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

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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.

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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