Curcumin and Its Oxidative Degradation Products: Their Comparative Effects on Inflammation

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Curcumin and Its Oxidative Degradation Products: Their Comparative Effects on Inflammation

A Thesis Presented

By

JULIA ZHU

Submitted to the Graduate School of the University of Massachusetts Amherst in partial fulfillment of the requirements for Master of Food Science

MASTER OF SCIENCE

May 2016

Food Science
Curcumin and Its Oxidative Degradation Products: Their Comparative Effects on Inflammation

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ABSTRACT
CURCUMIN AND ITS OXIDATIVE DEGRADATION PRODUCTS: THEIR COMPARATIVE EFFECTS ON INFLAMMATION
MAY 2016

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The anti-inflammatory agent curcumin degrades rapidly, leading to speculations that curcumin’s reported effects stem from its degradation products. Curcumin can degrade via hydrolysis, and more recently it was discovered that curcumin can degrade via oxidation at physiological pH. Additionally, bicyclopentadione is the major degradation product from this oxidation reaction. Evidence from the literature suggests that curcumin degrades primarily through oxidation. However, the biology of the oxidation products is not well characterized, and there is debate on whether oxidation intermediates or curcumin itself is more biologically active. To further elucidate the biology of the oxidation products, their effects on inflammation were examined. RAW264.7 murine macrophage cells were stimulated with E. coli lipopolysaccharide (LPS) and treated with curcumin, curcumin’s total oxidative degradation products, and bicyclopentadione. Curcumin strongly decreased LPS-induced nitric oxide production and iNOS expression in a dose dependent manner; total degradation products slightly decreased nitric oxide
production and iNOS expression, while bicyclopentadione failed to decrease either. Additionally, curcumin was significantly more effective than either bicyclopentadione or total degradation products in inhibiting COX-2 expression. iNOS and COX-2 arise from the activation of the NF-κB pathway, which curcumin is known to modulate; thus, the oxidation products’ effect on key proteins in this pathway was also examined. Neither total degradation products nor bicyclopentadione inhibited translocation of NF-κB into the nucleus, prevented degradation of IκBα, nor inhibited phosphorylation of IKK as effectively as curcumin. In conclusion, curcumin is significantly more effective at inhibiting inflammation than the oxidation degradation products.
# TABLE OF CONTENTS

<table>
<thead>
<tr>
<th>Page</th>
</tr>
</thead>
<tbody>
<tr>
<td>ACKNOWLEDGEMENTS ................................................................. iii</td>
</tr>
<tr>
<td>ABSTRACT ................................................................................... iv</td>
</tr>
<tr>
<td>LIST OF FIGURES ....................................................................... viii</td>
</tr>
<tr>
<td>CHAPTER</td>
</tr>
<tr>
<td>1. LITERATURE REVIEW .................................................................. 1</td>
</tr>
<tr>
<td>1.1 Introduction ........................................................................... 1</td>
</tr>
<tr>
<td>1.2 Chemistry of alkaline hydrolysis of curcumin ....................... 4</td>
</tr>
<tr>
<td>1.3 Biology of hydrolysis products ............................................. 5</td>
</tr>
<tr>
<td>1.3.1 Ferulic acid ........................................................................ 5</td>
</tr>
<tr>
<td>1.3.2 Vanillin .............................................................................. 7</td>
</tr>
<tr>
<td>1.4 Chemistry of autoxidation products of curcumin .................. 9</td>
</tr>
<tr>
<td>1.5 Biology of autoxidation products of curcumin ..................... 12</td>
</tr>
<tr>
<td>1.6 Conclusion ............................................................................ 13</td>
</tr>
<tr>
<td>2. CURCUMIN, BUT NOT ITS OXIDATIVE DEGRADATION PRODUCTS, ATTENUATES INFLAMMATORY RESPONSES AND NF-κB SIGNALING ................................................................. 15</td>
</tr>
<tr>
<td>2.1 Introduction ........................................................................... 15</td>
</tr>
<tr>
<td>2.2 Materials and Methods ......................................................... 16</td>
</tr>
<tr>
<td>2.2.1 Curcumin degradation products and cell culture ............... 16</td>
</tr>
<tr>
<td>2.2.2 Nitric oxide measurement and cell viability ....................... 16</td>
</tr>
<tr>
<td>2.2.3 Sample preparation for iNOS and COX-2 Western blots ....... 17</td>
</tr>
<tr>
<td>2.2.4 Sample preparation for IκBα and p-IKK Western blots ........ 17</td>
</tr>
<tr>
<td>2.2.5 Sample preparation for NF-κB translocation ..................... 17</td>
</tr>
<tr>
<td>2.2.6 Western Blotting ................................................................. 18</td>
</tr>
<tr>
<td>2.2.7 Statistical Analysis ............................................................. 18</td>
</tr>
<tr>
<td>2.3 Results ................................................................................ 19</td>
</tr>
</tbody>
</table>
2.3.1 Curcumin, not its degradation products, attenuates LPS-induced nitric oxide production.......................................................... 19
2.3.2 Curcumin, but not its degradation products, inhibits LPS-induced iNOS expression ............................................................ 19
2.3.3 Curcumin, but not its degradation products, inhibits LPS-induced COX-2 expression............................................................. 20
2.3.4 Curcumin, but not its degradation products, inhibits LPS-induced p65 translocation.............................................................. 21
2.3.5 Curcumin, but not its degradation products, inhibits LPS-induced IκBα degradation and IKK phosphorylation.......................... 22

