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Oxidative transformation of demethoxy- and bisdemethoxycurcumin: Products, mechanism of formation, and poisoning of human topoisomerase II α

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Abstract

Extracts from the rhizome of the turmeric plant are widely consumed as anti-inflammatory dietary supplements. Turmeric extract contains the three curcuminoids, curcumin ($\approx 80\%$ relative abundance), demethoxycurcumin (DMC; $\approx 15\%$), and bisdemethoxycurcumin (BDMC; $\approx 5\%$). A distinct feature of pure curcumin is its instability at physiological pH resulting in rapid autoxidation to a bicyclopentadione within 10–15 min. Here, we describe oxidative transformation of turmeric extract, DMC, and BDMC, and the identification of their oxidation products using LC-MS and NMR analyses. DMC autoxidized over the course of 24 h to the expected bicyclopentadione diastereomers. BDMC was resistant to autoxidation, and oxidative transformation required catalysis by horseradish peroxidase and H₂O₂ or potassium ferricyanide. The product of BDMC oxidation was a stable spiroepoxide that was equivalent to a reaction intermediate in the autoxidation of curcumin. The ability of DMC and BDMC to poison recombinant human topoisomerase II α was significantly increased in the presence of potassium ferricyanide indicating that oxidative transformation was required to achieve full DNA cleavage activity. DMC and BDMC are less prone to autoxidation than curcumin and contribute to the enhanced stability of turmeric extract at physiological pH. Their oxidative metabolites may contribute to the biological effects of turmeric extract.

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Supporting Information Available: Tables with NMR data for compounds **1a–c** and **2a,b**. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Graphical abstract

Keywords

Curcuminoids; turmeric; topoisomerase; oxidative activation; bicyclopentadione; spiroepoxide; degradation; metabolism; peroxidase

Introduction

Curcumin, in the form of an extract from the rhizomes of the turmeric plant, is widely consumed as a dietary supplement or as part of curry dishes. Turmeric also has a long history of use in traditional Asian medicine.¹ A large number of preclinical studies implicate that curcumin exerts antioxidant, anti-inflammatory, anti-neoplastic, and other beneficial biological activities.^{2–5} The past decade has seen a marked increase in testing curcumin in clinical trials. The database www.clinicaltrials.gov listed 106 studies when a search for the keyword “curcumin” was conducted at the time of submission of this article (January 2015). Only two studies were listed for the year 2001 in the database.⁶ The results from completed studies show positive effects in an open-label study in patients with osteoarthritis⁷ but less effects in placebo-controlled double blinded trials.^{8, 9}

Curcumin is unstable and degrades in aqueous solution at physiological pH, resulting in rapid disappearance of its orange-yellow color.^{10, 11} This degradation is well recognized but incompletely understood.^{11–14} We are interested in defining the products and mechanism of degradation of curcumin *in vitro*. Our long-term goal is to determine whether and how these products are mediating the biological activities of curcumin *in vitro* and *in vivo*. A recent study demonstrated that the unstable oxidation products of curcumin are topoisomerase II poisons *in vitro*.¹⁵ DNA cleavage was negligible under reaction conditions in which curcumin was stable, but significantly induced upon oxidative transformation of curcumin using potassium ferricyanide.¹⁵

The primary degradation pathway of curcumin is an autoxidation reaction that results in the stable incorporation of two atoms of oxygen in the final bicyclopentadione product (Chart 1).¹³ So far, a detailed analysis of the autoxidation reaction and its products and mechanisms has only been described for pure curcumin.¹⁶ Extracts from turmeric used in traditional medicine, dietary supplements, and in the curry spice mixture also contain the less abundant curcuminoids demethoxycurcumin (DMC) and bisdemethoxycurcumin (BDMC).² Here we describe analysis of the autoxidative and enzymatic transformation of a curcuminoid mixture

and the individual curcuminoids, including the isolation and identification of the major products, and studies on their formation and ability to poison human DNA topoisomerase II α .

Experimental Procedures

Materials

Curcumin, *d*₆-curcumin, and *d*₆-bicyclopentadione were synthesized as described.^{17, 18} For synthesis of DMC and BDMC the procedure was modified to use a 1:1 molar ratio of vanillin and 4-hydroxybenzaldehyde or only 4-hydroxybenzaldehyde, respectively. Stock solutions of curcumin, DMC (purity >93%), BDMC (purity >90%), and turmeric extract (5 mM in ethanol) were stored at -20°C. Curcumin from *Curcuma longa* (Turmeric) powder (>65% purity [i.e., curcumin]; product number C1386) and horseradish peroxidase (HRP; Type-II; 5 kU/mL; 25.9 mg/mL; product number P8250) were purchased from Sigma-Aldrich.

