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Stability and anti-inflammatory activity of the reduction-resistant curcumin analog, 2,6-dimethyl-curcumin

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Abstract

The efficacy of the curry spice compound curcumin as a natural anti-inflammatory agent is limited by its rapid reductive metabolism in vivo. A recent report described a novel synthetic derivative, 2,6-dimethyl-curcumin, with increased stability against reduction in vitro and in vivo. It is also known that curcumin is unstable at physiological pH in vitro and undergoes rapid autooxidative transformation. Since the oxidation products may contribute to the biological effects of curcumin, we tested oxidative stability of 2,6-dimethyl-curcumin in buffer (pH 7.5). The rate of degradation was similar to curcumin. The degradation products were identified as a one-carbon chain-shortened alcohol, vanillin, and two isomeric epoxides that underwent cleavage to vanillin and a corresponding hydroxylated cleavage product. 2,6-Dimethyl-curcumin was more potent than curcumin in inhibiting NF- κ B activity but less potent in inhibiting expression of cyclooxygenase-2 in LPS-activated RAW264.7 cells. 2,6-Dimethyl-curcumin and some of its degradation products covalently bound to a peptide that contains the redox-sensitive cysteine of IKK β kinase, the activating kinase upstream of NF- κ B, providing a mechanism for the anti-inflammatory activity. In RAW264.7 cells vanillin, the chain-shortened alcohol, and reduced 2,6-dimethyl-curcumin were detected as major metabolites. These studies provide new insight into the oxidative transformation mechanism of curcumin and related compounds. The products resulting from oxidative transformation contribute to the anti-inflammatory activity of 2,6-dimethyl-curcumin in addition to its enhanced resistance against enzymatic reduction.

Graphical abstract

Degradation products are linked to anti-inflammatory activity of 2,6-dimethyl-curcumin, a synthetic analog of curcumin stable against metabolic reduction in vivo.

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Electronic Supplementary Information (ESI) available: Tables and spectra of NMR data of products 3-5. See DOI: 10.1039/x0xx00000x

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Introduction

The biological activities described for the spice compound curcumin include antioxidant, anti-inflammatory, anti-microbial, and anti-tumorigenic effects ^{1, 2}. The majority of these effects have been established using cultured cells and other in vitro models, and some have been replicated in animal models ³⁻⁵. As a consequence of successful preclinical evaluation, a large and yet increasing number of clinical trials with curcumin has been registered at *clinicaltrials.gov*. Successful trials with curcumin or turmeric have been sparse ^{6, 7} although some recent controlled trials report improvements in cognitive function, joint arthritis, and inflammatory bowel disease ⁸⁻¹¹.

A key reason suggested for the lack of successful application in humans is the low bioavailability of curcumin ^{12, 13}, and rapid metabolism via reduction and conjugation of what little is absorbed is another ^{14, 15}. With the aim of impeding reductive metabolism, Koo and coworkers have placed methyl groups at double bond carbons 2 and 6 of the heptadienedione chain of curcumin (Fig. 1) ¹⁶. Introduction of steric bulk around the 1,2- and 6,7-double bonds was proposed to hinder enzymatic reduction (saturation) to di- and tetrahydro metabolites. The novel analog, 2,6-dimethyl-curcumin, showed enhanced anti-angiogenic activity in vitro and in an animal model compared to curcumin. It was less efficiently reduced by recombinant alcohol dehydrogenase, and there appeared to be less reductive metabolism in mice injected intraperitoneally with 2,6-dimethyl-curcumin than with curcumin ¹⁶. The authors concluded that inhibition of reductive metabolism accounted for the increased biological activity of 2,6-dimethyl-curcumin ¹⁶.

Curcumin is chemically unstable at physiological pH and undergoes rapid degradation ¹⁷. The degradation is an autoxidation involving reaction with oxygen to yield a bicyclopentadione as the final stable product (Fig. 1) ^{18, 19}. The initiating step of autoxidation is loss of a hydrogen atom from one of the phenolic hydroxyls followed by delocalization of the resulting radical into the heptadienedione chain. The radical facilitates cyclization and oxygenation reactions that transform the linear 7-carbon chain to a bicyclic cyclopentadione-tetrahydrofuran moiety ²⁰. The initial H-abstraction can be catalyzed by peroxidases ^{18, 21}, suggesting that oxidative transformation may occur in vivo although this has not been established yet. Oxidative activation of curcumin as well as the minor curcuminoids, demethoxy- and bisdemethoxycurcumin, is required for the inhibition of human topoisomerase II α and contributes to their anti-inflammatory effects ²²⁻²⁴. Inhibition of topoisomerase II α was mediated by reaction intermediates adducting to active site

cysteine residues since neither curcumin nor the final bicyclopentadione were active²². For the anti-inflammatory activity, a strong correlation between the rate of oxidative transformation and inhibition of NF- κ B was observed when synthetic analogs of curcumin were analyzed²⁴.

Considering the potential role of oxidative transformation products in the biological activity of curcumin¹⁹ we prepared 2,6-dimethyl-curcumin and tested its chemical stability. We found it to be unstable similar to curcumin, identified degradation products in buffer, and analyzed their possible role in mediating the anti-inflammatory effect of 2,6-dimethyl-curcumin.

