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Thiol Reactivity of Curcumin and Its Oxidation Products

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Abstract

The polypharmacological effects of the turmeric compound curcumin may be partly mediated by covalent adduction to cellular protein. Covalent binding to small molecule and protein thiols is thought to occur through a Michael-type addition at the enone moiety of the heptadienedione chain connecting the two methoxyphenol rings of curcumin. Here we show that curcumin formed the predicted thiol-Michael adducts with three model thiols, glutathione, N- acetylcysteine, and β -mercaptoethanol. More abundant, however, were respective thiol adducts of the dioxygenated spiroepoxide intermediate of curcumin autoxidation. Two electrophilic sites at the quinone-like ring of the spiroepoxide were identified. Addition of β -mercaptoethanol at the 5'-position of the ring gave a 1,7-dihydroxycyclopentadione-5'-thioether, addition at the 1'- position resulted in cleavage of the aromatic ring from the molecule, forming methoxyphenol- thioether and a tentatively identified cyclopentadione aldehyde. The curcuminoids demethoxy- and bisdemethoxycurcumin did not form all of the possible thioether adducts, corresponding with their increased stability towards autoxidation. RAW264.7 macrophage-like cells activated with phorbol ester formed curcumin-glutathionyl and the 1,7-dihydroxycyclopentadione-5'- glutathionyl adducts. These studies indicate that the enone of the parent compound is not the only functional electrophile in curcumin, and that its oxidation products provide additional electrophilic sites. This suggests that protein binding by curcumin may involve oxidative activation into reactive quinone methide and spiroepoxide electrophiles.

Graphical Abstract

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Author Contributions

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ASSOCIATED CONTENT

Supporting Information. Tables with NMR data of products 1-3. This material is available free of charge via the Internet at <http://pubs.acs.org>.

Notes

The authors declare that no competing financial interest exists.

Keywords

Michael-type addition; quinone methide; turmeric; curcuminoids; glutathione; spiroepoxide

INTRODUCTION

Research over the past two decades has uncovered a wealth of biological activities of curcumin, the major bioactive compound of the spice turmeric. Antioxidant, anti-inflammatory, antimicrobial, and anti-proliferative effects of curcumin have been at the center of attention.^{1,2} One hypothesis to explain the polypharmacologic effects of curcumin is covalent binding to cellular protein inducing a functional change to enzymes, protein kinases, transcription factors, and other proteins. An obvious mechanism for adduction is by Michael-like addition of a protein cysteine to the enone electrophile of the heptadienedione chain of curcumin (Scheme 1). This mechanism has been suggested to account, for example, for the inhibition of NF- κ B activity by adduction of curcumin to the upstream activating kinase, IKK β .³ There are numerous other examples where curcumin is proposed to adduct to proteins via its enone electrophile.^{4,5} Studies with alkynyl analogs indicate that binding of curcumin to cellular protein is extensive.^{6,7} Understanding thiol reactivity of curcumin is therefore key to understanding its biological effects. There is, however, little mechanistic information on how curcumin binds to protein or small molecule thiols except for the structural analysis of curcumin and a close analog binding to GSH or cysteamine, respectively, that showed the expected addition at C-1 of the heptadienedione chain.^{8,9}

The current study is based on the hypothesis that binding of curcumin to thiols may involve electrophiles different from the enone. Novel electrophiles may be formed upon autoxidation of curcumin (Scheme 1), an abundant reaction that is identical to the well recognized degradation of curcumin in buffer at physiological pH.¹⁰ Likely candidates for thiol reactive electrophiles are the enone of curcumin, the quinone methide formed upon H-abstraction, and the spiroepoxide intermediate resulting from cyclization and oxygenation (Scheme 1). In an attempt to estimate the contribution of oxidative transformation of curcumin to protein binding we have synthesized alkynyl-tagged derivatives of curcumin. Binding of the alkynyl derivative to protein was blocked by curcumin and an unstable analog but not by a stable analog.¹¹ Likewise, a model peptide containing the redox active Cys179 of the activation domain of IKK β was adducted by curcumin and unstable analogs but not by analogs that do not undergo autoxidation.¹¹ These findings suggested a key role of electrophiles derived

from autoxidation of curcumin in protein binding. The reacting electrophiles or the mechanism(s) of adduction to cysteine were not identified.