Stability of turmeric extract

For the spectrophotometric assay turmeric extract (10 μ g; 50 μ M) or curcumin (10 μ g; 50 μ M) were diluted in 500 μ L of NH₄OAc buffer (10 mM; pH 7.5) in a 1 cm cuvette. The solution was scanned every 2 min or analyzed by continuous recording at 430 nm for 20 min. For the LC-MS assay turmeric extract (5 μ g; 25 μ M) was diluted in 500 μ L of NH₄OAc buffer (10 mM; pH 7.5) at room temperature in a 1.5 mL plastic reaction tube. The reaction was allowed to proceed for 6 h before extraction with two 300 μ L aliquots of ethylacetate. The extracts were combined, evaporated under a stream of nitrogen, and dissolved in 100 μ L of MeOH/H₂O (1:1) for LC-MS analysis. For time course analysis autoxidation reactions were terminated at 0, 15, 30 min, 1, 2, 3, and 6 h by acidification to pH 4 with 1 N HCl, followed by addition of 50 ng each of *d*₆-curcumin and *d*₆-bicyclopentadione, and extraction with ethylacetate.

Preparative oxidation of DMC and BDMC

DMC (500 μ g; 30 μ M) was diluted in 50 mL of NH₄OAc buffer (10 mM; pH 7.5). Four parallel reactions were allowed to proceed at room temperature for 24 h. Oxidation of BDMC was performed on the same scale (4 $\times$ 50 mL buffer; 500 μ g BDMC; 30 μ M) in 4 parallel reactions. In addition, the reactions contained horseradish peroxidase (0.01 U/mL) and H₂O₂ (40 μ M) and proceeded for 10 min.

The DMC and BDMC oxidation reactions were acidified (pH 4, 1 N HCl) and loaded onto 2 preconditioned 500 mg Supelco Discovery DSC-18 cartridges (100 mL per cartridge) and eluted with 2 aliquots of 1 mL MeOH. The combined eluents from each reaction were concentrated under a stream of nitrogen to a final volume of 1 mL before HPLC isolation of the reaction products. The HPLC-collected peaks were further diluted 10-fold with water and acidified before loading on a preconditioned 50 mg Waters Oasis HLB (hydrophilic-lipophilic balance) cartridge. The products from DMC were eluted with 500 μ L of MeOH, dried, and dissolved in 150 μ L of *d*₆-acetone using a 3-mm sample tube. The products from

BDMC were eluted with 500 μL of D_2O /Acetonitrile- d_3 1:1 and concentrated to a final volume of 150 μL .

^{18}O incorporation

For analysis of ^{18}O incorporation during oxidation of DMC and BDMC, the reactions (30 μM , 5 μg each) were performed in 250 μL of 10 mM NH_4OAc buffer pH 7.5 buffer diluted with 250 μL of H_2^{18}O (99.5 atom-% ^{18}O). Reactions were initiated by the addition of HRP (0.01 U/mL) and H_2O_2 (40 μM), and allowed to proceed for 10 min before extraction and LC-MS analysis in the negative ion mode.

HPLC analyses

Reactions were analyzed using a Waters Symmetry C18 5- μm column (4.6 $\times$ 250 mm) eluted with a linear gradient of 20% to 80% acetonitrile in 0.01% aqueous acetic acid over 20 min. The samples were eluted at a flow rate of 1 mL/min and monitored using an Agilent 1200 diode array detector.

