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Cooking enhances curcumin anti-cancerogenic activity through pyrolytic formation of "deketene curcumin"

Indra N. Dahmke [a,e], Stefan P. Boettcher [b], Matthias Groh [c], Ulrich Mahlknecht [d,e,*]

[a] Institute of Clinical and Experimental Surgery, Saarland University, Homburg/Saar, Germany
[b] Institute of Pharmaceutical and Medicinal Chemistry, Saarland University, Saarbrucken, Germany
[c] Helmholtz-Institute of Pharmaceutical Research Saarland, Department of Drug Design and Optimization, Saarland University, Saarbrucken, Germany
[d] Department of Internal Medicine – Haematology/Oncology, St. Lukas Clinic, D-42697 Solingen, Germany
[e] Department of Internal Medicine, Division of Immunotherapy and Gene Therapy, Saarland University Medical Center, Homburg/Saar, Germany

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ABSTRACT

Curcumin is widely used in traditional Asian kitchen as a cooking ingredient. Despite its low bioavailability, epidemiological data, on low cancer incidence in Asia, suggest beneficial health effects of this compound. Therefore, the question arose whether cooking modifies the anti-cancerogenic effects of curcumin. To evaluate this, we pyrolysed curcumin with and without coconut fat or olive oil, and analysed the products by high-performance liquid chromatography (HPLC). A number of more hydrophilic curcumin isoforms and decomposition products, including a compound later identified by nuclear magnetic resonance spectroscopy (NMR) as "deketene curcumin" (1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one), formerly described as a synthetic curcumin derivative, were detected. Additionally, we proved that deketene curcumin, compared to curcumin, exhibits higher toxicity on B78H1 melanoma cells resulting in G2 arrest. In conclusion, deketene curcumin is formed as a consequence of pyrolysis during common household cooking, showing stronger anti-cancer effects than curcumin. Moreover, we propose a chemical reaction-pathway for this process.

1. Introduction

Curcumin (1,7-bis(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione), which is the main phenolic component found in *turmeric* prepared from the rhizome of *Curcuma longa*, is long known for its anti-inflammatory, anti-oxidant and anti-cancerogenic properties (Shehzad, Lee, & Lee, 2013). Based on epidemiological data, from the WHO, curcumin, as part of the traditional Indian diet, is associated with low incidence rates on colorectal, prostate and lung cancers in India (Mohandas, 2011; Sinha, Anderson, McDonald, & Greenwald, 2003). The low bioavailability of orally administered curcumin, which is described by numerous investigators, as well as the conversion into less active metabolites, is contradictory to this assumption (Cheng et al., 2001; Garcea et al., 2005; Sharma et al., 2001). Moreover, curcumin shows low buffer and plasma stability (Griesser et al., 2011; Tonnesen & Karlsen, 1985; Wang et al., 1997). Hence, we questioned whether the pre-consumptional processing, as performed in typical Indian cooking, changes the quality or bioavailability of curcumin. To address this question, we pyrolysed curcumin, with and without coconut fat, which is traditionally used on the Indian subcontinent, or olive oil, as in modern style western cooking, and analysed the products in reverse phase high-performance liquid chromatography (HPLC). Although we found the major part of curcumin unchanged, a number of more hydrophilic products were observed, including isoforms of curcumin derivatives, known degradation products, as well as an unknown product. This product was isolated by preparative HPLC and identified by nuclear magnetic resonance (NMR) spectroscopy as 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one), which has already been described as a potent synthetic curcumin derivative (Liang et al., 2009; Quincoces Suarez et al., 2010). We then evaluated the activity of this derivative, named "deketene curcumin" (DKC) and compared it to curcumin and pyrolysed curcumin, in different *in vitro* experiments. In conclusion, DKC showed significantly stronger cytotoxic activity on murine B78H1 melanoma cells when compared to the reactant. Therefore, we argue that traditional pre-consumptional processing of curcumin strongly enhances its bioactivity thus potentiating its beneficial effects. Furthermore, we propose a preferential reaction-pathway for the pyrolytic formation of DKC based on the identification of certain intermediates.