Results and discussion

Stability of 2,6-dimethyl-curcumin

Chemical stability of 2,6-dimethyl-curcumin was tested by incubation in pH 7.5 buffer. UV/Vis analysis indicated presence of both the enol tautomer (λ_{max} 410 nm) and the diketo tautomer with absorbance around 350 nm. Repetitive scanning using a UV/Vis spectrophotometer showed rapid disappearance of the absorbance at 410 nm of 2,6-dimethyl-curcumin in the first 2 min and then a decrease in rate until an apparent halt after about half of the absorbance had disappeared (Fig. 2a). There was a concomitant increase in absorbance around 340 nm. The rate of degradation during the initial 2 min of reaction was calculated as $2.8 \pm 0.1 \mu\text{M}/\text{min}$ (Fig. 2b), slightly less than the initial rate of degradation of curcumin ($4.0 \mu\text{M}/\text{min}$). The change in absorbance was not a mere slow shift in the equilibrium of the diketo and enol tautomers as was evident from analysis of the reaction products by HPLC.

Identification of degradation products

Autoxidation of 2,6-dimethyl-curcumin showed formation of 5 products (**1-5**) when the reaction was terminated after 1 h (Fig. 2c). Products **2** and **3** were unstable and underwent further transformation. After 5 h reaction time **1**, **4**, and **5** became the major products with **2** and **3** absent or decreased, respectively (Fig. 2d). Consistent with the UV/Vis assay, unreacted 2,6-dimethyl-curcumin was the most abundant compound in the analyses, chromatographing as two peaks representing the diketo and enol forms (λ_{max} 331 and 395 nm, respectively, recorded in RP-HPLC solvent). Large scale reactions for identification of the degradation products were conducted for 1 h or 24 h, respectively, in order to produce the intermediate and final products. Products were isolated by RP-HPLC and identified using UV/Vis, LC-MS, and homo- and heteronuclear 1D- and 2D-NMR analyses.

Product **1** eluting at 7.5 min retention time was identified as vanillin based on its UV/Vis spectrum and positive ion LC-ESI-MS analysis (m/z 153.1) in comparison to an authentic standard.

Product **2** and **3** appeared to be isomeric products. Due to differences in stability we were able to obtain clean NMR spectra only for more stable isomer **3**, and its identification is presented in detail. Product **3** gave a molecular ion of m/z 413.2 in positive ion LC-MS analysis, indicating incorporation of one atom of oxygen. The oxygen was present as a

C-6/C-7 epoxide[‡], based on diagnostic chemical shifts of H-7, C-6, and C-7 in the ¹H and heteronuclear NMR spectra. Consistent with the lack of a direct neighbor, H-7 was a singlet, and thus, it was not possible to determine *cis* or *trans* configuration of the epoxide. Absence of the 6,7 double bond was consistent with the UV/Vis spectrum with a λ_{max} at 360 nm, a hypsochromic shift compared to λ_{max} 420 nm of 2,6-dimethyl-curcumin. Based on these data product **2** was identified as 6,7-epoxy-2,6-dimethyl-curcumin.

Product **2** (λ_{max} 359 nm) had similar UV/Vis, LC-MS, and ¹H NMR data as **3** and was identified as an isomer of 6,7-epoxy-2,6-dimethyl-curcumin, likely with changed configuration of the epoxide group.

Product **4** showed a λ_{max} at 355 nm indicating a shortened chromophore as in **2** and **3**. The ¹H NMR spectrum showed the presence of only one methoxyphenol ring. The signals for H-1 and H-4 of the original heptadienedione chain were present as well as both original methyl groups at C-2 and C-6. One of the methyl groups, however, was shifted upfield to 1.47 ppm, indicating shielding by a hydroxyl. A C-6 hydroxyl was evident from the carbon chemical shift (82.3 ppm) and confirmed absence of the 6,7-double bond. The expected hydrogen signal (H-6), however, was not observed due to exchange with deuterated solvent. The NMR data identified **4** as 2,5-dihydroxy-7-(4-hydroxy-3-methoxyphenyl)-6-methylhepta-4*Z*,6*E*-dien-3-one. LC-MS in positive ion mode gave an abundant ion at *m/z* 261.1, which was 18 amu less than the predicted ion and indicated facile loss of water during the ionization process.

Product **5** shared features in the UV/Vis (λ_{max} at 358 nm) and NMR analyses with the other products indicating that one methoxyphenol ring and the attached carbon chain through C-5 were unchanged from the starting material. HMBC analyses indicated lack of a signal for C-7 of the original carbon chain. In order to provide further support for loss of this carbon, sufficient material was prepared for direct ¹³C-NMR analysis which confirmed absence of a signal for C-7. The upfield shift of the methyl group attached at C-6 was explained by a geminal hydroxyl, compatible with the absence of a hydrogen at this carbon. Additional evidence for the loss of C-7 came from the HMBC cross-peaks of H-2'' and H-6'' of the ring with C-6, placing this carbon one bond closer to the methoxyphenol ring than in 2,6-dimethyl-curcumin. Loss of a carbon from **5** was supported by LC-MS data. A prominent ion was obtained at *m/z* 383.2 which resulted from loss of water from the molecular ion which was not detected, similar to the facile dehydration observed with **4**. These analyses identified **5** as the chain-shortened alcohol, 2,5-dihydroxy-2,7-bis(4-hydroxy-3-methoxyphenyl)-6-methylhepta-4*Z*,6*E*-dien-3-one.

Thus, the immediate products of autoxidative degradation of 2,6-dimethyl-curcumin are alcohol **5**, the 6,7 (or 1,2)-epoxide isomers **2** and **3**, and vanillin **1** (Fig. 2e). The epoxides undergo secondary cleavage, forming vanillin and alcohol **4**.

