cells appear to be more readily detached and washed away than damaged Ishikawa and HepG2 cells.

In order to study the ability of DPs of CUR to interfere with the cell cycle of Ishikawa cells, CUR was kept in culture medium without cells for 24 h at 37°C. Subsequent extraction of the medium and HPLC analysis of the extract showed that the CUR had decomposed to ten products (Fig. 5), among which were ferulic acid (peak 2), vanillin (peak 3), and feruloylmethane (peak 5) as identified by cochromatography with authentic standards. The complete mixture of DPs obtained from two different CUR concentra-

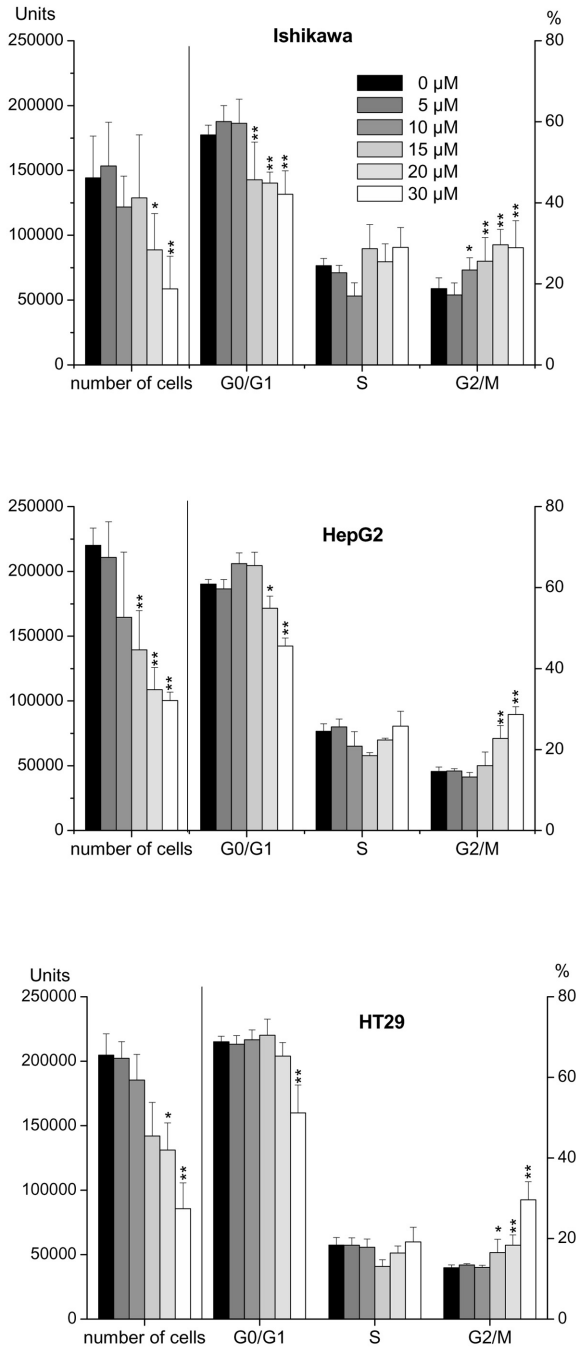

Figure 4. Effects of a 24 h-incubation with CUR on the cell number (left) and cell cycle distribution (right) in cultured human cancer cells. Data represent the mean ± SD of three independent experiments. The percentage of cells in different phases of the cell cycle was calculated separately in each individual experiment (100% is the sum of all cells). Statistical significance was determined using Student's *t*-test. Levels of significance: *, 0.01; **, 0.001.

tions, as well as a mixture of authentic ferulic acid, vanillin and feruloylmethane, were incubated with Ishikawa cells for 24 h and the cells subsequently analyzed by flow cytom-

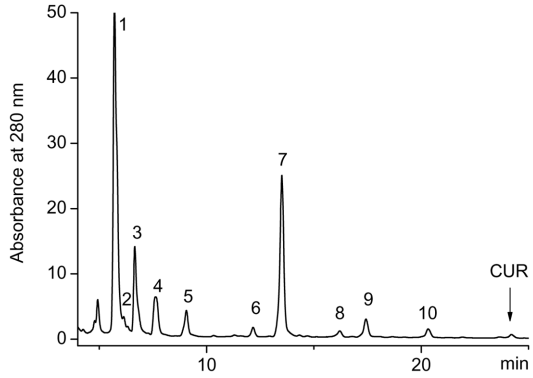

Figure 5. HPLC profile of the DPs of CUR. Peak 2, ferulic acid; 3, vanillin; 5, feruloylmethane.