2.4 Discussion.................................................................................................................. 24

APPENDICES

A. EFFECT OF TRICLOBARBAN ON ANIMAL BODY WEIGHT .......... 27
B. COMPARATIVE STABILITY OF CURCUMIN, DIDEHYXYCURCUMIN, AND DIMETHOXYCURCUMIN, AND THEIR EFFECTS ON NITRIC OXIDE PRODUCTION ................. 29
C. CURCUMIN STABILIZED BY ANTIOXIDANTS CAN ATTENUATE LPS-INDUCED NITRIC OXIDE PRODUCTION AND iNOS EXPRESSION ........................................................................ 33

REFERENCES .................................................................................................................. 35
## LIST OF FIGURES

<table>
<thead>
<tr>
<th>Figure</th>
<th>Pages</th>
</tr>
</thead>
<tbody>
<tr>
<td>1: The hydrolytic degradation pathway of curcumin. In the presence of (OH(_{-})), curcumin can be hydrolyzed into several different products, including ferulic acid, feruloyl methane, and vanillin.</td>
<td>4</td>
</tr>
<tr>
<td>2: The effects of curcumin, TDP, and BCPD on LPS-induced nitric oxide production.</td>
<td>20</td>
</tr>
<tr>
<td>3: The effects of curcumin, TDP, and BCPD on iNOS expression.</td>
<td>21</td>
</tr>
<tr>
<td>4: The effects of curcumin, TDP, and BCPD on COX-2 production.</td>
<td>22</td>
</tr>
<tr>
<td>5: The effects of curcumin, TDP, and BCPD on NF-κB (p65) nuclear translocation.</td>
<td>23</td>
</tr>
<tr>
<td>6: The effects of curcumin, TDP, and BCPD on IκBα degradation.</td>
<td>24</td>
</tr>
<tr>
<td>7: The effects of curcumin, TDP, and BCPD on IKK phosphorylation.</td>
<td>25</td>
</tr>
<tr>
<td>8: Weights of mice fed with 10% corn oil alone (Control) or with 10% corn oil supplement with 0.08% triclocarban (TCC). Student t-test was performed between the groups for each pair of data point (p&lt;0.05).</td>
<td>28</td>
</tr>
<tr>
<td>9: Nitric oxide production dose response at 24 hours and 48 hours for all treatments.</td>
<td>30</td>
</tr>
<tr>
<td>10: HPLC analysis of didemethylcurcumin, curcumin, and dimethoxycurcumin.</td>
<td>31</td>
</tr>
<tr>
<td>11: Nitric oxide production of LPS-induced RAW 264.7 cells treated with either curcumin and/or Trolox, sodium ascorbate (SA), caffeic acid (CA), or gallic acid (GA).</td>
<td>34</td>
</tr>
<tr>
<td>12: iNOS expression of LPS-induced RAW 264.7 cells treated with curcumin and/or Trolox, sodium ascorbate (SA), caffeic acid (CA), or gallic acid (GA).</td>
<td>34</td>
</tr>
</tbody>
</table>
CHAPTER 1