[‡]2,6-Dimethyl-curcumin is symmetric, and thus C-1 through C-3 are identical to C-7 through C-4. We decided to describe the reactions as occurring at C-6/C-7 because that avoided changing numbers of the carbon atoms in the 7-carbon linker chain of product **5**.

Time course of degradation

A time course for the degradation of 2,6-dimethyl-curcumin was established by analyzing reaction aliquots every 1 h for a total of 10 h (Fig. 3a). 2,6-Dimethyl-curcumin was decreased to $\approx 40\%$ within the first h of reaction and remained stable from there on. Product **5** was formed in parallel within the first h and likewise did not change in abundance during the remainder of the experiment. Epoxides **2** and **3** peaked at 1 h and disappeared within the next 2–5 h to undergo further transformation. Disappearance of **2** and **3** was paralleled by the formation of cleavage products **4** and vanillin **1**. Vanillin, however, was already present in the first aliquot analyzed ($t = 0$) and showed an earlier increase than **4**. This indicated that there was an alternative route of formation of vanillin that did not include epoxides **2** and **3**.

When isolated **2** and **3** were incubated in buffer both gave rise to vanillin **1** and product **4** (Fig. 3b,c). Several additional products were formed but these were not sufficiently abundant for isolation and structural identification. Transformation of the purified epoxides required longer incubation times than during the degradation reactions. After 24 h incubation in buffer about half of **2** and **3** remained that had not converted to **1**, **4**, and the less abundant by-products.

Anti-inflammatory activity

We analyzed the anti-inflammatory activity of 2,6-dimethyl-curcumin by quantifying the inhibition of NF- κ B in activated RAW264.7 mouse macrophage-like cells that stably express luciferase under the control of an NF- κ B response element. Treatment of the cells with LPS increased luciferase activity, and pretreatment with 2,6-dimethyl-curcumin for 45 min prior to LPS resulted in dose-dependent inhibition (Fig. 4a). The IC_{50} for inhibition of NF- κ B by 2,6-dimethyl-curcumin was 7 μ M, about half of the value observed for curcumin ($IC_{50} = 18 \mu$ M²⁴).

Conversely, 2,6-dimethyl-curcumin was less potent than curcumin in inhibiting the expression of COX-2 in LPS-stimulated RAW264.7 cells (Fig. 4b). Decrease of COX-2 expression required 2,6-dimethyl-curcumin at concentration $>20 \mu$ M whereas curcumin was effective at 10 μ M. These results showed that 2,6-dimethyl-curcumin has in vitro anti-inflammatory activity similar to curcumin.

Peptide adduction

Inhibition of NF- κ B activity by curcumin involves covalent adduction of oxidative metabolites of curcumin to IKK β , the upstream kinase that activates NF- κ B²⁴. To test whether 2,6-dimethyl-curcumin and/or its degradation products adducted to IKK β , we used a 27-amino acid peptide that mimics the activation domain of IKK β ^{24, 25}. The peptide contains a redox-active cysteine (Cys179) that has been well characterized as a target of covalent modification by small molecule electrophiles^{26, 27}.

The peptide was added to autooxidation reactions of 2,6-dimethyl-curcumin, and peptide adducts were analyzed using LC-MS (Fig. 5a). The most prominent adduct was formed by addition of 122 amu, indicating adduction of the methoxyphenol terminal group of 2,6-dimethyl-curcumin. An identical adduct was observed when the same peptide was incubated

with curcumin²⁴. Less abundant adducts were formed by the addition of 396 amu, representing the relative mass of 2,6-dimethyl-curcumin, and 412 amu, the relative mass of epoxides **2** and **3**. A fourth adduct was formed by the addition of 166 amu, likely representing a hydroxy-ethyl-methoxyphenol cleavage product. The latter may result by β -fragmentation of an intermediate C-6 alkoxyl radical during the formation of chain-shortened alcohol **5** (see a proposed mechanism in Scheme 1).

Using a 5-fold molar excess of 2,6-dimethyl-curcumin over peptide, there was no unreacted peptide left, although some peptide had dimerized under these conditions (Fig 5a, b). When a Cys179Ala mutant peptide was used, no adducts with 2,6-dimethyl-curcumin were detectable (Fig. 5b). This suggested that the mechanism of adduct formation was by a Michael-type addition of electrophilic degradation products with the nucleophilic cysteine (Cys179) of the peptide.

Metabolism of 2,6-dimethyl-curcumin in RAW264.7 cells

We analyzed whether the degradation products and reduced metabolites were present in the supernatant of RAW264.7 cells incubated with 2,6-dimethyl-curcumin. LC-ESI-SRM analyses were developed based on characteristic MS2 fragment ions of the major degradation products identified above. A standard of the reduced metabolite (tetrahydro-2,6-dimethyl-curcumin) was generated by catalytic hydrogenation of 2,6-dimethyl-curcumin. RAW264.7 cells transformed 2,6-dimethyl-curcumin to vanillin **1** and chain shortened alcohol **5** whereas the other oxidative products, epoxides **2** and **3** as well as cleavage product **4** were not detected (Fig. 6). The reduced metabolite tetrahydro-2,6-dimethyl-curcumin was also detected. This indicated that 2,6-dimethyl-curcumin underwent oxidative as well as reductive metabolic transformation in the presence of RAW264.7 cells.