Thus, identification of the functionally relevant electrophiles in protein binding is important for understanding the mechanisms by which curcumin achieves its biological effects. In order to address this question we analyzed adduct formation of curcumin and its naturally occurring analogs, demethoxy- (DMC) and bisdemethoxycurcumin (BDMC), with the small molecule thiols GSH, NAc, and β ME.

EXPERIMENTAL PROCEDURES

Materials.

Curcumin, DMC, and BDMC were synthesized according to Pabon¹² with modifications.¹³ Stock solutions of curcuminoids (5 mM in ethanol) were stored at -20°C . Turmeric extract (curcumin from *Curcuma longa* powder; C1386), HRP (type-II, 5 kU/mL, P8250), and phorbol ester (12-O-tetradecanoylphorbol-13-acetate, PMA) were obtained from Sigma. Spiroepoxide, vinyl ether, and bicyclopentadione derivatives of curcumin were prepared by autoxidation of curcumin followed by RP-HPLC purification as described.¹⁴ RAW264.7 cells were purchased from ATCC.

Incubations with thiols.

Curcumin (50 μM) was added to 50 mM ammonium acetate buffer (pH 7.5) and incubated for 20 min to initiate autoxidation. GSH, NAc, or β ME (1 mM) were added either before or 20 min after initiation of autoxidation, and the samples were allowed to react for a total time of 1 h. Samples were extracted using 30-mg Waters HLB cartridges preconditioned with MeOH, water, and cold 50 mM ammonium acetate buffer (pH 7.5) and eluted with MeOH (350 μL). The eluates were evaporated to dryness under a stream of nitrogen and reconstituted in methanol for analysis by RP-HPLC or analyzed directly (without concentration) by LC-MS, respectively.

Reactions of DMC and BDMC with thiols were conducted as autoxidations or enzymatic oxidations. Autoxidations of DMC and BDMC (50 μM) in 50 mM ammonium acetate buffer (pH 8) were conducted at 20°C for 19 h followed by addition of thiol (1 mM) and incubation for 1 h. Enzymatic oxidations (10 ml of 50 mM ammonium acetate buffer, pH 8) of DMC and BDMC (50 μM) used HRP (200 μl of a 1:10,000,000 dilution; 0.1 mU) and H_2O_2 (40 μM). Reactions were conducted for 30 min before addition of thiols (1 mM) and continued for 30 min.

Synthesis of products 1–3.

Curcumin (50 μM) was dissolved in 50 ml of 20 mM ammonium acetate buffer (pH 8) and allowed to react for 17 min. β -ME (10 mM) was added and allowed to react for 3.5 h. Products were extracted using methyl-tert. butyl ether followed by dichloromethane and evaporated to dryness. RP-HPLC used a Waters Symmetry C18 column (4.6×250 mm; 5 μm) eluted with a gradient of solvent A (50 mM ammonium acetate buffer, pH 8) and B (acetonitrile) at 1 mL/min flow rate. The gradient was programmed from 98% A to 70% A

in 15 min and then to 20% A in 15 min. Chromatography was monitored using an Agilent 1200 diode array detector.

Cell incubations.

RAW 264.7 cells were maintained in DMEM supplemented with 10% fetal bovine serum at 37°C in 5% CO₂ and constant humidity. Cells were seeded at a density of 1×10^6 cells/mL in 6-well plates and allowed to adhere overnight. Cells were activated with PMA (500 nM) for 5 min followed by incubation with curcumin (10 µM) for 10 min. Incubations were terminated by removing media and placing on ice followed by immediate extraction. For analysis of curcumin metabolites, media were extracted without acidification using HLB cartridges as described.

LC-MS.

LC-MS analysis was performed using a Thermo Scientific TSQ Vantage triple stage quadrupole MS using electrospray ionization in the negative ion mode. Chromatographic separation of metabolites was achieved using a Waters Symmetry Shield C18 column (2.1 × 50 mm, 1.7 µm) eluted at a flow rate of 0.4 mL/min. Samples were eluted from the column using a linear gradient of 5–95% acetonitrile containing 0.1% formic acid over 2 min followed by 1 min of isocratic elution with 95% acetonitrile with 0.1% formic acid. Analysis of samples shown in Fig. 6 used a different gradient. The column was eluted with a gradient of 5–20% over 4 min, 20–95% over 2 min and 95% isocratic elution for 1 min using acetonitrile containing 0.1% formic acid. In vitro incubations of curcumin, DMC, and BDMC with thiols were analyzed in the MS1 mode, and extracted ion chromatograms are shown in Figs. 1A-C and 5. The incubations of curcumin with RAW264.7 cells (Fig. 6) were analyzed in the SRM mode using m/z 674.3 to m/z 306.0 for curcumin-GS adduct; and m/z 706.0 to m/z 272.2 for the dioxygenated curcumin-GS adduct (1,7-dihydroxy-cyclopentadione-5'-GS).