LITERATURE REVIEW

1.1 Introduction

Substantial studies have shown that curcumin, a bis-α,β-unsaturated β-diketone compound found in turmeric, has potent anti-cancer and anti-metastatic actions. Indeed, many animal studies have shown that curcumin suppressed primary tumor growth of many types of cancers, including but not limited to pancreatic cancer, breast cancer, cervical cancer, lung cancer, and colon cancer. Tumor metastasis, the process by which tumor cells spread from the primary tumor site to other organs, causes approximate 90% of human cancer deaths. Animal experiments have also shown that curcumin inhibited tumor metastasis of prostate cancer, breast cancer, colorectal cancer, and pancreatic cancer. Human studies also support the cancer risk reducing effects of curcumin. In a Phase II clinical trial of colorectal cancer, daily intake of 4 grams of curcumin for a month caused a ~40% reduction of aberrant crypt foci. In another Phase II human clinical trial, daily intake of 8 grams of curcumin demonstrated anti-cancer efficacy in some patients with advanced pancreatic cancer. Together, these results make curcumin a promising dietary compound for cancer prevention and/or treatment.

Curcumin inhibits tumor progression and metastasis through multiple possible mechanisms. Previous studies demonstrated that curcumin inhibited cancer cell proliferation by inducing cell cycle arrest and apoptosis and inhibiting cancer cell migration and invasion, which are critical cellular processes involved in tumor progression and metastasis. Curcumin has also been shown to inhibit inflammation by suppressing NF-kB signaling and modulating the expressions of inflammatory cytokines.
and microRNAs in cancer cells, leading to reduced cancer progression and metastasis.

Besides directly targeting cancer cells, curcumin can also target tumor-associated endothelial cells, resulting in reduced tumor angiogenesis and associated tumor progression and metastasis. In addition, a recent study showed that curcumin potently inhibited lymphangiogenesis (de novo lymphatic formation), which is a critical process in mediating tumor metastasis. In summary, curcumin can inhibit tumor growth and metastasis through targeting several critical cellular processes involved in tumorigenesis, including cancer cell proliferation and motility, tumor inflammation, angiogenesis and lymphangiogenesis.

Although the cancer risk-reducing effects of curcumin have been well characterized, the molecular mechanisms by which curcumin inhibits cancer are not well understood. Curcumin has poor metabolic and chemical stability, making it difficult to determine whether the observed anti-cancer effects are mediated by curcumin itself or its metabolites. Indeed, many studies have shown that curcumin has a poor pharmacokinetics profile: one study showed that after a single oral dose of 10 or 12 grams of curcumin, free form of curcumin was barely detected in human plasma. The low levels of curcumin in circulation make it difficult to understand how curcumin exerts systematic anti-cancer effects, particularly in its inhibitory effect on non-gastrointestinal tract cancer types. This leads to hypothesis that the biological effects of curcumin are mediated by its metabolites. However, many studies have shown that most of the enzymatic metabolites of curcumin are either biologically less active or inactive compared to curcumin. For example, curcumin sulfate and curcumin glucuronide, which are the major metabolites of curcumin after oral consumption, have been shown to have

2
dramatically reduced biological activities. These results suggest that the biological activities of curcumin are unlikely to be mediated by these enzymatic metabolites.