Instability of 2,6-dimethyl-curcumin

Introduction of methyl groups at the 2- and 6-position of the heptadienedione chain of curcumin increases stability against enzymatic reduction¹⁶. The gain in metabolic stability was suggested to account for the increased anti-angiogenic potency of 2,6-dimethyl-curcumin compared to curcumin¹⁶. Reduction is not the only possible metabolic transformation of curcumin. Curcumin is chemically unstable at physiological pH and undergoes rapid autoxidative degradation^{19, 20}. Here we show that the addition of methyl groups to curcumin at C-2 and C-6 did not increase chemical stability, in contrast to the gain in stability against enzymatic reduction. Similar to curcumin 2,6-dimethyl-curcumin was subject to spontaneous autoxidative degradation. The chemical transformation showed differences but also similarities with the autoxidation of curcumin.

Differences in degradation to curcumin

The major outcome of oxidative transformation of curcumin is cyclization of C-2 and C-6 of the heptadienedione chain to form a cyclopentadione ring and incorporation of two atoms of oxygen into the final products²⁰. With 2,6-dimethyl-curcumin only one atom of oxygen is incorporated in the final and intermediate products (Fig. 2e). Cyclopentadione formation was not observed, and instead, cleavage of the methyl-substituted double bond was a prevalent outcome. As with curcumin, the oxidation reaction of 2,6-dimethyl-curcumin is

initiated by H-abstraction from the phenolic hydroxyl. H-abstraction occurs as a sequential proton loss-electron transfer process (SPLET)²⁸, and the methyl groups at C-2 and C-6 that stabilize the diketo form may influence the kinetics of the initial oxidation reaction²⁹. H-abstraction yields a phenoxyl radical that is stabilized at C-2 (C-6) by the electron-donating effect of the methyl group. Radical cyclization of the tertiary radical C-2 to C-6 is disfavored, and instead O₂ is added to form a peroxy radical (ROO•). Two possibilities for further reaction of the peroxy radical appear plausible based on the products we have identified.

Proposed mechanisms of degradation

The first proposed reaction of the peroxy radical is intramolecular addition to the exocyclic double bond of the quinone methide (Scheme 1a). This gives a 1,2-dioxetane with the radical transferred to the ring. β -Cleavage yields two aldehydic fragments, one of which is vanillin **1**, the other is the carbonyl equivalent of **4**. The latter was not among the products identified, possibly due to the reactive nature of the 1,2,4-tricarbonyl moiety. Dioxetane formation and fragmentation provides a direct pathway to vanillin in the degradation of 2,6-dimethyl-curcumin.

Alternatively, the peroxy radical can add to the 6,7 (1,2) double bond of a second molecule of 2,6-dimethyl-curcumin (Scheme 1b). This results in the formation of a peroxide-linked dimer. The radical can add to either C-6 or C-7 (C-1, C-2) forming two isomeric dimers that will eventually yield the same products, namely epoxides **2** and **3**. Epoxide formation is achieved (1) by intramolecular homolytic substitution (S_{HI}) of the carbon-centered radical at the dimer peroxide bond and (2) by the resulting alkoxyl radical (RO•) reacting with the exocyclic double bond of the quinone methide (Scheme 1b). Thus, both parts of the dimer give the same epoxides (**2** and **3**) when the peroxide bond breaks, regardless of whether the peroxy radical added initially to C-6 or C-7. Equivalent dimerization pathways to epoxide formation are well precedented in radical reactions during non-enzymatic and enzymatic lipid oxidation³⁰⁻³⁵.

Epoxides **2** and **3** are unstable intermediates. Upon isolation and further incubation, they both transformed to vanillin and product **4** (Fig. 2e). This reaction likely involves the addition of water to introduce a hydroxyl in **4** although a detailed reaction mechanism could not be proposed.

Product **5** was an unusual product due to the loss of C-7 from the molecule. A satisfactory explanation for formation of a new bond between C-6 and the methoxyphenol ring is provided by radical 1,2-migration of the phenolic ring in the peroxide-linked dimer (Scheme 1c)³⁷. Following migration of the methoxyphenol ring, breaking of the C-6/C-7 bond to form one-carbon chain shortened alcohol **5** proceeds through a mechanism that was not further probed but likely involves additional reaction with oxygen.

Formation of vanillin

Vanillin is formed by two mechanisms during autoxidative degradation of 2,6-dimethyl-curcumin (Fig. 2). One is a direct pathway through a dioxetane radical intermediate. The

predicted complementary cleavage product, a keto analog of **4**, has not been identified. The other, indirect pathway involves epoxides **2** and **3** as reaction intermediates. Vanillin is the most abundant product of degradation of 2,6-dimethyl-curcumin, whereas in the autoxidation of curcumin it is only a very minor product³⁸. The mechanism of how vanillin is formed from curcumin has not been established although cleavage of a dioxetane intermediate³⁹ as well as retro Claisen condensation⁴⁰ have been suggested. It is conceivable that the increased stability of the tertiary radical at C-6 or C-2, respectively, formed upon H-abstraction, favors reaction with oxygen in the case of 2,6-dimethyl-curcumin. At the same time cyclization of the carbon-centered radical with the double bond is disfavored due to steric bulk provided by the methyl groups. In the degradation of 2,6-dimethyl-curcumin formation of the peroxy radical is a key step toward subsequent carbon bond cleavage to vanillin, through either the direct (dioxetane) or indirect (epoxide) pathway. With curcumin, there is no evidence for formation of a peroxy radical at C-2 (C-6) immediately following the initial hydrogen abstraction and prior to 5-*exo* cyclization²⁰. This may explain why vanillin is not an abundant product of the degradation of curcumin.