NMR.

Samples were dissolved in 150 µL of D₂O/CD₃CN (9:1), CD₃OD, or CD₂Cl₂ using a 3 mm sample tube. NMR analyses were performed on a Bruker AV-II 600 MHz spectrometer equipped with a cryoprobe using pulse frequencies taken from the Bruker library. Chemical shifts are reported relative to residual non-deuterated acetonitrile (δ = 1.97), methanol (δ = 3.30 ppm), or dichloromethane (δ = 5.32 ppm) in the solvents. Carbon chemical shifts were determined from the HSQC and HMBC experiments.

RESULTS

Reaction of curcumin with thiols.

Curcumin (50 µM) was added to buffer pH 7.5 to initiate autooxidation. The reaction proceeded for 20 min before addition of a 20-fold molar excess of GSH, NAc, or βME. After 40 min adduct formation was analyzed using LC-MS in the negative ion mode. Unreacted curcumin (m/z 367) accounted for the most abundant ion chromatogram in all reactions (Fig. 3). The BCP oxidation product (m/z 399) was about half as abundant, indicating that efficient oxidation had occurred by the time the reaction was terminated.

Adducts with GSH were detected at m/z 674 for a curcumin-GSH adduct and at m/z 706 indicating that GSH had also formed an adduct with a dioxygenated metabolite of curcumin (Fig. 1A).

The reaction of curcumin with NAc did not show a distinct curcumin-NAc adduct (m/z 530) (Fig. 1B).¹⁴ Instead, two NAc adducts with a dioxygenated curcumin metabolite (m/z 562) were detected. Based on ion intensities, β ME reacted more readily than GSH or NAc to form adducts with curcumin (m/z 445) and a dioxygenated curcumin metabolite (m/z 477), respectively (Fig. 1C). The appearance of two peaks in the chromatograms of the dioxygenated curcumin-thiol adducts was an artifact of the chromatographic conditions and did not indicate that there were additional adducts. Di-thiol adducts were not observed under these reaction conditions.

MS2 analyses of the curcumin-thiol adducts showed loss of the thiol component indicating that these were likely formed by Michael-type addition to C-1 of the heptadienedione chain (data not shown). MS2 fragmentation of the dioxygenated curcumin-thiol adducts with GSH, NAc, and β ME, respectively, showed each loss of 250, 170 (vanillin + H₂O), 152 (vanillin), and 62 (H₂O + CO₂) (Fig. 1D-F). Loss of 250 was compatible with cleavage of the C-1/C-2 carbon bond in a C-7 hydroxylated cyclopentadione, a structural moiety that is present in many of the oxidation products of curcumin.¹⁴ This suggested that the thiol was attached either at one of the methoxyphenol rings or at C-1 of the former heptadienedione chain. The loss of 152 and 170 was compatible with loss of vanillin and vanillin + water, respectively, which also indicated C-7 hydroxylation. The matching fragmentation patterns suggested that the adducts with the three thiols were structurally similar and that the adducts were formed by the same dioxygenated curcumin metabolite.

Previous analyses of peptide adduct formation by curcumin revealed not only the expected mass increase of 368 amu (curcumin) and 400 amu (dioxygenated curcumin) but also a mass increase of 122 amu.¹¹ The latter was attributed to addition of a methoxyphenol ring to the cysteine residue of the peptide. Therefore, we analyzed whether GSH, NAc, and β ME had formed the corresponding methoxyphenol thioethers. LC-MS analyses in the negative ion mode showed a product with the expected m/z 428, 284, and 199 in the incubations with GSH, NAc, and β ME, respectively (Fig. 1A-C), indicating that all three thiols formed the corresponding methoxyphenol thioether adducts. The detected adduct masses (+122, +368, and +400) of the small molecule thiols (i.e., GSH, NAc, and β ME) were identical to the adduct masses detected for the 27-amino acid peptide,¹¹ indicating that the model reactions with thiols mimicked the reactions of the larger peptide.