Besides its poor metabolic stability, curcumin also has poor chemical stability: it rapidly degraded in aqueous solution at physiological pH with a half-life of several minutes, leading to rapid formation of various degradation products. These chemical degradation products include two classes of compounds: (1) alkaline hydrolysis products, where the hydroxyl ion (OH⁻) attacks the carbonyl group of curcumin, generating hydrolyzed products such as ferulic acid, vanillin, ferulaldehyde, and feruloyl methane; (2) autoxidation products, where curcumin is first converted to a phenolic radical, which then migrates to the conjugated heptadienedione chain and initiates a chain reaction of curcumin degradation to generate cyclized compounds such as bicyclpentadione. Recently, several studies have suggested that the biological activities of curcumin could be mediated by its degradation products. In this chapter, the chemistry and biology of the curcumin degradation products will be discussed.
1.2 Chemistry of alkaline hydrolysis of curcumin

Wang et al. discovered that curcumin rapidly degraded in aqueous solution (e.g. phosphate, citrate-phosphate, carbonate buffers and serum-free cell culture medium) in a pH-dependent manner: in acidic environments (pH = 3-6.5), the half-life of curcumin in aqueous buffer was around 100-200 min, while at neutral and alkaline environments (pH >7), the half-life of curcumin was around 10 min. HPLC analysis showed that the degradation products included vanillin, ferulic acid, ferulic aldehyde, feruloyl methane, and trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal, which are breakdown products of curcumin. Together, these results suggest that these degradation products are formed through a hydroxyl ion (OH\(^{-}\))-dependent mechanism (see Fig. 1) \(^{32}\).

Of these degradation products, ferulic acid and feruloyl methane are produced from the alkaline hydrolysis of the diketone group \(^{32}\), and feruloyl methane can be hydrolyzed further to generate vanillin \(^{31}\).

*Figure 1:* The hydrolytic degradation pathway of curcumin. In the presence of (OH\(^{-}\)), curcumin can be hydrolyzed into several different products, including ferulic acid, feruloyl methane, and vanillin.

Recent research suggests that the alkaline hydrolysis pathway has a limited contribution to curcumin degradation in aqueous buffers. First, the hydrolysis-derived products are minor compounds in curcumin degradation products. Wang et al found that vanillin, ferulic acid, and feruloyl methane were minor degradation products compared to trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexenal at 280 nm \(^{29}\). Second, the
comparative stabilities of curcumin and its analogues (such as dimethoxycurcumin, demethoxycurcumin, and didemethoxycurcumin) further support that the hydrolysis mechanism is not a major pathway in mediating curcumin degradation. Since the curcumin analogues have the same conjugated heptadienedione chain as curcumin; it follows that if alkaline hydrolysis mechanism is the primary mediator of curcumin degradation, these compounds should have similar stability. However, the analogues are much more stable than curcumin \(^{33,34}\). For example, after 1 hour incubation in phosphate buffer, HPLC analysis showed that curcumin rapidly degraded, while dimethoxycurcumin was very stable \(^{33}\), suggesting that the alkaline hydrolysis mechanism has a limited contribution in mediating curcumin degradation.

1.3 Biology of hydrolysis products

Of the aforementioned hydrolysis products, the biological effects of vanillin and ferulic acid have been well studied. Both compounds have been shown to have anti-cancer effects \textit{in vitro} and \textit{in vivo}. Some of the other hydrolysis degradation products have also been studied, though not to the extent of vanillin and ferulic acid. For example, ferulaldehyde has been shown to inhibit inflammatory responses in mice \(^{35}\). In the section below, we will discuss the anti-cancer effects and mechanisms of vanillin and ferulic acid.