Conclusions

The experiments with peptide adduction and metabolism in RAW264.7 cells suggested a key role of oxidative degradation products in mediating the anti-inflammatory activity of 2,6-dimethyl-curcumin. The cell incubations showed that 2,6-dimethyl-curcumin undergoes reductive as well as oxidative metabolic transformation. Based on LC-MS ion intensities (a non-quantitative measure of abundance) vanillin was the most prominent product, about 10-fold higher than **5** and reduced tetrahydro-2,6-dimethyl-curcumin, respectively.

A recent analysis of the anti-inflammatory activity of curcumin suggested that inhibition of NF- κ B may be mediated by the degradation products in addition to or possibly even instead of the parent compound²⁴. The same may be the case for 2,6-dimethyl-curcumin. This was supported by the peptide binding assay that showed more prominent adduction with degradation products, especially methoxyphenol, than with 2,6-dimethyl-curcumin (Fig. 7). Even for 2,6-dimethyl-curcumin it was not apparent whether adduction occurred via the enone of the parent molecule or the quinone methide oxidation product (Fig. 7).

The anti-inflammatory activity of curcumin involves covalent adduction to Cys179 of IKK β ^{24, 41}. Adduction of small molecule electrophiles to Cys179 prevents phosphorylation of neighboring serines 177 and 181 and inhibits kinase activity that is required for the phosphorylation of I κ B α and activation of NF- κ B⁴². For curcumin, there is evidence to support the hypothesis that adduction to Cys179 occurs by oxidative metabolites as well as parent curcumin⁴³. This implies that the enones in curcumin and 2,6-dimethyl-curcumin are not the (only) functionally relevant electrophilic moiety. The methyl groups at the 2- and 6-positions of 2,6-dimethyl-curcumin likely further decrease the ability to form a Michael-type adduct due to increased steric bulk and decreased electrophilic character at C-1 (C-7) by the electron-donating effect of the methyl group at the adjacent carbon. Thus, it is unlikely that 2,6-dimethyl-curcumin is more potent than curcumin in inhibiting NF- κ B if the 1,2-enone were the electrophile reacting with Cys179 of IKK β . This supports the hypothesis in which

not the parent molecule but its degradation products are the electrophilic agents and thus the ultimate anti-inflammatory agents.

Experimental

Materials

Curcumin and 2,6-dimethyl-curcumin were synthesized according to published procedures^{16, 44}. The IKK β peptide (E¹⁷²LDQGSLSFVGTLLQYLAPELLEQQK¹⁹⁸; M_r 3012.43 g/mol, obtained by in silicotryptic digest of human IKK β) was synthesized by United Biosystems Inc. (Herndon, VA). RAW264.7 cells were from ATCC. For quantification of NF- κ B activity RAW264.7 cell stably transfected with a *pNFkB-TA-MetLuc* with TB vector (Clontech) were used²⁴.

Degradation reactions and HPLC analysis

For the UV/Vis assay, 2,6-dimethyl-curcumin (25 μ M final concentration) was dissolved in 20 mM NH₄OAc buffer (pH 7.5) in a 500 μ L-cuvette. The sample was analyzed in time-drive mode following the chromophore at 410 nm and by repetitive scanning from 700 – 220 nm every 1 min using a Perkin-Elmer Lambda 35 spectrophotometer. The time-course analysis was performed using 2,6-dimethyl-curcumin (25 μ M) dissolved in 20 mM NH₄OAc buffer (500 μ L) in an autosampler vial. The autosampler was maintained at 25°C, and aliquots were injected every 1 h over a period of 10 h.

HPLC analysis of product formation was performed using an Agilent Eclipse XDB-C183.5 μ m column (3.0 $\times$ 100 mm) eluted a flow rate of 0.6 mL/min with a gradient of acetonitrile/water, 20:80 (by volume) to 80:20 (by volume), containing 0.01% acetic acid, within 20 min. Elution of products was monitored using an Agilent 1200 diode array detector.

Large-scale reactions were conducted by dissolving 2,6-dimethyl-curcumin (25 μ M) in 2 L of 20 mM NH₄OAc buffer (pH 8). The reaction was left at 25 °C for 16 h and extracted with CH₂Cl₂ (4 $\times$ 100 mL). The organic extracts were combined and dried with anhydrous MgSO₄ (s). The solvent was evaporated under a stream of N₂. The crude products were dissolved in a mixture of methanol/water (50:50) and isolated by RP-HPLC using a Waters Symmetry C18, 5 μ m column (4.6 $\times$ 250 mm) with a gradient of 20:80 (by volume) to 80:20 (by volume) containing 0.01% acetic acid, within 20 min. The flow rate was 1 mL/min. The products were monitored using an Agilent 1200 diode array detector. Products were recovered from the solvent by lyophilization.

NF- κ B inhibition

RAW264.7 cells stably expressing luciferase under the control of an NF- κ B response element were used²⁴. Cells were grown at 37°C in 5% CO₂ in DMEM containing glucose (4.5 g/L) and fetal bovine serum (10%, v/v) in the presence of geneticin (1 mg/mL). Cells were treated with 2,6-dimethyl-curcumin at the concentrations indicated from a stock solution in DMSO (10 mM) for 45 min prior to stimulation with LPS (100 ng/mL). The medium was removed after 4 h, and 50 μ L were analyzed using a Ready-to-glow Secreted Luciferase Reporter assay (Clontech). Statistical analysis was performed using GraphPad

Prism 7.00 software using an unpaired non-parametric t-test (Mann-Whitney) with significance of $p < 0.05$.