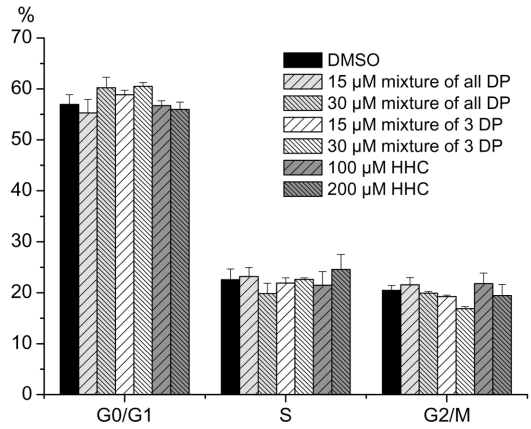

Figure 6. Effects of a 24 h-incubation with CUR DPs and HHC on the cell cycle distribution in cultured Ishikawa cells. Data represent the mean ± SD of three independent experiments. The percentage of cells in different phases of the cell cycle was calculated separately in each individual experiment (100% is the sum of all cells).

etry. The reductive CUR metabolite HHC was also included in this experiment. Neither the complete nor partial mixture of DPs or HHC were able to affect the cell cycle of Ishikawa cells (Fig. 6).

4 Concluding remarks

The concentration-dependent induction of mitotic arrest and mitotic catastrophe in Ishikawa, HepG2, and HT29 cells by CUR, shown in the present study, is in accordance with numerous reports on the same effects of CUR in other cell types [18 – 23]. However, our study is the first one to report that the major reductive metabolite, *i.e.*, HHC, and the products of the spontaneous degradation of CUR, are devoid of activity, implying that the active compound is the CUR molecule *per se*. It should be pointed out that the induction of mitotic catastrophe must not be confused with

Table 2. Effects of a 24 h-incubation with CUR on the number of mitotic cells and the formation of fragmented nuclei and disrupted microtubules

Concentration of CUR (μM)	0	10	20	30
Ishikawa cells				
Mitotic cells (*per* 2000 cells)	55.0 ± 4.58	63.7 ± 13.05	14.4 ± 5.59[a)]	1.7 ± 2.08[a)]
Fragmented nuclei	–	+++	++	++
Disrupted microtubules	–	+	++	+++
HepG2 cells				
Mitotic cells (*per* 2000 cells)	64.8 ± 9.98	10.0 ± 5.20[a)]	1.3 ± 1.53[a)]	0[a)]
Fragmented nuclei	–	++++	+++	++
Disrupted microtubules	–	+	++	+++
HT29 cells				
Mitotic cells (*per* 2000 cells)	69.7 ± 7.51	56.0 ± 27.18	7.3 ± 3.51[a)]	0.7 ± 0.58[a)]
Fragmented nuclei	–	+	+	(+)
Disrupted microtubules	–	(+)	+	++

Data represent the mean ± SD of three independent experiments.
a) Statistically different from untreated cells (CUR concentration 0) with a level of significance <0.001 as determined using Student's *t*-test.

the well-known apoptotic effect of CUR [35], although the cellular morphology of the two events is similar [34]. Moreover, the induction of mitotic catastrophe by CUR but not HHC is not related to the antioxidant activities of these curcuminoids, because HHC has been shown to exhibit even higher antioxidant activity than CUR [36]. In contrast to HHC, the parent CUR is rapidly taken up by the cells and transiently reaches high concentrations in the cytosol and, in particular, in the ER (Fig. 2). This is consistent with the observations by Jaruga *et al*. [37] that CUR readily penetrates into the cytoplasm of rat thymocytes and accumulates in membraneous structures including the ER. Its membraneous localization in cells appears to protect CUR from spontaneous chemical degradation: without cells, more than 90% of a 30 μM solution of CUR in culture medium has decomposed after 24 h; in contrast, no significant amounts of DPs were detected in the medium and subcellular fractions when CUR was incubated with cells under the same conditions, and the amounts of CUR metabolites accounted for the decline of parent CUR over time. In HT29 cells, it is assumed that the concentration of CUR in the ER is limited by rapid glucuronidation, mediated by membrane-bound UGTs localized at the luminal side of the ER. For the formation of the reductive metabolites in Ishikawa and HepG2 cells, transition of CUR from the ER into the cytosol must be assumed, because only cytosolic enzymes are capable of reducing CUR to HHC [26]. The propensity of CUR to accumulate in the membranes of subcellular organelles is proposed to account for the induction of mitotic catastrophe and subsequent cell death. However, it remains to be elucidated whether disruption of membrane function, *e.g*., changes of lipid fluidity, or interaction with membrane proteins, *e.g*., factors involved in signal transduction, is the ultimate reason for CUR-induced cell death.

The authors have declared no conflict of interest.

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