1.3.1 Ferulic acid

Ferulic acid has been shown to inhibit cancer and inflammation in cell culture models. After 24 h treatment, 500-1000 µM ferulic acid suppressed 20-25% of cell proliferation in lung, colon and oral cancer cells \(^{36,37}\); after 48 hour treatment, curcumin at a lower dose (~30 µM) inhibited approximately 50% and 25% of cellular proliferation in HepG2 hepatoma cells \(^{38}\) and PC-3 prostate cancer cells, respectively \(^{39}\). Ferulic acid has also
been shown to inhibit cell cycle progression and induce apoptosis in cancer cells. At a dose of 300-1000 µM, ferulic acid can decrease expression of genes involved in G1 cell cycle arrest in PC-3 cells\(^{39}\) and cause G2 arrest in malignant oral cancer cells\(^{37}\). It has also been shown to inhibit cellular migration and invasion in cancer and endothelial cells, which are critical processes involved in tumor metastasis. A wound healing assay in A549 lung cancer cells showed that ferulic acid at 200 µM inhibited ~65% of cell migration\(^{36}\). At a dose range of 2.5-10 µM, ferulic acid inhibited fibroblast growth factor-1 (FGF1)-induced migration and invasion, as well as expressions of matrix metalloproteinase-2 (MMP-2) and MMP-9, in human umbilical vein endothelial cells (HUVEC)\(^{40}\). Ferulic acid has also been shown to suppress inflammation: In RAW 264.7 macrophage cells, ferulic acid inhibited IKK-α/β phosphorylation and subsequent nuclear translocation of NF-kB, suggesting that ferulic acid inhibited inflammation by suppressing NF-kB pathway\(^{41}\).

Animal experiments have also shown the anti-cancer effects of ferulic acid. In an azoxymethane (AOM)-induced colon cancer model in F334 rats, continuous oral administration of 500 mg/kg ferulic acid reduced aberrant crypt foci formation per colon by 30.5%\(^{42}\). In a 7,12-dimethylbenz[a]anthracene (DMBA)-induced breast cancer model in Sprague-Dawley rats, oral administration of 40 mg/kg ferulic acid for 1 week before DMBA induction and throughout the 16 week experimental period reduced mammary cancer incidence by 80%\(^{43}\). In a DMBA-induced skin tumor model in Swiss albino mice, oral administration of 40 mg/kg ferulic acid abolished tumor progression\(^{44}\). Intraperitoneal injection of 50 mg/kg ferulic acid 1 hour prior to exposure to UV light
decreased tumor number and volume by 66% and 80% respectively in Swiss albino mice.

However, the biological activities of ferulic acid are less potent compared to curcumin. For example, curcumin has been shown to be >160-fold more potent in inhibiting cellular proliferation of human peripheral blood mononuclear cells. In addition, ferulic acid at a dose range of 100-1000 µM did not have any effect on cellular proliferation of MDA-MB-231 breast cancer cells, while curcumin at a dose as low as 30 µM inhibited ~50% of MDA-MB-231 proliferation. In a 12-O-tetradecanoylphorbol-13-acetate (TPA)-induced skin cancer model in CD-1 mice, topical application of 10 µM suppressed the number of skin tumors by 98%, whereas at the same dose ferulic acid only caused a 35% inhibition. In a mouse edema inflammation model, topical application of 1 µM curcumin almost abolished TPA-induced inflammation, while ferulic acid at the same dose only caused a 16% inhibition. Many other studies have also shown that ferulic acid was dramatically less active compared with curcumin.

1.3.2 Vanillin

Similar to ferulic acid, vanillin has also been shown to have anti-cancer and anti-inflammatory effects. However, the doses of vanillin used in these studies were far higher than the amount that would be found as a curcumin degradation product. For example, HepG2 remained viable at concentrations as high as 10 mM. In other words, as a degradation product, vanillin would not be in a biologically relevant concentration that could induce anti-cancer or anti-inflammatory effects. Despite this setback, vanillin nevertheless has been found to have some biological effects, provided that high doses were used.
Vanillin has been shown to modulate cell cycle, and apoptosis in several different cell lines, albeit at high doses. For example, the compound has been shown to modulate cell cycle by inducing G1 and G2 arrest in HT-29 cells\textsuperscript{54}, downregulating genes associated with cell cycle such as cyclin A2 in HepG2 cells\textsuperscript{53}, and by increasing expression of p21, a protein that inhibits cyclin B and leads to G2 arrest\textsuperscript{55}. Vanillin also induced apoptosis in HT-29 cells\textsuperscript{54} and further sensitized HeLa cervical cancer cells to tumor necrosis factor-related apoptosis-inducing ligand (TRAIL), a cytokine that is capable of inducing apoptosis in cancer cell lines\textsuperscript{56}. In the same cell line, vanillin enhanced cleavage of caspase-3 and PARP, both of which are apoptotic proteins that are activated by cleavage\textsuperscript{56}.