COX-2 expression

RAW264.7 cells were treated with 2,6-dimethyl-curcumin or curcumin (1-50 μM) for 1 h prior to treatment with LPS (100 ng/mL). Cells were harvested after 5 h, and protein (5 μg each) was resolved on a 10% SDS-PAGE gel. Gels were blotted onto nitrocellulose membranes and probed with antibodies for COX-2 (mouse polyclonal, catalog-# 160126, Cayman Chemical) and β -actin (mouse monoclonal, 8H10D10, catalog-# 3700, Cell Signaling Technology) as loading control.

Peptide adduction and analysis

IKK β peptide (10 μM) was incubated with 2,6-dimethylcurcumin (50 μM) at 37°C in 20 mM NH_4OAc buffer, pH 8, for 1 h. Aliquots were analyzed directly using a Waters Synapt HDMS Q-TOF instrument in positive ionization mode following separation of the sample using a Waters XSelect C18 3.5 μm column (2.1 $\times$ 50 mm) with a gradient of acetonitrile/water, 15:85 (by volume) to 65:35 (by volume), containing 0.1% formic acid, within 10 min at a flow rate of 0.25 mL/min.

Metabolism in RAW264.7 cells

RAW264.7 cells were seeded in 6-well plates using DMEM containing 10% fetal bovine serum. After 24 h 2,6-dimethyl-curcumin (10 μM ; from a 10 mM stock solution in DMSO) was added to the cells and incubated for 30 min. Media (800 μL) were removed, acidified (pH 4-5), and extracted using 30-mg Waters HLB cartridges. The cartridges were eluted with ethylacetate (350 μL) and methanol (700 μL), and the combined eluates were evaporated under a stream of nitrogen. The residue was dissolved in 700 μL acetonitrile and 100 μL water, and 10 μL were analyzed by LC-SRM-MS.

LC-MS

A TSQ Vantage Triple Quadrupole LC-MS instrument with electrospray ionization was used. Chromatographic separation was achieved using a Waters Symmetry Shield C18 column (2.1 $\times$ 50 mm, 1.7 μm) eluted at 0.4 mL/min flow rate with a gradient of water (solvent A) and acetonitrile (solvent B) (both containing 0.1% formic acid, v/v). A linear gradient was programmed from 85% A to 15% A in 3 min, and then to 5% in 1 min followed by isocratic elution for 1 min before returning to starting conditions. The SRM ion transitions (collision energy) for the products were as follows: for 2,6-dimethyl-curcumin: m/z 397.2 $\rightarrow$ 191.1 (15 eV); for vanillin **1**: m/z 153.1 $\rightarrow$ 92.9 (10 eV); for **2**, **3**: m/z 413.2 $\rightarrow$ 137.1 (15 eV); for **4**: m/z 261.1 ($-\text{H}_2\text{O}$) $\rightarrow$ 137.1 (20 eV); for **5**: m/z 383.2 ($-\text{H}_2\text{O}$) $\rightarrow$ 259.1 (15 eV); for tetrahydro-2,6-dimethyl-curcumin: m/z 401.5 $\rightarrow$ 137.0 (15 eV).

NMR

Samples were dissolved in 150 μL of methanol- d_4 or D_2O in 3-mm sample tubes. NMR spectra were recorded using a Bruker DRX 600-MHz spectrometer equipped with a cryoprobe at 295 K. Chemical shifts are reported in ppm calibrated by the residual non-

deuterated solvent signals (δ 3.31 for methanol- d_4 and 4.79 ppm for D_2O). Pulse frequencies for the H,H-COSY, heteronuclear single quantum coherence (HSQC), and heteronuclear multiple bond correlation (HMBC) experiments were taken from the Bruker library. Carbon chemical shifts were determined from HSQC, HMBC, and, for product **5**, from ^{13}C NMR experiments.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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References

1. Esatbeyoglu T, Huebbe P, Ernst IM, Chin D, Wagner AE, Rimbach G. *Angew Chem Int Ed Engl*. 2012; 51:5308–5332. [PubMed: 22566109]
2. Heger M, van Golen RF, Broekgaarden M, Michel MC. *Pharmacol Rev*. 2014; 66:222–307. [PubMed: 24368738]
3. Funk JL, Oyarzo JN, Frye JB, Chen G, Lantz RC, Jolad SD, Solyom AM, Timmermann BN. *J Nat Prod*. 2006; 69:351–355. [PubMed: 16562833]
4. Killian PH, Kronski E, Michalik KM, Barbieri O, Astigiano S, Sommerhoff CP, Pfeffer U, Nerlich AG, Bachmeier BE. *Carcinogenesis*. 2012; 33:2507–2519. [PubMed: 23042094]
5. Yu J, Peng Y, Wu LC, Xie Z, Deng Y, Hughes T, He S, Mo X, Chiu M, Wang QE, He X, Liu S, Grever MR, Chan KK, Liu Z. *PloS one*. 2013; 8:e55934. [PubMed: 23457487]
6. Epstein J, Sanderson IR, Macdonald TT. *Br J Nutr*. 2010; 103:1545–1557. [PubMed: 20100380]
7. Chandran B, Goel A. *Phytotherapy research : PTR*. 2012; 26:1719–1725. [PubMed: 22407780]
8. Small GW, Siddarth P, Li Z, Miller KJ, Ercoli L, Emerson ND, Martinez J, Wong KP, Liu J, Merrill DA, Chen ST, Henning SM, Satyamurthy N, Huang SC, Heber D, Barrio JR. *Am J Geriatr Psychiatry*. 2017; doi: 10.1016/j.jagp.2017.10.010
9. Daily JW, Yang MN, Park S. *J Med Food*. 2016; 19:717–729. [PubMed: 27533649]
10. Liu X, Machado GC, Eyles JP, Ravi V, Hunter DJ. *Br J Sports Med*. 2018; 52:167–175. [PubMed: 29018060]
11. Lang A, Salomon N, Wu JC, Kopylov U, Lahat A, Har-Noy O, Ching JY, Cheong PK, Avidan B, Gamus D, Kaimakliotis I, Eliakim R, Ng SC, Ben-Horin S. *Clin Gastroenterol Hepatol*. 2015; 13:1444–1449 e1441. [PubMed: 25724700]
12. Holder GM, Plummer JL, Ryan AJ. *Xenobiotica; the fate of foreign compounds in biological systems*. 1978; 8:761–768. [PubMed: 726520]
13. Metzler M, Pfeiffer E, Schulz SI, Dempe JS. *BioFactors*. 2013; 39:14–20. [PubMed: 22996406]
14. Pan MH, Huang TM, Lin JK. *Drug Metab Dispos*. 1999; 27:486–494. [PubMed: 10101144]
15. Ireson C, Orr S, Jones DJ, Verschoyle R, Lim CK, Luo JL, Howells L, Plummer S, Jukes R, Williams M, Steward WP, Gescher A. *Cancer Res*. 2001; 61:1058–1064. [PubMed: 11221833]
16. Koo HJ, Shin S, Choi JY, Lee KH, Kim BT, Choe YS. *Sci Rep*. 2015; 5:14205. [PubMed: 26391485]
17. Wang YJ, Pan MH, Cheng AL, Lin LI, Ho YS, Hsieh CY, Lin JK. *J Pharm Biomed Anal*. 1997; 15:1867–1876. [PubMed: 9278892]