* Corresponding author at: Department of Internal Medicine – Haematology/Oncology, St. Lukas Clinic, D-42697 Solingen, Germany. Tel.: +49 212 705 2150; fax: +49 212 705 2152.
E-mail address: mahlknecht@gmx.de (U. Mahlknecht).

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2. Materials and methods

2.1. Chemicals

Curcumin that was used for pyrolysis was purchased from Sigma Aldrich (Deisenhofen, Germany). Curcumin employed for the preparative HPLC was obtained from Sabinsa Cooperation (East Windsor, USA). Both samples contained about 80% curcumin (CUR), 17% demethoxycurcumin (DMC) and 3% bisdemethoxycurcumin (BDMC). All chemicals were purchased from Sigma Aldrich (Deisenhofen) if not indicated otherwise.

2.2. Pyrolysis procedure

5.0 mg of curcumin was filled into a glass tube (⌀ 20 mm, height 40 mm) for each single sample. For the fat treated samples, 50.0 mg of the particular fat was added. The samples were subsequently heated to 250 °C for 20 min, using a hot plate. For the isolation of DKC 100 mg curcumin were heated to the same temperature for 120 min in a crystallizing dish.

2.3. Instruments and reagents

The quantitation of the pyrolysis products was carried out using a *ThermoFisher SpectraSystem HPLC–UV–MS* (Thermo Fisher Scientific, Waltham), equipped with a degasser, a quaternary pump, an autosampler, a MWD (254 and 278 nm) and a MSQ ESI mass spectrometer in positive mode (source temperature 350 °C, capillary voltage 3.9 kV, nitrogen sheath gas pressure 4.0×10^5 Pa, sheath gas flow: 58 ml/min according to descriptions of the manufacturer, auxiliary gas flow: 29 ml/min). *Xcalibur* software was deployed for data acquisition and plotting. The isolation of DKC, from the pyrolysis mixture, was performed using a *Waters Autopurification HPLC–DAD–MS*. For the chromatographic methods bidistilled water (Elix® water, pure, Millipore Corporation, Billerica) was used as aquatic phase. All solvents used for HPLC were of chromatographic grade.

NMR spectra were recorded on a *Bruker Fourier* 300 (Bruker Corporation, Billerica) (^{1}H, 300 MHz; ^{13}C, 75 MHz) spectrometer at 300 K. Chemical shifts are recorded as δ values in ppm units by reference to the hydrogenated residues of deuterated solvent as internal standard (DMSO-d_6: δ = 2.50, 39.99). Splitting patterns describe apparent multiplicities and are designated as s (singlet), br s (broad singlet), d (doublet), dd (doublet of doublet). Coupling constants (J) are given in Hertz (Hz).

2.3.1. LC–UV–MS

The pyrolysis residues were diluted in 1 ml acetone. All samples were injected by an autosampler (Surveyor®, Finnigan™, Thermo Fisher Scientific) with an injection volume of 25 µl. A RP C18 NUCLEODUR® 100–5 (125 × 3 mm) column (Macherey–Nagel GmbH, Düren) was used as the stationary phase. The solvent system consisted of water (A) and acetonitrile (B), each containing 0.1% trifluoroacetic acid (TVA) (v/v).

The flow rate was set to 800 µl/min. The percentage of B started at an initial of 5%, was increased to 100% during 16 min, kept at 100% for 2 min and flushed back to the 5% in 2 min.

2.3.2. Preparative HPLC–MS

In 30 single runs each time 1 ml of methanol, containing 3–4 mg of pyrolysis residue was injected and separated on a C-18 Waters X-Bridge OBD 19 × 150 mm, 5 µM column. The solvent system consisted of water (A) and methanol (B), each containing 0.1% TFA (v/v).

The flow rate was set to 20 ml/min. The percentage of B started at an initial of 10%, was increased up to 95% during 7 min, kept at that percentage for 1 min and flushed back to 10% in 1 min. The fraction collector was triggered by the mass spectrometer in SIM mode, collecting the m/z 327.2 with a width of 1.0 m/z.