Plasmid DNA cleavage

DNA cleavage reactions were performed as described by Fortune and Osheroff.¹⁹ Reaction mixtures contained 150 nM recombinant human topoisomerase I α (prepared as described previously^{20–22}), 10 nM negatively supercoiled pBR322 DNA, and 0–100 μM DMC or BDMC in 20 μL of cleavage buffer (10 mM Tris-HCl pH 7.9, 5 mM MgCl_2 , 100 mM KCl, 0.1 mM EDTA, and 2.5% (v/v) glycerol). In some cases, reactions also contained 50 μM $\text{K}_3\text{Fe}(\text{CN})_6$. Reactions were incubated for 6 min at 37°C, and enzyme-DNA cleavage complexes were trapped by adding 2 μL of 5% SDS followed by 2 μL of 250 mM EDTA (pH 8.0). Proteinase K was added (2 μL of a 0.8 mg/mL solution), and reaction mixtures were incubated for 30 min at 45°C to digest topoisomerase I α . Samples were mixed with 2 μL of agarose loading dye (60% sucrose in 10 mM Tris-HCl pH 7.9, 0.5% bromophenol blue; and 0.5% xylene cyanol FF), heated for 2 min at 45°C, and subjected to electrophoresis in 1% agarose gels in 40 mM Tris-acetate pH 8.3 and 2 mM EDTA containing 0.5 $\mu\text{g/mL}$ ethidium bromide. DNA cleavage was monitored by the conversion of the negatively supercoiled plasmid to linear molecules. DNA bands were visualized by ultraviolet light and quantified using an Alpha Innotech digital imaging system.

LC-MS

LC-MS analysis was conducted using a TSQ Vantage Triple Quadrupole instrument equipped with an electrospray interface. The instrument was operated in the negative ion mode, and mass spectra were acquired at a rate of 2 s/scan. The settings for the heated capillary (300°C), spray voltage (4.0 kV), spray current (0.22 μA), auxiliary (37 mTorr) and sheath gas (16 mTorr) were optimized using direct infusion of a solution of the bicyclopentadione formed from curcumin oxidation (20 ng/ μL) in acetonitrile/water 50/50 (v/v), containing 10 mM NH_4OAc . Samples were introduced into the instrument using a Waters Symmetry Shield C18 1.7- μm column (2.1 $\times$ 50 mm) eluted with a linear gradient of 5% to 95% acetonitrile in 0.01% aqueous acetic acid over 3 min followed by isocratic

elution with 95% acetonitrile in 0.01% aqueous acetic acid for 2 min at a flow rate of 200 $\mu\text{L}/\text{min}$.

NMR

Samples were dissolved in 150 μL of d_6 -acetone or D_2O /acetonitrile- d_3 in a 3 mm-sample tube and analyzed using a Bruker AV-II 600 MHz spectrometer equipped with a cryoprobe. Chemical shifts are reported relative to residual acetone ($\delta = 2.05$ ppm) or acetonitrile ($\delta = 1.94$ ppm).

Results

Stability and degradation of turmeric extract

The turmeric extract was a preparation extracted from turmeric and obtained as “curcumin >65%” from Sigma-Aldrich. The relative abundance of the curcuminoids in the turmeric extract were 82% curcumin, 15% DMC, and 3% BDMC as determined from peak areas in HPLC analyses.

The rates of degradation of turmeric extract and pure curcumin at pH 7.5 were compared using a spectrophotometric assay. The chromophore of the turmeric extract disappeared at an initial rate of 42 ± 1 mAU/min in the first 1 min, followed by a slower rate of 4 ± 1 mAU/min over the next 20 min (Fig. 1A). In contrast, the chromophore of pure curcumin disappeared at a rate of 75 ± 1 mAU/min ($3 \mu\text{M}/\text{min}$; $\epsilon_{430} = 25,000 \text{ M}^{-1}\text{cm}^{-1}$), which was similar to previously reported values (Fig. 1B).¹³ Comparison of the rates of degradation indicated that DMC and/or BDMC have a stabilizing effect in the curcuminoid mixture. An artificial mixture of curcumin, DMC, and BDMC in a ratio of 80:15:5 was prepared and showed a similar slow disappearance of the chromophore as turmeric extract (initial rate of 46 ± 1 mAU/min for the first 1 min; 11 ± 2 mAU/min over the next 20 min). Because adding DMC and BDMC was sufficient to slow down degradation it could be excluded that other compounds present in the turmeric extract were responsible for inhibition. Mechanisms leading to enhanced stability of curcumin by DMC or BDMC were not further investigated.