The spiroepoxide intermediate of curcumin autoxidation¹⁴ was isolated by RP-HPLC and incubated with the thiols. LC-MS analyses showed formation of adducts that were identical to the dioxygenated curcumin-thiol adducts formed in the autoxidation reactions of curcumin (data not shown). HPLC-isolated vinyl ether or bicyclopentadione autoxidation products did not form adducts with the thiols.

Structural identification of β ME adducts.

β ME formed the most abundant adducts with curcumin and its dioxygenated metabolite(s), and, therefore, the reaction was performed on a large scale to isolate products for structural identification. RP-HPLC analysis gave 3 prominent products that were identified as β ME adducts upon LC-MS analysis (Fig. 4). Product **1** had a molecular ion at m/z 477, compatible with a dioxygenated curcumin- β ME product. Product **2** had a molecular ion at m/z 199, compatible with a methoxyphenol- β ME adduct. Product **3** had molecular ions at m/z 445 and 523, indicating a mixture of products with one or two β ME moieties attached to curcumin, respectively. The fourth major product in the reaction was identified as the BCP product of curcumin autoxidation.¹⁵

Product **1** was dissolved in a mixture of D₂O and CD₃CN (9:1; buffered at pD $\approx$ 7.2) to obtain NMR spectra with the best resolved signals. ¹H NMR spectra of **1** showed 5 aryl signals (rather than 6 as in curcumin) indicating that a substitution had occurred at one of the aromatic rings. The substitution was located at C-5', and the two protons remaining in the ring were identified as H-2' and H-6' due to long-range coupling with each other as well as H,C cross-peaks of both to C-1 (70.9 ppm) and C-4' in the HMBC experiment (Fig. 5 and Table S1). The β ME moiety at C-5' was bound through the sulfide since H-1'' of β ME showed an H,C cross-peak to C-6' (119.4 ppm). C-1 and C-7 of the former heptadienedione chain carried a hydroxyl group while C-2 through C-6 formed a cyclopentadione ring. There was only a weak coupling between C-2 and C-6 that was apparent in the H,H-COSY but not in the ¹H NMR spectrum ($J_{2,6}$ = 0 Hz). The $J_{2,6}$ coupling adopts a range of values in different cyclopentadiones derived from the same cyclization reaction¹⁴ and therefore appears an insufficient measure to determine the relative configuration (cis/trans) of the substituents at the cyclopentadione. The NMR analyses identified product **1** as a 5'- β ME thioether adduct of a 1,7-dihydroxy-cyclopentadione of curcumin.

Product **2** showed only 6 signals in the ¹H NMR spectrum. Three aromatic hydrogen signals and a singlet comprised of 3 hydrogens were derived from the methoxyphenol ring. The two other signals, located at 3.61 ppm and 2.91 ppm, were from β ME. Chemical shift and multiplicity of the aromatic hydrogen signals were similar to curcumin indicating that the substitution at the methoxyphenol ring was unchanged and that β ME was connected at C-1'. β ME was bound to the ring via the sulfide since H-1 (2.91 ppm) of β ME gave a cross-peak with C-1' of the ring in the HMBC experiment. This identified **2** as the β ME adduct of methoxyphenol with the thiol attached in the para position relative to the phenol. LC-MS analyses gave a peak with m/z 199 in negative ion mode, confirming the calculated MW (200.25 g/mol).

As explained in the Discussion section, formation of the methoxyphenol adduct predicted a cyclopentadione aldehyde as the other cleavage product. If C-1 and C-7 are in cis configuration the cyclopentadione aldehyde is likely to undergo acetal formation which does not change the predicted MW of 278.26 g/mol. LC-MS analyses in the negative ion mode showed formation of a product with m/z 277 in the reactions of curcumin with all three thiols (not shown). Elution of the putative cyclopentadione aldehyde was not apparent in the

HPLC-diode array chromatograms, and therefore, the product could not be isolated and identified by NMR analysis.

NMR analysis of product **3** showed the presence of two β ME moieties. These were located at C-1 and C-7 of the 7-carbon linker between the two methoxyphenol rings. Addition of the thiol group to C-1 and C-7 resulted in saturation of the double bonds such that C-2 and C-6 were found as methylenes ($\delta_{\text{H}} = 2.77$ ppm; $\delta_{\text{C}} = 45.6$ ppm). This identified product **3** as a 1,7-di- β ME adduct of curcumin. Multiplicity of the signals for H-1, -2, -6, and -7 indicated that product **3** was a mixture of isomers, most likely consisting of the 4 predicted diastereomers.