2.4. Cell culture

Murine amelanotic B78H1 melanoma cells, employed for the *in vitro* experiments, were a kind gift from the Laboratory of Immunology and Biology of Metastasis of the Department of Experimental Pathology at the University of Bologna (Lollini et al., 1987). The B78H1 cells were cultured in Dulbecco's modified Eagle's medium (DMEM; PAA, Cölbe) supplemented with 10% fetal calf serum (FCS), 100 U/ml penicillin and 0.1 mg/ml streptomycin (PAA) at 37 °C in a humidified atmosphere containing 5% CO_2. They were grown to 80–90% confluence and subjected to no more than five cell passages after cryostorage.

2.5. Fluorescence microscopy of cells

For qualitative analysis of the incorporation of curcumin derivatives by B78H1 cells 1×10^5 cells were seeded per well into a 24-well plate. After 24 h, cells were incubated either with 20 µM of curcumin derivatives or DMSO at 37 °C and 5% CO_2 and microscopic pictures were taken after 1 and 24 h with the BIOREVO BZ-8000 (Keyence, Osaka). The auto-fluorescent curcumin derivatives incorporated by the cells were detectable in the *GFP fluorescence channel*. All substances were tested in triplicate.

2.6. Water-soluble tetrazolium (WST)-1 assay

To assess the effect of curcumin derivatives on the viability of B78H1 cells, a WST-1 assay (Roche Diagnostics, Mannheim) was performed according to the manufacturer's instructions. Briefly, 5×10^3 cells were seeded into 96-well plates, and were treated either with vehicle (DMSO) or serial dilutions of curcumin derivatives (Santa Cruz Biotechnology, Heidelberg). All derivatives were tested in quadruplicate. After 24 h, 10 µl of WST-1 reagent per 100 µl medium was added to each well. After 30 min incubation at 37 °C, the absorbance of each well was measured at a wavelength of 450 nm (reference at 620 nm) and corrected against blanks (medium ± curcumin derivatives without cells).

2.7. Analysis of apoptotic cells by flow cytometry

The number of apoptotic versus necrotic cells was assessed by flow cytometry. In short, B78H1 cells (3×10^6 cells per well) were seeded into a 12-well plate and allowed to adhere overnight. Cells were incubated with either serial dilutions of curcumin derivatives or vehicle for 24 h at 37 °C, 5% CO_2. They were cropped, washed twice with PBS, transferred to 5 ml round bottom polystyrene tubes (BD Falcon, REF. 352054) and incubated for 15 min at room temperature in the dark with APC-Annexin V (1:24 in Annexin Binding Buffer, Immunotools, Friesoythe). Immediately prior to analysis on a FACSCanto™ (BD Biosciences, San Jose) 2 µl propidium iodide [1 mg/ml] was added to each tube. Three independent experiments were performed; each sample was analysed in duplicate.

2.8. Cell cycle analysis

B78H1 were plated as described above and incubated in the presence of 20 µM curcumin derivatives or DMSO for 24 h at 37 °C and 5% CO_2. Subsequently, cells were cropped, washed twice with PBS and resuspended in 200 µl NaCl (0.9%). With the help of a

1 ml syringe and a 25 gauge needle the resuspended cells were squirted into 1800 µl 100% methanol and stored at −20 °C overnight. Cells were shortly thawed, centrifuged and washed with PBS. Supernatant was carefully removed, cells were resuspended in PBS with RNAse A (1:400, 10 mg/ml stock solution; Macherey–Nagel GmbH, Düren), transferred to 5 ml round bottom polystyrene tubes and incubated for 1 h at room temperature. Afterwards, an equal volume of PBS containing propidium iodide (1:100, 1 mg/ml stock solution) was added to the suspension and incubated for 1 h, at room temperature in the dark. Samples were analysed on a FACSCanto™. Three independent experiments were performed; each sample was analysed in duplicate.