Degradation reactions of DMC and BDMC

The slow degradation of turmeric extract relative to pure curcumin suggested different degradation rates for curcuminoids in the turmeric mixture compared to the pure compounds. Thus, DMC and BDMC were prepared by chemical synthesis^{17, 18} and their rates of degradation were determined. The degradation of the pure compounds was too slow for continuous recording using the UV/Vis spectrophotometric assay.¹² Therefore, degradation reactions of DMC and BDMC ($30 \mu\text{M}$ each) were extracted at 0 and 24 h and the remaining starting material quantified by RP-HPLC with diode array detection. 57% of the initial amount of DMC remained unchanged at 24 h which gave a calculated degradation rate of $0.013 \mu\text{M}/\text{min}$ assuming a linear reaction. BDMC was unchanged after 24 h incubation in buffer.

The addition of HRP and H₂O₂ to the degradation reactions of DMC and BDMC resulted in rapid transformation of both compounds (Fig. 2). Addition of an equimolar amount of the oxidizing agent potassium ferricyanide likewise resulted in rapid transformation of DMC and BDMC, respectively (not shown).

DMC oxidation products

RP-HPLC analysis of a degradation reaction of DMC at 48 h reaction time gave three distinct product peaks **1a–c** (Fig. 3A). The peaks had identical UV/Vis spectra (λ_{max} at 225 nm; Fig. 3A, inset) that were similar in character to the curcumin-derived bicyclopentadione.¹⁴ LC-ESI-MS analyses in the positive ion mode gave a molecular ion of m/z 371 for all three product peaks, and collision-induced fragmentation of m/z 371 gave major product ions at m/z 325 and 249 for all three products.

A large-scale degradation reaction starting with 2 mg of DMC was performed and the products were isolated using RP-HPLC for structural identification by NMR. One- and two dimensional mono- and heteronuclear NMR analyses confirmed that **1a–c** were diastereomeric isomers of a bicyclopentadione derivative of DMC (Tables S1 and S2). The main product **1a** showed characteristic signals for oxygen substitution at C-1 (97.0 ppm) and C7 (79.2 ppm) (Fig. 3B). The bicyclic ring structure was evident from a C-C bond in the *cis* configuration connecting C-2 and C-6 ($^3J_{2,6}$ = 6.5 Hz) and further supported by HMBC crosspeaks, for example, of H-6 and C-3. The two minor isomers **1b** and **1c** could not be separated on a preparative scale and were analyzed as a mixture. **1b** and **1c** were identified as demethoxy bicyclopentadione diastereomers with alternate configuration of C-1 and C-7 (Table S2).

BDMC oxidation products

The degradation reactions indicated that BDMC is stable at physiological pH and does not undergo spontaneous oxidation. In order to structurally characterize potential enzymatic oxidation products we incubated BDMC with HRP in the presence of H₂O₂ and observed rapid disappearance of the chromophore (cf. Fig. 2B). Likewise, addition of K₃Fe(CN)₆ as the oxidizing agent resulted in rapid transformation of BDMC and formation of the same products.

RP-HPLC analysis of the HRP/H₂O₂-catalyzed transformation of BDMC gave two prominent product peaks **2a** and **2b** (Fig. 4A). The UV/Vis spectra of the products were notably different from the bicyclopentadiones and showed a λ_{max} of 258 nm suggesting the presence of a more extended conjugated system (Fig. 4A inset). Both products were isolated and analyzed by NMR (Tables S3 and S4). The major product **2a** was cyclized between C-2 (44.3 ppm) and C-6 (57.7 ppm) to form a cyclopentadione ring. The ring was evident for the H2,H6 coupling constant ($^3J_{2,6}$ = 2.5 Hz) and also from HMBC crosspeaks, e.g., between H-1 and C-6 (Fig. 4B). The two carbonyls of the cyclopentadione ring (C-3/C-5) were detected at 202.9 ppm and 205.1 ppm, respectively. C-6 of the ring was connected via C-7 (70.1 ppm; carrying a hydroxyl) to the phenol ring. The methoxyphenol ring was oxidized to an epoxyquinone methide (spiroepoxide) as evident from the chemical shift for C-4' (a carbonyl at 188.4 ppm) and the epoxide at C-1 and C-1' (68.7 and 58.3 ppm, respectively).

In addition, carbons 2', 3', and 6' of the ring had chemical shifts compatible with double bond rather than arene signals. Thus, **2a** is a demethoxy spiroepoxide and is equivalent to the spiroepoxide intermediate in curcumin autoxidation.¹⁶ Product **2b** was identified as a diastereomeric demethoxy spiroepoxide.