Further support for the structural identification of **3** was obtained by LC-MS analysis after derivatization. Since the adduct with the thiol was prone to undergo reverse Michael-type reaction we attempted to stabilize **3** by derivatization of the carbonyl groups. Treatment with methoxyamine hydrochloride gave the predicted increase of the curcumin-di- β ME adduct (m/z 525; positive ion mode) to m/z 583 ($+ 2 \times 29$) indicating that two carbonyls (C-3 and C-5) were present in **3** and had been derivatized.

Autoxidation progress and adduct formation.

We compared adduct formation depending on the time point of thiol addition to curcumin autoxidation reactions. Thiols were added before curcumin with the goal of inhibiting autoxidation and providing the enone as the predominant electrophile. Alternatively, thiols were added after 20 min when autoxidation of curcumin was in full progress, providing reactive intermediates as additional electrophiles. Total reaction time for both experiments was 1 h. The reactions were analyzed comparing absolute peak intensities since quantification using d_6 -curcumin and d_6 -BCP as internal standards was problematic. Quantitative recovery of the standards required acidification which increased degradation of the thiol adducts.

Presence of GSH, NAc, and β ME from the start inhibited autoxidation such that curcumin was between 100- and 200-fold more abundant than BCP (Fig. 4A). Upon delayed addition of thiols BCP was the major product at about 2- to 3-times the amount of curcumin. The time point of addition of thiol did not change the ratio of curcumin to the respective curcumin-thiol adducts (Fig. 4B). β ME formed adducts with curcumin and dioxygenated curcumin more effectively than GSH and NAc. Adduct **1** became 10-times more abundant relative to dioxygenated curcumin upon delayed addition of β ME whereas GSH and NAc did not change the ratio of dioxygenated curcumin to the respective thiol adduct (i.e., the GSH and NAc analogs of product **1**) (Fig. 4B). Thus, both BCP and the respective thiol adducts of the spiroepoxide were markedly increased when autoxidation was allowed to initiate and proceed before thiol addition. Control reactions that were injected directly (i.e., without extraction) or terminated with *N*-ethylmaleimide to capture remaining thiol showed that adduct formation did not occur during sample work-up procedures.

Thiol adducts of curcuminoids DMC and BDMC.

Natural turmeric extract is a mixture of curcumin and its isomers DMC and BDMC in a ratio of about 80:15:5. Turmeric extract, rather than chemically pure curcumin, is used preferentially in clinical studies as well as in many in vitro studies of “curcumin”. Therefore, we analyzed the reaction of synthetic pure DMC and BDMC with the thiols. DMC is chemically more stable than curcumin with an estimated half life of about 24 h versus ≈ 15 min in buffer pH 7.5. BDMC is even more resistant to autoxidation and shows little to no degradation upon incubation for 24 h.¹⁶ We analyzed the reaction of DMC and BDMC with thiols both in the presence of horseradish peroxidase (HRP) and H_2O_2 to catalyze oxidative transformation as well as under autoxidation conditions. Enzymatic oxidations were conducted for 30 min, autoxidation reactions for 19 h with the goal of achieving a similar extent of oxidative transformation.

HRP-catalyzed oxidation of DMC resulted in about 1% abundance of dioxygenated products (demethoxy-spiroepoxide and -bicyclopentadione analogs; m/z 369),¹⁶ and about three times as much was formed during extended autoxidation (Fig. 5A, B). Both reactions gave a similar amount of DMC- β ME adduct (m/z 415; $\approx 10\%$ abundance relative to DMC). The adduct of dioxygenated DMC with β ME (m/z 447) was about as abundant as dioxygenated DMC (m/z 369) in the enzymatic oxidation but about 4-fold less abundant in the autoxidation. The DMC- β ME adduct was about 10-times more abundant than the dioxygenated DMC- β ME adduct, regardless whether oxidation was enzyme-catalyzed or spontaneous.