2.9. Statistical analyses

All values are shown as mean ± standard error of the mean (SEM). Data was first analysed for normal distribution and equal variance. Differences between the two experimental groups were calculated by unpaired Student's *t*-test (SigmaStat, Jandel Corporation, San Rafael). A value of $P < 0.05$ was considered statistically significant.

3. Results and discussion

3.1. Pyrolysis with a fatty matrix changes composition of products

After mixing curcumin with fatty acids a reduction of spontaneously formed hydrophilic isoforms was detected (Fig. 1). After pyrolysis of curcumin, we measured an increase of more hydrophilic CUR-, BMC- and BDMC-isoforms, both with and without a fatty matrix. By performing curcumin pyrolysis, without coconut fat or olive oil, the formation of curcumin- and dehydrocurcumin-dimers with a higher retention time (RT) compared to curcumin were observed. Furthermore, we discovered a yet unidentified product at RT 9.6 min with a molecular weight of 326 g mol^{-1} (*m*/*z*: 327). Since a high deviation among the same preparations was noticed, probably due to unequal heating processes, this analysis was primarily of qualitative nature.

In 1986 Nagabhushan and Bhide demonstrated a dose-dependent reduction of chilli induced mutagenicity by pyrolysed curcumin (Nagabhushan & Bhide, 1986). It was also shown that heating curcumin for 15 min in water increased its solubility and also pharmacological activity for example on toxic lipid peroxidation products or human auto-antibodies *in vitro* (Kurien, D'Souza, & Scofield, 2010; Kurien, Singh, Matsumoto, & Scofield, 2007). In our HPLC analysis unmodified CUR showed a retention time of 11.7 min. Roughly, about 30% of CUR was converted by the pyrolytic process into more hydrophilic products with a shorter retention time compared to the educts.

The formation of CUR dimers under slightly more radical conditions (1 h heating at 70 °C with 2,2′-azobis(isobutyronitrile)) was described by Masuda et al. (1999), and the authors suggest the dimers importance for the antioxidant activity of CUR (Masuda et al., 1999).

3.2. Identification of the pyrolysis product with RT 9.6 and m/z 327 by NMR spectroscopy

The pyrolysis product of interest was isolated in sufficient quantity by the use of preparative HPLC as described above and yielded 4.2 mg. Based on the molecular structure and atomic composition mass of curcumin, the substance could be identified by NMR spectroscopy as 1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadiene-3-one), named "deketene curcumin" (DKC). The NMR data showed good accordance to a formerly published reference spectrum (Masuda, Jitoe, Isobe, Nakatani, & Yonemori, 1993).

^{1}H NMR (300 MHz, DMSO-d_6): δ = 9.63 (br s, 2 H), 7.65 (d, J = 15.8 Hz, 2 H), 7.37 (d, J = 1.7 Hz, 2 H), 7.20 (dd, J = 8.1, 1.7 Hz, 2 H), 7.15 (d, J = 15.8 Hz, 2 H), 6.84 (d, J = 8.1 Hz, 2 H), 3.85 (s, 6 H) ppm. ^{13}C NMR (75 MHz, DMSO-d_6): δ = 188.0, 149.4, 148.0, 142.8, 126.4, 123.3, 123.0, 115.7, 111.4, 55.7 ppm.

3.3. Reaction-pathway for the pyrolytic formation of deketene curcumin (DKC)

In Fig. 2, two plausible pathways (A and B) for the formation of DKC are displayed. The 1,3 diketone bridge of curcumin but also the unsaturated keto group represent labile parts responsible for its instability. After the initial formation of acetyl fragments a subsequent addition of vanillin, originating from curcumin decomposition, should lead to the formation of the α,β-di-unsaturated