Since the curcumin-derived spiroepoxide is acid-labile¹⁶ we tested the stability of BDMC spiroepoxide **2a** and found that it was stable to treatment with HCl to pH 3. When **2a** was placed in organic solvent, however, it quickly degraded, and a number of products were detected using RP-HPLC; these products were not further analyzed. Thus, NMR analyses of **2a** and **2b** were performed on samples dissolved in D₂O containing a small amount of acetonitrile-*d*₃.

Oxygen incorporation from H₂¹⁸O

Previous studies on the origin of oxygen in the bicyclopentadione from curcumin showed that one of the two oxygen atoms inserted was derived from water.¹³ To test whether the same exchange occurs during DMC and BDMC oxidation both were oxidized using HRP/H₂O₂ in 10 mM NH₄OAc buffer pH 7.5 containing 50% H₂¹⁸O. The oxidation mixtures were analyzed using LC-ESI-MS in the negative ion mode. The molecular ion for the DMC bicyclopentadione **1a** showed a ≈1:1 ratio of *m/z* 369 and *m/z* 371, indicating that the incorporation of ¹⁸O from H₂¹⁸O into **1a** was near quantitative (Fig. 5A). In contrast, the spiroepoxide **2a** from BDMC oxidation gave a peak with *m/z* 339 only, indicating that it did not incorporate ¹⁸O from H₂¹⁸O (Fig. 5B). Thus, both oxygen atoms introduced into **2a** are derived from O₂.

Time course of degradation of turmeric extract

The time-course for the degradation of the individual components contained in turmeric extract was determined (Fig. 6A–C). Degradation reactions of turmeric extract were terminated at time points ranging from 15 min to 6 h, and the levels of curcumin, DMC, and BDMC were quantified. The samples were analyzed by LC-ESI-MS in the positive ion mode using MRM transitions determined from purified starting materials and products. In the turmeric extract curcumin was degraded 75% within 2 h whereas DMC was degraded only 35% at the same time point. BDMC was reduced by 10% after 6 h reaction time. The corresponding bicyclopentadiones were formed in parallel. The slight degradation of BDMC in the mixture was different from the findings with pure BDMC. It is likely that BDMC was affected by radicals generated during degradation of curcumin and DMC in the mixture.

Effect on DNA cleavage by topoisomerase IIα

Type II topoisomerases are enzymes that regulate DNA supercoiling and remove knots and tangles from the genetic material by generating transient enzyme-linked breaks in the double helix.^{23, 24} Beyond their critical physiological functions, these enzymes are the targets for some of the most widely prescribed anticancer drugs in clinical use.^{23, 25–27} Drugs act by poisoning (*i.e.*, increasing levels of DNA cleavage mediated by) the type II enzymes. A number of compounds with chemopreventative properties, including curcumin, also are topoisomerase II poisons.²⁸ However, in order to induce DNA cleavage by recombinant human topoisomerase IIα and IIβ, curcumin must undergo oxidative transformation.¹⁵

Here, we analyzed the ability of pure DMC, BDMC, and their oxidation products generated by exposure to $K_3Fe(CN)_6$ to poison topoisomerase II α (Fig. 7). DMC induced a small increase (≈ 1.5 -fold) in DNA cleavage at high concentration ($>50 \mu M$) in the absence of $K_3Fe(CN)_6$. The addition of $K_3Fe(CN)_6$ resulted in DNA cleavage occurring at a lower ($10 \mu M$) concentration of DMC, with the maximum effect of about a 2-fold increase being observed at $25 \mu M$. Higher concentrations of DMC did not result in increased DNA cleavage. BDMC was inactive in the absence of $K_3Fe(CN)_6$, and the addition of the oxidizing agent resulted in a 1.5-fold increase in relative DNA cleavage. In comparison, $5 \mu M$ curcumin resulted in 3-fold increased DNA cleavage in the presence of $K_3Fe(CN)_6$.¹⁵

Discussion

Degradation of curcumin and curcuminoids

The bright yellow-orange color of an aqueous solution of $50 \mu M$ curcumin at pH 7.5 changes to a faint hue within minutes. This instability of curcumin at physiological pH is well recognized, and it is due to a chemical transformation of the compound.^{10, 11, 29} Mechanistic studies have established that the degradation reaction is an autoxidation initiated by hydrogen abstraction from one of the phenolic hydroxyl groups.¹³ The final autoxidation product is a bicyclopentadione formed by a series of intramolecular radical reactions that result in the formation of a new C-C bond and the incorporation of oxygen in the form of two ether bridges.¹⁶