Oxidative transformation of BDMC stalls at the spiroepoxide¹⁶ and a respective product (m/z 339; ca. 2% relative abundance) was present in the enzymatic oxidation but there was no discernible oxidation product in the autoxidation (Fig. 5C,D). A BDMC- β ME adduct (m/z 385) was present in about 1% abundance relative to BDMC in both autoxidation and enzymatic reactions. A dioxygenated BDMC- β ME adduct (m/z 417) was detected in the enzymatic reaction (ca. 1% relative to BDMC) and, unexpectedly, also in the autoxidation reaction (ca. 0.1% relative to BDMC). The resistance of BDMC toward autoxidation correlated with decreased adduction of β ME to BDMC and its dioxygenated spiroepoxide metabolite compared to DMC. Adduct formation was not detected in the reactions of BDMC with NAc and GSH (Table 1).

Curcumin- and spiroepoxide-GS adducts in RAW264.7 cells.

Oxidative transformation of curcumin to BCP occurred spontaneously in cell culture medium and was enhanced by activation of leukocytes with phorbol ester (PMA).¹⁷ We wanted to test whether oxidative transformation of curcumin in activated cells results in formation of GS-adducts of curcumin and its oxidation products. Mouse RAW264.7 macrophage-like cells were stimulated with PMA to induce the oxidative burst¹⁸ and incubated with curcumin. GS-adducts of curcumin and spiroepoxide (1,7- dihydroxy-cyclopentadione-5'-GS) were readily detected in RAW264.7 cells (Fig. 6). The adducts were absent in cells not incubated with curcumin.

DISCUSSION

The α,β -unsaturated carbonyl (enone) has been considered the sole electrophile of curcumin,^{19,20} responsible for mediating the interaction with cellular protein. Our study has uncovered additional electrophiles, formed upon autoxidative transformation of curcumin. Autoxidation is inevitable when curcumin is added to buffer or cell culture medium at physiological pH.¹⁰ We tested for electrophiles in curcumin and its autoxidation products by analyzing adduct formation with the thiol nucleophiles GSH, NAc, and β ME. All three thiols formed adducts with curcumin as well as with dioxygenated autoxidation products of curcumin, the latter providing evidence for formation of novel electrophiles different from the enone. The spiroepoxide intermediate of oxidative transformation of curcumin was identified as the key electrophile.¹⁴ HPLC-isolated spiroepoxide formed the same thiol adducts, indicating that addition of O₂ was not secondary to adduct formation. Of note, adducts derived from the spiroepoxide were more abundant than the respective thiol adducts with curcumin in the autoxidation reactions. This suggested that oxidative transformation of curcumin produces electrophiles that are equally or more relevant than the enone when it comes to reaction with thiol nucleophiles.

The spiroepoxide formed two different thioether adducts (Scheme 2). Reaction of the thiol at the 5' position of the quinone ring yielded a 1,7-dihydroxycyclopentadione-5'-thioether as a result of re-aromatization of the ring and opening of the epoxide to a hydroxyl. Alternatively, addition of the thiol to the 1' position resulted in cleavage of the epoxide C-C bond and loss of the ring from the rest of the molecule. The products were methoxyphenol thioether and a cyclopentadione aldehyde, the latter only tentatively identified by LC-MS analyses. Addition of the thiol to the 1' position was reminiscent of the addition of water to the same position of the spiroepoxide that likewise resulted in cleavage of the epoxide C-C bond, a key step during transformation to the final bicyclopentadione.¹⁴ Thus, the spiroepoxide contains two electrophilic sites for reaction with thiol nucleophiles. Unfortunately, we were unable to determine the relative preference of the thiols to react at either electrophilic site due to different ionization efficiencies of the methoxyphenol and 1,7-dihydroxycyclopentadione thioether adducts.

In the case of curcumin, i.e., for thiol adduct **3** and its analogs with an increase in mass by 368 amu, the reacting electrophile is less readily identified. Thiol adduction not only to the enone of parent curcumin but also to two structurally distinct quinone methide oxidation products (I and II in Scheme 1) appears a possibility and will result in an increase of 368 amu. To further increase complexity, curcumin and quinone methide I are predicted to form the identical adduct such that even elucidating the structure of the adduct did not help identify the reacting electrophile. With the caveat of relying on indirect evidence, our data support both the enone as well as the quinone methide as reacting electrophiles. The curcumin adduct with two thiol moieties argues for a Michael-type reaction at the enone. Formation of the monothiol adduct (by whichever mechanism) disrupts the conjugated heptadienedione that is required for efficient autoxidation and formation of a quinone methide. This suggests that addition of the second thiol occurred as a Michael-type addition to the remaining enone and not to a quinone methide, formation of which is unlikely after disruption of the conjugated system. The first addition of the thiol may also occur via