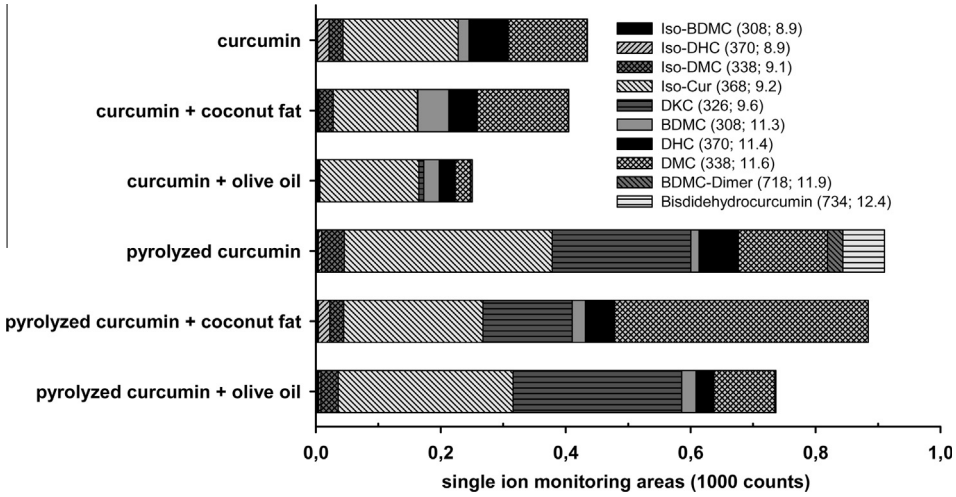

Fig. 1. Pyrolysis of curcumin leads to formation of hydrophilic products. Products of pyrolysed and unpyrolysed samples with and without a fatty matrix were analysed *via* reverse phase HPLC. The content of products is displayed in *single ion monitoring areas* (1000) and was normalised to unchanged curcumin found in the samples after pyrolysis. Compared to unpyrolysed controls the content of isoforms of curcumin derivatives and decomposition products with a shorter retention time (RT) in HPLC elution compared to curcumin was considerably higher in pyrolysed samples. In the pyrolysed curcumin sample, we also detected dimers with a RT of 11.9 and 12.4 min. Note the new product deketene curcumin (DKC) at RT 9.6 min with a molecular weight of 326 g mol^{-1} (*m*/*z*: 327) in all pyrolysed samples. In the parenthesis behind every derivative the molecular weight (g mol^{-1}) as well as the RT (min) is included. Curcumin (Cur), bisdemethoxycurcumin (BDMC), demethoxycurcumin (DMC), dihydrocurcumin (DHC).