BDMC and DMC are less soluble than curcumin in aqueous buffer at pH 7.5. When an aqueous solution of BDMC ($30 \mu M$) is scanned repeatedly in a UV/Vis spectrophotometer the yellow color will also disappear within minutes. In this case, however, the loss of color is not due to a chemical transformation of BDMC but due to the compound coming out of solution and precipitating. DMC is slightly more soluble, with the color disappearing slowly over the span of a couple of hours. In the case of DMC, the color disappearance is partially due to a chemical transformation, as indicated by the recovery of autoxidation products over time when the solution is extracted and analyzed by HPLC. Thus, in order to generate oxidation products of BDMC, the reaction was catalyzed by the addition of HRP and H_2O_2 . Products from DMC were generated both from autoxidation and enzymatic (HRP/ H_2O_2) reactions.

Oxidation products

The major oxidation products of DMC were the expected demethoxy bicyclopentadione diastereomers (**1a–c**), whereas the products isolated from BDMC oxidation were two diastereomeric spiroepoxides (**2a** and **2b**). The mechanism of formation of **1** and **2** appears to follow the steps proposed for curcumin autoxidation,¹⁶ i.e., formation of a phenoxyl radical, delocalization of the radical into the chain, 5-*exo* cyclization, oxygen addition to give a peroxy radical, formation of an endoperoxide, and its cleavage through S_H1 attack by a tertiary radical resulting in a spiroepoxide intermediate (Scheme 1). For BDMC oxidation, the reaction stalls at this point; for DMC, the reaction continues to give the demethoxy bicyclopentadione diastereomers equivalent to the bicyclopentadiones formed from curcumin. Similar to curcumin autoxidation, we found that one of the two oxygen atoms

inserted into the demethoxy bicyclopentadione was derived from H₂O; the other, by exclusion, must have come from molecular oxygen. Our analyses did not allow to pinpoint the step at which water exchange occurred but it is predicted to be the same as during curcumin autoxidation,¹⁶ i.e., during the conversion of the spiroepoxide intermediate to the demethoxy bicyclopentadione. In the case of curcumin further transformation of the spiroepoxide proceeds through a vinyl ether intermediate,¹⁶ and the same can be assumed for DMC oxidation. We did not attempt to isolate the corresponding spiroepoxide or vinyl ether intermediates of DMC.

A spiroepoxide has first been postulated as a reaction intermediate in curcumin autoxidation¹³ and was subsequently isolated and confirmed by NMR structural analysis.¹⁶ As predicted, the BDMC spiroepoxide **2** did not incorporate H₂O from the buffer, consistent with the proposed mechanism in which both of its oxygens are derived from an endoperoxide precursor (Scheme 1). Successful isolation of the curcumin spiroepoxide required strict maintenance of pH of 7.5 at all steps because even brief incubation at acidic pH was sufficient to catalyze its transformation to the bicyclopentadione.¹⁶ In comparison, the BDMC derived spiroepoxide **2** was stable and was readily isolated as the final product. Even when acidified to pH 3 the spiroepoxide was not further converted to a bicyclopentadione or a diol hydrolysis product. The fact that the transformation of BDMC stalls at the spiroepoxide rather than continuing on to a bicyclopentadione supports our suggestion on a crucial role of the methoxy group in contributing to S_N1 opening of the epoxide and the subsequent exchange of water.¹⁶

Role of the methoxy group

The presence or absence of a methoxy group in the phenol rings is relevant not only to opening of the epoxide, it also appears to be crucial for the ability of curcumin, DMC, and BDMC to undergo oxidative transformation. DMC, with one methoxy group remaining, was slow to autoxidize when compared to curcumin, and BDMC, lacking both methoxy groups, did not autoxidize at all. As explained in the following paragraph, in both DMC and curcumin the initiating H-abstraction occurs at the methoxyphenol ring. Why the lack of a methoxy group at the far away side of the molecule has an influence on the rate of H-abstraction on the proximal side is not immediately obvious but points toward a crucial electronic balance in the molecules that holds the key to whether and at what rate they undergo autoxidation. Substituents that increase or decrease the electron density of the aromatic rings have a decisive influence on the rate of hydrogen abstraction. It should be pointed out that “hydrogen abstraction” is an imprecise term since the reaction is likely to occur as a sequential proton loss electron transfer (SPLET) process that may involve an initial event at the β-diketo moiety.^{30, 31}