Vanillin is also capable of attenuating cancer metastasis by modulating angiogenesis, cell invasion and migration \textit{in vitro}. In A549 lung cancer, 4T1 mammary adenocarcinoma, and HepG2 hepatocarcinoma cells, vanillin was found to decrease cell invasion and migration at a dose range of 0.5-1 mM without affecting cell viability\textsuperscript{57-59}; vanillin also suppressed angiogenic activity in A549 cells\textsuperscript{57}. Vanillin inhibited metastasis through suppressing MMP-9 and phosphoinositide 3-kinase (PI3K)/Akt pathway, which play critical roles in cell migration\textsuperscript{59}.

In addition to its anti-cancer effects, vanillin has also been shown to have anti-inflammatory effects. In a dose range of 100-500 µM, vanillin inhibited COX-2 mRNA expression in RAW 264.7 mouse macrophage cells stimulated with lipopolysaccharide (LPS)\textsuperscript{60}. Like ferulic acid, vanillin suppressed inflammation through the NF-κB pathway. In a dose range of 0.25-5 mM, vanillin was shown to inhibit phosphorylation of IκBα in
HepG2 cells, phosphorylation of p65 in HeLa cells, and NF-κB binding to the consensus sequence in RAW 264.7 cells.

Despite vanillin’s potential as an anti-cancer and anti-inflammatory agent, even when compared to ferulic acid, the concentration of vanillin required in the studies were often in the millimolar range, while the dosage used for ferulic acid was generally lower; since ferulic acid has been shown to be biologically less effective than curcumin, it is unlikely that vanillin contributes to the biological effects of curcumin. Indeed, the IC\textsubscript{50} for vanillin for suppressing peripheral-blood mononuclear cell (PBMC) proliferation was 260 times higher than that curcumin. Curcumin at a dose of 30\(\mu\)M inhibited proliferation of human breast cancer MDA-MB-231 cells by 50%, while vanillin was unable to inhibit proliferation of the same cell line, even at a dose as high as 1 mM.

1.4 Chemistry of autoxidation products of curcumin

Griesser et al. found that besides alkaline hydrolysis, curcumin can also be degraded via an autoxidation process. The autoxidation process was pH-dependent with the highest rate at pH = 8, which was consistent with a previous study. During this process, equimolar amount of oxygen was consumed to degrade curcumin, suggesting that an oxidation reaction is involved in curcumin degradation. Co-addition of COX-2 enzyme accelerated curcumin degradation, and co-addition of hydrogen peroxide further increased COX-2-mediated curcumin degradation.

Structural analysis of the autoxidation products have shown that the most abundant degradation product was bicyclopentadione, which has incorporated two oxygen atoms into the structure of curcumin, consistent with the stoichiometric consumption of oxygen.
in curcumin degradation. Further structural analysis of other degradation products suggests that curcumin degradation in aqueous buffer could happen through a mechanism comparable to that of lipid peroxidation. The first step of curcumin degradation is the hydrogen dissociation from the phenolic group to form a phenolic radical, which then migrates to the conjugated heptadienedione chain and leads to the formation of cyclized compounds such as bicyclopentadione derivatives of curcumin. The radical can then be transferred to another curcumin molecule, resulting in a chain reaction that causes curcumin to rapidly degrade.