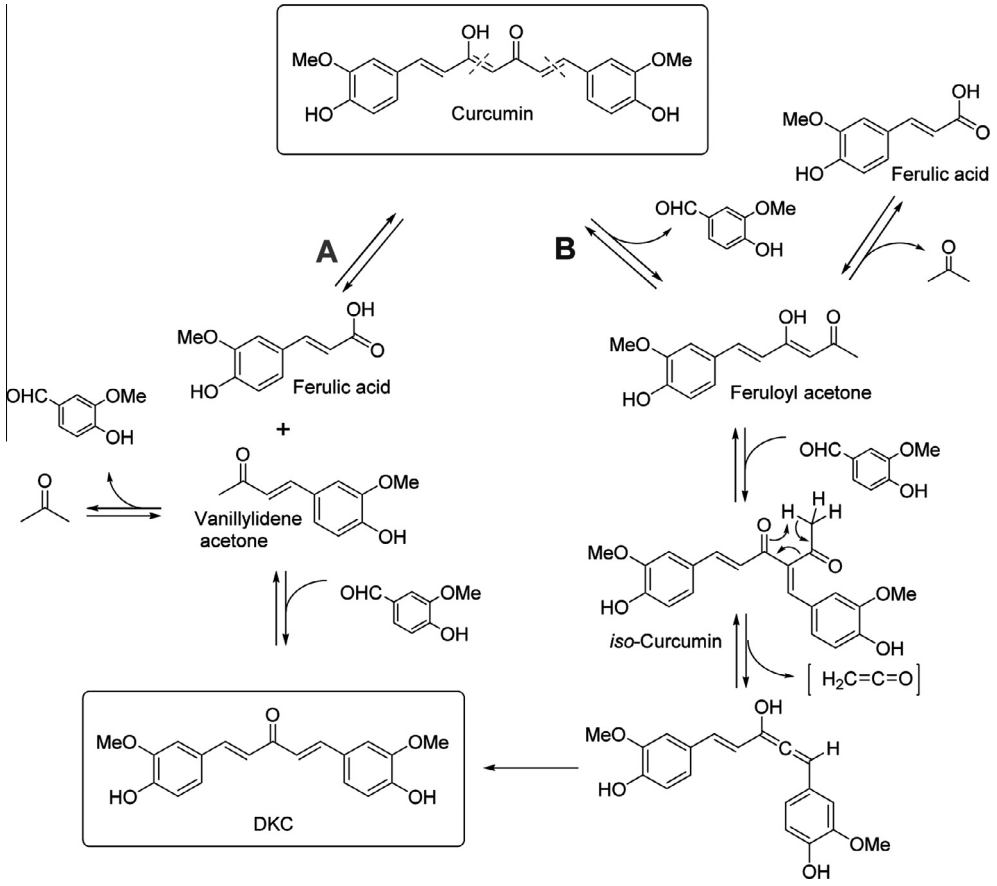

Fig. 2. Plausible reaction-pathway for the formation of deketene curcumin (DKC) from curcumin. (A) Cleavage of the 1,3 diketone bridge *via* retro aldole reaction yielding ferulic acid and vanillylidene acetone. After cleavage of acetone the resulting 4-hydroxy-3-methoxy-benzaldehyde (vanillin) reacts with the vanillylidene acetone in an aldole condensation to form DKC. (B) Cleavage of the α,β-unsaturated bridge *via* retro aldole reaction yielding feruloyl acetone and vanillin. A subsequent aldol condensation of vanillin and feruloyl acetone affords iso-curcumin. A formal ketene elimination, *via* a cyclic transition state, leads to the di-unsaturated DKC.

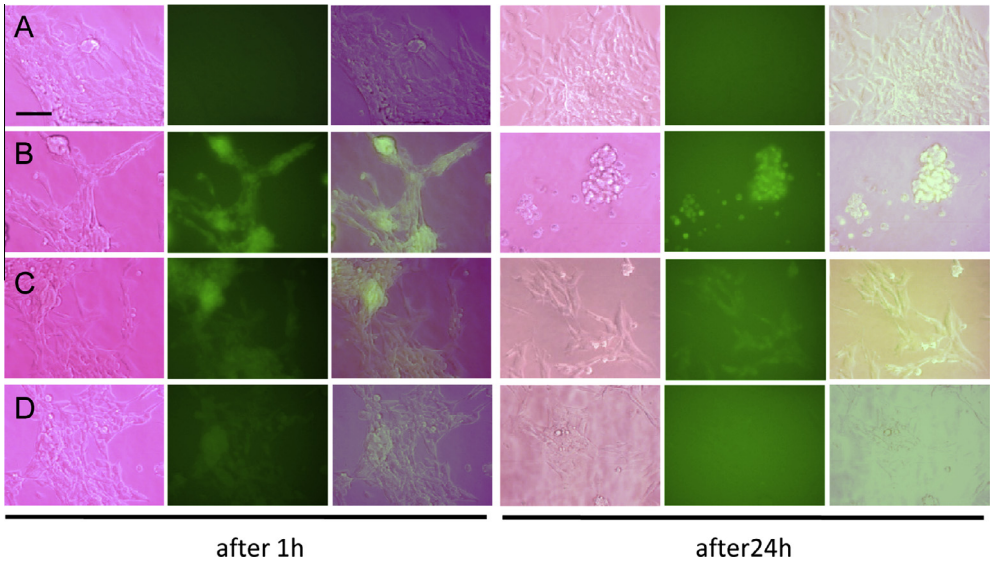

Fig. 3. Pyrolysis of curcumin enhances incorporation into B78H1 melanoma cells. Fluorescence microscopic images of murine B78H1 melanoma cells after 1 and 24 h incubation with 20 μM deketene curcumin (B), pyrolysed curcumin (C), curcumin (D) or DMSO vehikel (A) show autofluorescence of test substances. The first column includes the bright field, the second the GFP channel and the third column the overlay images of both. Note the stronger fluorescence signal of deketene curcumin at 24 h plus the change in cell morphology in this sample. Scale bar: 100 μM.

bridge. Among others, Suresh, Gurudutt, & Srinivasan (2009) identified vanillin and ferulic acid as two major degradation products of curcumin after pyrolysis, both of which were also detected herein. In a chromatographic comparison of the postulated intermediates with the pyrolytic products, the presence of feruloylacetone and *iso*-curcumin hinted towards pathway B.