Using the asymmetrical DMC we found that the methoxyphenol ring was always located on the same side of the bicyclopentadione product, namely, it was connected through the ether bridge at C-1 and not at C-7. Thus, there was a directionality during the autoxidation reaction of DMC such that the distribution of the methoxyphenol and phenol rings in the DMC bicyclopentadiones **1a–c** was not random. This is best explained when the initial H-abstraction occurs on only one side of the molecule, i.e., at the methoxyphenol ring. The

ensuing steps of the proposed autoxidation mechanism (radical delocalization, 5-*exo* cyclization, oxygenation, endoperoxide formation, homolytic substitution and water exchange; see Scheme 1) predict that the methoxyphenol ring will be linked through the ether bond, and this was confirmed by heteronuclear NMR analyses. Thus, the proposed mechanism is sufficient to explain formation of the demethoxy bicyclopentadione isomers that were experimentally found.

Products compatible with cleavage of the 7-carbon chain connecting the phenolic rings were not observed in the DMC or BDMC degradation reactions. Such cleavage products, in the case of curcumin described to be vanillin, ferulic acid, and feruloylmethane,¹¹ were either absent or of very minor abundance compared to the bicyclopentadione.¹⁴

Biological implications

Our studies show that curcumin is more stable to degradation when in the presence of DMC and BDMC as it occurs in turmeric extract, the natural product used in most applications and formulations. Do the differences in stability of curcumin versus turmeric extract result in differences in biological activities, irrespective of the absence or presence of DMC and BDMC? On the face of it, enhanced stability of curcumin should result in enhanced biological activities, and there is little reason to assume this would not be the case for the effects that are caused by curcumin. Our underlying hypothesis, however, is that certain biological effects of curcumin require oxidative activation. This was shown to be the case for the effects of curcumin on topoisomerase II α . Although curcumin had no significant effect on the activity of the enzyme, short-lived intermediates formed during the oxidation of the compound poisoned the type II enzyme.¹⁵ Here we similarly observed that oxidative activation of DMC and BDMC enhanced their ability to poison recombinant human topoisomerase II α .

Whether this in vitro effect plays a role as an outcome of turmeric consumption is not clear. Studies using cultured cells show DNA topoisomerase poisoning by curcuminoids, and our analyses invoke the formation of electrophilic reaction intermediates adducting to topoisomerase II α as a mechanistic explanation.^{15, 32, 33} Topoisomerase poisoning can lead to DNA damage and cell death as a means of killing cancer cells.²³ Curcuminoids have anti-cancer activities in mouse models, and there is circumstantial evidence from the dietary consumption of turmeric over thousands of years implying a cancer chemopreventive effect.³⁴⁻³⁸ Due to the polypharmacological nature of curcumin, however, it is difficult to tell whether topoisomerase poisoning is a major contributors to the overall effect.

The electrophilic intermediates formed in the oxidative transformation of curcumin and turmeric are prone to react with nucleophiles in a cellular environment.³⁹ Trapping of intermediates reduces the yield of the final oxidation product.¹⁶ Scavenging of reactive electrophiles is a cellular defense mechanism against oxidative damage and at the same time a mechanism to sense oxidative stress and change protein function to induce a response.^{40, 41} Therefore, the net result of oxidative transformation of curcuminoids on cellular function is likely a consequence of divergent and possibly competing effects.

Based on the “oxidative activation” hypothesis, one might predict reduced or absent biological activities for turmeric extract, DMC, and BDMC due to their increased stability. However, a simple and linear correlation between oxidizability and biological effects may not be the case, if one considers, for example, enzymatic oxidation of the curcuminoids as a means of activation. It is also conceivable that for effects mediated by early electrophilic intermediates curcumin may oxidize too quickly (rapidly transforming into less or inactive end products), and that upon slower oxidation, as would occur with DMC and BDMC, there is a better chance that electrophilic intermediates are formed “at the right time and at the right place”. There is evidence that DMC and BDMC can have bioactivities similar to curcumin^{34, 36, 42, 43} but in the absence of any detailed studies it is difficult to decide whether their oxidative metabolites are involved in these effects. Future studies in our laboratory will focus on such analyses.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BDMC	bisdemethoxycurcumin
DMC	demethoxycurcumin
ESI	electrospray ionization
HRP	horseradish peroxidase
MRM	multiple reaction monitoring

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Figure 1.