18. Griesser M, Pistis V, Suzuki T, Tejera N, Pratt DA, Schneider C. *J Biol Chem*. 2011; 286:1114–1124. [PubMed: 21071447]
19. Schneider C, Gordon ON, Edwards RL, Luis PB. *J Agric Food Chem*. 2015; 63:7606–7614. [PubMed: 25817068]
20. Gordon ON, Luis PB, Sintim HO, Schneider C. *J Biol Chem*. 2015; 290:4817–4828. [PubMed: 25564617]
21. Luis PB, Gordon ON, Nakashima F, Joseph AI, Shibata T, Uchida K, Schneider C. *Biochem Pharmacol*. 2017; 132:143–149. [PubMed: 28274615]
22. Ketron AC, Gordon ON, Schneider C, Osheroff N. *Biochemistry*. 2013; 52:221–227. [PubMed: 23253398]
23. Gordon ON, Luis PB, Ashley RE, Osheroff N, Schneider C. *Chem Res Toxicol*. 2015; 28:989–996. [PubMed: 25806475]
24. Edwards RL, Luis PB, Varuzza PV, Joseph AI, Presley SH, Chaturvedi R, Schneider C. *J Biol Chem*. 2017; 292:21243–21252. [PubMed: 29097552]
25. Reynaert NL, van der Vliet A, Guala AS, McGovern T, Hristova M, Pantano C, Heintz NH, Heim J, Ho YS, Matthews DE, Wouters EF, Janssen-Heininger YM. *Proc Natl Acad Sci USA*. 2006; 103:13086–13091. [PubMed: 16916935]
26. Straus DS, Pascual G, Li M, Welch JS, Ricote M, Hsiang CH, Sengchanthalangsy LL, Ghosh G, Glass CK. *Proc Natl Acad Sci USA*. 2000; 97:4844–4849. [PubMed: 10781090]
27. Ji C, Kozak KR, Marnett LJ. *J Biol Chem*. 2001; 276:18223–18228. [PubMed: 11359792]
28. Litwinienko G, Ingold KU. *J Org Chem*. 2004; 69:5888–5896. [PubMed: 15373474]
29. Foti MC, Slavova-Kazakova A, Rocco C, Kancheva VD. *Org Biomol Chem*. 2016; 14:8331–8337. [PubMed: 27530442]
30. Miyashita K, Fujimoto K, Kaneda T. *Agr Biol Chem Tokyo*. 1982; 46:751–755.
31. Porter NA, Zuraw PJ, Sullivan JA. *Tetrahed Lett*. 1984; 25:807–810.
32. Miyashita K, Fujimoto K, Kaneda T. *Agr Biol Chem Tokyo*. 1984; 48:2511–2515.
33. Schneider C, Boeglin WE, Brash AR. *J Biol Chem*. 2004; 279:4404–4414. [PubMed: 14594816]
34. Schneider C, Boeglin WE, Yin H, Porter NA, Brash AR. *Chem Res Toxicol*. 2008; 21:895–903. [PubMed: 18324788]
35. Schneider C, Porter NA, Brash AR. *J Biol Chem*. 2008; 283:15539–15543. [PubMed: 18285327]
36. Tedder JM, Walton JC. *Acc Chem Res*. 1976; 9:183–191.
37. Lowry, TH., Richardson, KS. *Mechanism and theory in organic chemistry*. Harper Collins; New York: 1987.
38. Gordon ON, Schneider C. *Trends Mol Med*. 2012; 18:361–363. author reply 363–364. [PubMed: 22652257]
39. Esparan V, Krings U, Struch M, Berger RG. *Molecules*. 2015; 20:6640–6653. [PubMed: 25875042]
40. Masuda T, Hidaka K, Shinohara A, Maekawa T, Takeda Y, Yamaguchi H. *J Agric Food Chem*. 1999; 47:71–77. [PubMed: 10563852]
41. Jobin C, Bradham CA, Russo MP, Juma B, Narula AS, Brenner DA, Sartor RB. *J Immunol*. 1999; 163:3474–3483. [PubMed: 10477620]
42. Delhase M, Hayakawa M, Chen Y, Karin M. *Science*. 1999; 284:309–313. [PubMed: 10195894]
43. Luis PB, Boeglin WE, Schneider C. *Chem Res Toxicol*. 2018; in revision. doi: 10.1021/acs.chemrestox.1027b00326
44. Pabon HJJ. *Recl Trav Chim Pays Bas*. 1964; 83:379–386.