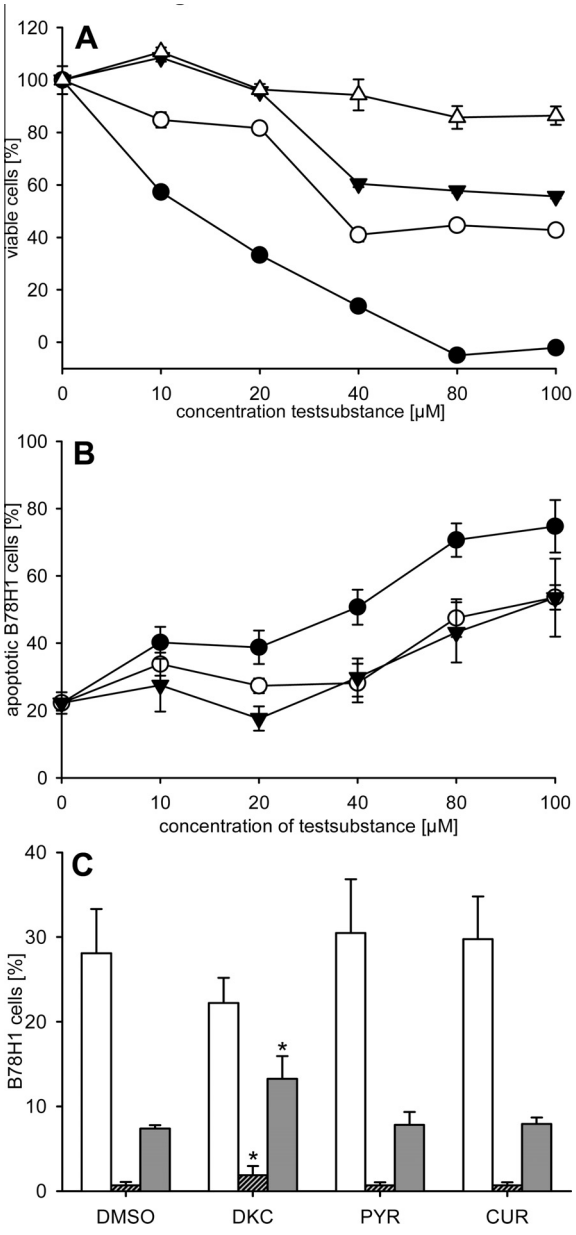

Fig. 4. Pyrolysis of curcumin enhances cytotoxicity on B78H1 melanoma cells. (A) Percentage of viable cells as detected with WST-1 assay after 24 h incubation of B78H1 melanoma with different dilutions of deketene curcumin (black dots), pyrolysed curcumin (white dots), curcumin (black triangles) and DMSO control (white deltas). Note the reduced cell viability at equivalent molar concentrations of pyrolysed curcumin and deketene curcumin compared to the curcumin sample. (B) Percentage of apoptotic B78H1 cells after 24 h incubation with different dilutions of deketene curcumin (black dots), pyrolysed curcumin (white dots) and curcumin (black triangles) as measured by flow cytometry of annexin V positive cells. Percentage of apoptotic cells in the DMSO control was set as zero point. (C) Cell cycle analysis after 24 h incubation of B78H1 cells with 20 µM of deketene curcumin (DKC), pyrolysed curcumin (PYR), curcumin (CUR) and DMSO control. Displayed are number of cells [%] in G1 phase (white bars), S phase (striated gray bars) and G2 phase (dark gray bars) as measured by flow cytometry after fixation with methanol and staining with propidium iodide. $^{*}P < 0.05$ vs. control.