Michael-type addition to the enone. This was suggested by the finding that the relative abundances of the curcumin-thiol adducts were not increased when autoxidation was allowed to initiate and progress to quinone methide formation prior to addition of thiols as compared to thiols being present from the start. On the other hand, there is contrasting evidence to support a role of the quinone methide in addition of the first thiol. Major support comes from the reduced reactivity of DMC and especially BDMC with thiols compared to curcumin. Although both compounds contain the same enone as curcumin, reactivity of DMC and BDMC with thiols correlated with their ability to undergo autoxidation, i.e., to form a quinone methide.¹⁶ The same correlation had been found for synthetic curcumin derivatives that contain an enone moiety when comparing the rate of autoxidation with the inhibition of NF- κ B,¹¹ an effect that involves covalent binding to a regulatory cysteine in the upstream kinase, IKK β .^{21–23} Thus, currently available indirect evidence supports both the enone and quinone methide as electrophiles involved in thiol adduct formation by curcumin.

Quinone methides act as potent DNA and protein alkylating agents,^{24, 25} a property exploited by many anti-cancer drugs in clinical use.^{26, 27} Although we did not find evidence that the thiols react at the ring of a curcumin quinone methide intermediate, others have described that such reactions can occur. Studying the reaction of quercetin with thiol nucleophiles Awad and colleagues found addition of GSH to electrophilic carbons of the flavonoid A-ring ring after enzymatic oxidation to an ortho-quinone and rearrangement to a quinone methide.^{28, 29} In the quercetin oxidation products GSH reacted with the quinone ring rather than the methide carbon whereas we found that GSH reaction with curcumin or its quinone methide did not result in addition to the ring. Binding of GSH to the quinone ring was also observed with catechol estrogens that form an ortho-quinone but not a quinone methide.³⁰ Capsaicin, which shares a methoxyphenol ring with curcumin, showed addition of GSH at electrophilic carbons at the ring as well as the exocyclic double bond of its quinone methide oxidation products.³¹ Detection of these metabolites in vivo implied that they may have been formed enzymatically, possibly through action of GSH transferase(s). Eugenol, which shares even greater structural similarity with curcumin, bound GSH upon oxidative activation to a quinone methide only at the alkyl chain but not at the ring,³² consistent with our findings for curcumin. Oxidative transformation of eugenol has been suggested to occur in vivo but was not proven experimentally.

Some aspects of this study might be relevant for understanding the biological effects of curcumin. Our previous studies using alkynyl-derivatives of curcumin showed that oxidation markedly contributes to protein binding.¹¹ Thus, similar to the reaction with GSH in cells (Fig. 6), adduction to cellular protein may involve not only parent curcumin but also its oxidative metabolites, specifically, the spiroepoxide.¹¹ As shown here, protein modification by oxidative metabolites may entail adduction of the entire molecule or the methoxyphenol ring only. Adduct formation, if it occurs at a functionally relevant cysteine, is likely to result in a change in protein function, with the possibility of more subtle or different effects upon adduction of the smaller methoxyphenol than of the entire molecule, be it curcumin or spiroepoxide. While abundant in vitro, an underlying question is whether oxidative transformation takes place at all in vivo. It is conceivable that protein adduction by oxidative metabolites of curcumin is abundant in vitro but less so or even absent in vivo. Inefficient oxidative transformation in vivo could therefore contribute to the lack of

therapeutic effects of curcumin, at least regarding effects mediated by covalent modification of cellular protein. Our studies, aimed at identifying the BCP major and final oxidation product in plasma or urine as a marker of oxidative transformation, have not been successful so far (PBL and CS, unpublished). Other metabolites, like the GSH adducts of spiroepoxide or methoxyphenol identified here, may be more suitable candidates in the quest for evidence of oxidative transformation of curcumin in vivo.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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ABBREVIATIONS

BCP	bicyclopentadione
BDMC	bis-demethoxycurcumin
βME	β-mercaptoethanol
DMC	demethoxycurcumin
HMBC	heteronuclear multiple bond correlation
HRP	horseradish peroxidase
HSQC	heteronuclear single quantum coherence
NAc	<i>N</i> -acetylcysteine
PMA	phorbol 12-myristate 13-acetate

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Figure 1.