Figure 1. Chemical structures of curcumin, 2,6-dimethyl-curcumin, and the curcumin metabolites tetrahydrocurcumin and bicyclopentadione.

Fig. 2.

Degradation of 2,6-dimethyl-curcumin. (a) Stability of 2,6-dimethyl-curcumin at pH 7.5. 2,6-Dimethyl-curcumin (25 μ M) was diluted in 20 mM phosphate buffer pH 7.5, and the sample was analyzed by repetitive scanning every 1 min or (b) in the time drive mode at 410 nm in a UV/Vis spectrophotometer. (c) Incubation reactions of 2,6-dimethyl-curcumin in 20 mM phosphate buffer pH 8 were extracted after 1 h or (d) 5 h and analyzed using RP-HPLC with diode array detection. The chromatograms were recorded at UV 205 nm. (e) Isolated and identified products of degradation of 2,6-dimethyl-curcumin.

Fig. 3.

Time course of the degradation of 2,6-dimethyl-curcumin. (a) The disappearance of 2,6-dimethyl-curcumin is plotted as % remaining of the starting amount on the right axis. Formation of products in % of total products is plotted on the left axis. 2,6-Dimethyl-curcumin (25 μ M) was dissolved in 20 mM phosphate buffer pH 8 in an HPLC autosampler vial. Aliquots (10 μ l) were injected every 1 h for 10 h. The mean $\pm$ SD of three independent replicates is shown. (b) RP-HPLC analysis of the incubation of isolated epoxide **2** in buffer pH 7.5 for 1 h. (c) RP-HPLC analysis of the incubation of isolated epoxide **3**. The chromatograms were recorded at UV 205 nm.

Fig. 4. Anti-inflammatory activity of 2,6-dimethyl-curcumin. (a) RAW264.7 cells stably expressing luciferase downstream of an NF- κ B response element were treated with vehicle or with 2,6-dimethyl-curcumin (1-50 μ M) 1 h prior to LPS (100 ng/mL). Cells were harvested 4 h after LPS addition, and luciferase activity was measured in the cell supernatant. (b) Western blot detection of COX-2 in RAW264.7 cells treated with 2,6-dimethyl-curcumin or curcumin 1 h prior to stimulation with LPS (100 ng/mL). The cells were harvested after 5 h, and protein was resolved by SDS-PAGE. The image shown is representative of three replicates with identical results.

Fig. 5.

Peptide adduction by 2,6-dimethyl-curcumin and its degradation products. A 27-amino acid peptide containing redox-regulated Cys179 of Inhibitor of nuclear factor κ -B kinase subunit β (IKK β) (10 μ M) was reacted with 2,6-dimethyl-curcumin (50 μ M) at 37°C for 1 h. An aliquot of the reaction was analyzed using LC-MS. (a) The first panel shows the total ion chromatogram (TIC) and the following panels show extracted ion chromatograms for peptide adducts with 2,6-dimethyl-curcumin (+396; m/z 1703), epoxides **2** and **3** (+412; m/z 1711), methoxyphenol (+122; m/z 1567), 1-hydroxy-ethyl-methoxyphenol (+166; m/z 1589), and for the unreacted peptide and its dimer, respectively, (m/z 1506). (b) Total ion chromatogram for analysis of the IKK β peptide before incubation with 2,6-dimethyl-curcumin and of a Cys179Ala mutant IKK β peptide (10 μ M) reacted with 2,6-dimethyl-curcumin (50 μ M) for 1 h.

Fig. 6. Metabolism of 2,6-dimethyl-curcumin in RAW264.7 cells. RAW264.7 cells were incubated with 2,6-dimethyl-curcumin (10 μ M) for 30 min and extracted. LC-SRM-MS analysis was performed in the positive ion mode. The ion chromatograms for detection of vanillin, product **5**, tetrahydro-2,6-dimethyl-curcumin, and 2,6-dimethyl-curcumin are shown.

Fig. 7.
Proposed structures of 2,6-dimethyl-curcumin and its degradation products adducting to the IKK β peptide. The red arrow indicates predicted sites of reaction with Cys179 of the 27-amino acid peptide of IKK β .

Scheme 1.

Proposed mechanisms of oxidative transformation of 2,6-dimethyl-curcumin. (a) Direct pathway of formation of vanillin **1** via β -cleavage of a dioxetane radical intermediate. (b) Formation of epoxides **2** and **3** via peroxide mediated dimerization of 2,6-dimethyl-curcumin. The first step is formation of a peroxy radical ($\text{ROO}^\bullet$). In the second step the peroxy radical adds to the double bond of a second molecule of 2,6-dimethyl-curcumin (blue). Addition can occur at either C-6 or C-7³⁶ and results in the formation of the same epoxides, **2** and **3** via homolytic intramolecular substitution S_{HI} or addition of the alkoxy radical to the double bond, respectively. (c) Formation of chain-shortened alcohol **5** by phenyl 1,2-migration rearrangement of a peroxide-linked dimer of 2,6-dimethyl-curcumin.