3.4. Formerly reported biological activity of deketene curcumin (1,5-bis(4-hydroxy-3-methoxyphenyl)-1,4-pentadien-3-one)

Deketene curcumin was described so far either as a component isolated from plant extracts or as a synthesised curcumin analogue. Testing of biological activity showed DKC and analogues to inhibit auto-oxidation of lipids, scavenge radicals and inhibit TPA-induced activator protein-1 production, as well as TPA-induced edema in mice (Dinkova-Kostova, Abeygunawardana, & Talalay, 1998; Masuda et al., 1993; Sardjiman, Reksohadiprodja, Hakim, van der Goot, & Timmerman, 1997; Weber et al., 2006). Furthermore, DKC protected cells from beta-amyloid insults, reduced mast-cell mediated allergy and inhibited growth of different tumor cell lines (Cen et al., 2009; Liang et al., 2009; Nugroho, Yuniarti, Istyastono, Supardjan, & Hakim, 2009; Park & Kim, 2002; Quincoces Suarez et al., 2010). Additionally, Quincoces Suarez et al. (2010) found a low toxicity rate after 14 days of intra-peritoneal injection of DKC in rats. In most publications DKC was described to exhibit similar or higher bioactivity compared to curcumin. Liang et al. (2009) also found DKC to be more stable in the plasma of rats after oral administration.

3.5. Pyrolysis enhances curcumin absorption by B78H1 melanoma cells

Qualitative analysis of fluorescence microscopy reveals that isolated deketene curcumin as well as pyrolysed curcumin are found to be incorporated to a higher extent by murine B78H1 melanoma cells compared to unpyrolysed curcumin (Fig. 3). After 24 h B78H1 cells treated with 20 µM curcumin and pyrolysed curcumin appeared spindle-like and unchanged in morphology compared to the DMSO control, whereas DKC treated cells were clearly rounded in all samples, indicating initiation of cell death. Moreover, there was no curcumin-based fluorescence detected in cells that were treated with curcumin and barely some in cells treated with pyrolysed curcumin. In contrast, fluorescence was emitted clearly visible by B78H1 melanoma cells treated with DKC after 24 h.

Our findings about cellular incorporation of DKC and PYR support the hypothesis of Kurien and colleagues, stated above, regarding the increase in bioavailability of curcumin by heat.

3.6. Deketene curcumin shows enhanced cytotoxicity in B78H1 melanoma cells and induces G2 arrest

Pyrolysis of curcumin enhances cytotoxicity when compared to unpyrolysed curcumin as confirmed by a reduction of cell viability in WST-1 assay as well as increased induction of apoptosis (Fig. 4A and B). Deketene curcumin reduces cell viability more pronounced which is supported by the strong induction of apoptosis by this compound in murine B78H1 melanoma cells. Cell cycle analysis based on flow cytometry shows a significant induction of G2 arrest in the presence of 20 µM DKC compared to the other substances that were tested in B78H1 melanoma cells (Fig. 4C).

Other investigators already found enhanced cytotoxicity for synthetic DKC in human and murine cancer cell lines, which is also true for the DKC that we isolated after curcumin pyrolysis. In addition, we were able to show that DKC induces G2 arrest in B78H1 melanoma cells comparable to curcumin and other curcumin derivatives (Liu et al., 2012; Luthra, Kumar, & Prakash, 2009).

4. Conclusion

Curcumin is traditionally consumed on the Indian subcontinent. Epidemiological, as well as experimental findings, imply an anti-cancerogenic property of this compound albeit its low bioavailability. Our data suggest that the traditional cooking process enhances the well-known beneficial effects of curcumin by forming more hydrophilic products. Deketene curcumin, which we found to be formed during pyrolysis of curcumin, shows higher stability and exhibits higher toxicity against tumor cells compared to curcumin. From these findings we conclude that pre-consumptional pyrolysis of curcumin, during common cooking, strongly enhances the beneficial health effects of curcumin.

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