Stability of turmeric extract and curcumin at pH 7.5. A 50 μ M solution of (A) turmeric extract or (B) curcumin in 10 mM NH_4OAc buffer pH 7.5 was scanned from 700 to 200 nm every 2 min for 20 min.

Figure 2.

Horseradish peroxidase (HRP)-catalyzed transformation of DMC and BDMC. (A) DMC or (B) BDMC (50 μ M) were diluted in 500 μ l 10 mM NH_4OAc buffer pH 7.5 in a spectrophotometer cuvette and the UV/Vis spectra (700 to 200 nm) were recorded every min. HRP and H_2O_2 (40 μ M) were added after the first (A) or second (B) scan.

Figure 3.

RP-HPLC analysis of DMC autoxidation products. (A) 100 μ l of a reaction of 50 μ M DMC in 1.5 ml 10 mM NH₄OAc buffer pH 7.5 were injected at 48 h reaction time without prior extraction. The chromatogram was recorded at UV 205 nm using a diode array detector. The inset shows the UV spectrum of peak **1a** recorded online during HPLC analysis. (B) Selected carbon chemical shifts and HMBC correlations (*arrows*) of demethoxy-bicyclopentadione diastereomer **1a**.

Figure 4.

RP-HPLC analysis of HRP-H₂O₂-catalyzed transformation of BDMC. (A) 100 μ l of a reaction of 50 μ M DMC in 1.5 ml 10 mM NH₄OAc buffer pH 7.5 containing horseradish peroxidase (0.01 U/ml) and H₂O₂ (40 μ M) were injected at 10 min reaction time without prior extraction. The chromatogram was recorded at UV 205 nm using a diode array detector. The inset shows the UV spectrum of peak **2a** recorded online during HPLC analysis. (B) Selected carbon chemical shifts and HMBC correlations (*arrows*) of demethoxy-spiroepoxide diastereomer **2a**.

Figure 5.

Negative ion LC-ESI-MS analysis of ^{18}O incorporation in (A) demethoxy bicyclopentadione **1a** (B) bisdemethoxy spiroepoxide **2a**. DMC and BDMC, respectively, were oxidized using HRP/ H_2O_2 in 10 mM NH_4OAc buffer pH 7.5 containing 50% H_2^{18}O . Expanded MS1 spectra (negative ion mode) of (A) **1a** from m/z 360 to 380, and (B) **2a** from m/z 330 to 350 are shown.

Figure 6.

Degradation of turmeric extract analyzed by LC-ESI-MS. Turmeric extract (25 μ M) was incubated in 10 mM NH_4OAc buffer pH 7.5 for the indicated time points, extracted, and analyzed using LC-ESI-MRM-MS in the positive ion mode. The time course of the degradation and formation of (A) curcumin and bicyclopentadione, (B) DMC and DMC bicyclopentadione, and (C) BDMC were quantified using d_6 -curcumin and d_6 -bicyclopentadione, respectively, as internal standards. The average of three independent reactions is shown with the error bars representing standard deviations.

Figure 7.

Effects of DMC and BDMC on DNA cleavage mediated by human topoisomerase II α . Reactions were carried out in the absence of oxidant (open symbols) or in the presence of 50 μ M K₃Fe(CN)₆ (closed symbols). The left panel shows the effects of DMC (circles) and BDMC (squares) on DNA cleavage. The right panel shows control reactions carried out in the absence of compounds (No Drug) or in the presence of 100 μ M etoposide (Etop) or curcumin (Curc). Error bars represent the standard deviations for at least three independent experiments. The top shows a representative ethidium bromide-stained agarose gel of DNA cleavage reactions that contain 0–100 μ M DMC and 50 μ M K₃Fe(CN)₆. The first lane contains only negatively supercoiled DNA. The positions of negatively supercoiled (FI), nicked (FII), and linear (FIII) DNA are indicated. Baseline levels of enzyme-mediated DNA cleavage in the absence of oxidant were ~2%.

Scheme 1.

Proposed mechanism of oxidative transformation of DMC and BDMC.

Chart 1.

Structures of curcumin, demethoxycurcumin (DMC), bisdemethoxycurcumin (BDMC), and curcumin bicyclopentadione.