Negative ion LC-MS analysis of the reaction of curcumin with GSH, NAC, and βME. Autoxidation reactions of curcumin (50 μM) were supplemented with thiols (1 mM), extracted, and analyzed using LC-MS as described in “Experimental Procedures”. The traces are the extracted ion chromatograms for curcumin, BCP, and the respective thiol adducts of curcumin, dioxycurcumin, and methoxyphenol with (A) GSH, (B) NAC, and (C) βME. Elution of two peaks in the traces representing dioxycurcumin-thiol adducts was due to a chromatographic artifact. (D-F) LC-MS/MS product ion spectra of the adducts of dioxycurcumin with (D) GSH, (E) NAC, and (F) βME obtained by collision-induced dissociation of the peaks marked with an asterisk in the corresponding ion chromatograms. BCP, bicyclopentadiene.

Figure 2.

RP-HPLC analysis of products **1-3** and BCP formed by autoxidation of curcumin in the presence of β ME. The reaction was conducted, extracted, and analyzed as described in “Experimental Procedures”. Elution of the products was monitored using a diode array detector, and the chromatogram shown was recorded at UV235 nm. BCP, bicyclopentadione.

Figure 3.
(A) Structure of **1** and (B) diagnostic HMBC interactions (blue arrows).

Figure 4. Curcumin autoxidation and adduct formation in relation to the time point of thiol addition. GSH, NAc, and βME (1 mM) were either present from the start (0 min) or added 20 min after the start (20 min) of autoxidation of curcumin (50 μM) for a total reaction time of 1 h. (A) Peak areas for curcumin and dioxygenated curcumin (BCP and other products). (B) Peak areas of curcumin-thiol and dioxygenated curcumin-thiol adducts. Negative ion LC-MS analyses were performed as described in “Experimental Procedures”. The mean of three independent experiments (± SD) is shown.

Figure 5.

Negative ion LC-MS analyses of the reactions of DMC and BDMC with β ME. DMC was incubated with HRP and H_2O_2 for 30 min (A) or autoxidized for 19 h (B) prior to the addition of β ME (1 mM) and additional reaction for 30 min or 1 h, respectively. (C,D) Similar reactions were conducted with BDMC.

Figure 6.

Adduct formation in RAW264.7 cells. RAW264.7 cells were activated with PMA for 5 min and incubated with curcumin (50 μ M) or vehicle for 10 min. (A) Curcumin-glutathione and dioxygenated curcumin-glutathione adducts were detected in cells treated with curcumin. (B) Co-chromatography of the sample in (A) with an autoxidation reaction of curcumin in the presence of GSH. Negative ion LC-SRM-MS analyses were used to analyze curcumin-glutathione (m/z 674.3 to m/z 306.0) and dioxygenated curcumin-glutathione adducts (m/z 706.0 to m/z 272.2).

Scheme 1.

Electrophilic sites (marked with an asterisk) in curcumin and its autoxidation products.

Scheme 2.

Reaction of curcumin and its autooxidation products with thiols.

Table 1.

LC-ESI-MS molecular and fragment ions detected for free and thioether adducts of curcumin, demethoxy-(DMC), and bisdemethoxy-(BDMC) curcumin and their dioxygenated products (*m/z*, negative ion mode).

Compound	free	Adduct with		
		β ME (+78 amu)	NAc (+163 amu)	GSH (+307 amu)
Curcumin	367	445.2($\rightarrow$ 367.2)	530.3($\rightarrow$ 162.4)	674.3($\rightarrow$ 306.0)
Dioxygenated curcumin (spiroepoxide, BCP) ¹	399 ²	477.0($\rightarrow$ 226.8)	562.0($\rightarrow$ 370.9)	706.0($\rightarrow$ 272.2)
DMC	337	415.2($\rightarrow$ 337.1)	500.0($\rightarrow$ 163.0)	644.0($\rightarrow$ 305.8)
Dioxygenated DMC (spiroepoxide, BCP) ¹	369	447.3($\rightarrow$ 226.8)	532.0($\rightarrow$ 341.5)	n.d. ⁴
BDMC	307	385.1($\rightarrow$ 306.5)	n.d.	n.d.
Dioxygenated BDMC (spiroepoxide) ³	339	417.0($\rightarrow$ 277.4)	n.d.	n.d.

¹Thiol adducts are formed by the spiroepoxide but not by the bicyclopentadione (BCP).

²Spiroepoxide and BCP give the same molecular ion.

³Oxidative transformation of BDMC stalls at the spiroepoxide, no BCP is formed.¹⁶

⁴n.d., not detected.