Recent research further suggests that this autoxidation mechanism is the major pathway in mediating curcumin degradation. It was recently shown that a wide range of redox-active antioxidants with diverse structures, including gallic acid, ascorbate (vitamin C), tert-butylhydroquinone (TBHQ), caffeic acid, rosmarinic acid, and Trolox (a water-soluble analogue of vitamin E), dramatically enhanced curcumin stability in aqueous buffer, supporting the hypothesis that the autoxidation pathway is the major process mediating curcumin degradation. Antioxidant could also enhance curcumin stability in vivo: when mice were treated with both curcumin and TBHQ for one hour, the amount of curcumin present in plasma was approximately 4-times higher compared to that treated with curcumin alone. As an antioxidant itself, curcumin can scavenge free radicals by donating an electron to the free radical, thus becoming a stable radical itself. Antioxidants themselves can be stabilized by other antioxidants if the electron-donating potential of one of the them is higher than the other. The electrochemical potential of curcumin has been reported as 0.66V; comparatively, the electrochemical potential of antioxidants such as ascorbate, Trolox, and caffeic acid have been reported as
0.282V $^{64}$, 0.48V $^{64}$, and 0.45V $^{66}$ respectively. In other words, the aforementioned antioxidants are able to stabilize curcumin due to their higher electron-donating potential.

Structure and activity relationship study showed that several structural moieties of curcumin are critical for curcumin stability. These structural moieties include: (1) the phenolic group: previous studies have shown that dimethoxycurcumin, in which the radical-initiating phenolic (-OH) groups are converted to methoxy groups (-OCH$_3$), was highly stable in aqueous buffer, cultured cells and animals $^{30,33}$, suggesting the critical role of the phenolic group in curcumin stability. Indeed, only ~20% of dimethoxycurcumin dissolved in phosphate buffer degraded after 60 minutes, whereas curcumin had already degraded by 80% within the first 10 minutes $^{33}$. In addition, several studies have shown that dimethoxycurcumin exerts more potent anti-proliferative and anti-inflammatory effects compared to curcumin $^{67-69}$. The enhanced stability of dimethoxycurcumin can be explained by the autoxidation mechanism: since the hydrogen dissociation from the phenolic group to form a phenolic radical is the first step to initiate curcumin degradation, blocking this group would dramatically enhance curcumin stability; (2) ortho-substitution of phenolic group: recent research has shown that the ortho-substitution groups also play important roles in curcumin stability, though more studies are needed to clarify the structure and activity relationship. For example, demethoxycurcumin, in which the ortho-substitution group is changed from methoxy to hydrogen, showed dramatically enhanced stability compared to curcumin $^{30,34}$. This can also be explained by the autoxidation mechanism: as discussed earlier, the first step of curcumin degradation is the formation of the phenolic radical (Ph-$\text{O}^\cdot$), and the rate of p radical formation is dependent on the bond dissociation energy of the phenol group (Ph-
OH), which is affected by the electron-donating properties of the substitution groups on the aromatic ring. Compared to hydrogen, methoxy group has stronger electron-donating properties; therefore, curcumin, which possess an ortho-substitution of methoxy group, will generate the phenolic radical more easily than demethoxycurcumin. The same mechanism can also explain the enhanced stability of bisdemethoxycurcumin compared to curcumin; (3) the conjugated heptadienedione chain: previous studies have shown that tetrahydrocurcumin, in which the carbon-carbon double bonds in the conjugated heptadienedione chain were reduced to single bonds, has enhanced stability compared to curcumin. This enhanced stability could also be explained by the autoxidation mechanism, as structural changes of the heptadienedione chain block the radical migration and subsequent formation of bicyclopentadione derivatives of curcumin. Together, these structure and activity relationship studies could help us to better understand the mechanism of curcumin stability, leading to design of better curcumin-based therapeutics.

1.5 Biology of autoxidation products of curcumin

To date, the biological activities of the curcumin oxidation products are largely unknown. Ketron et al have suggested that the reactive intermediates generated during curcumin degradation could increase topoisomerase II-mediated DNA cleavage. Topoisomerase II is an enzyme responsible for preventing under- or over-winding of DNA during replication by covalently binding to the double stranded DNA, cleaving both strands, unwinding them, and finally ligating them. Ketron et al found that treatment with curcumin alone or potassium ferricyanide alone could not enhance topoisomerase II mediated-DNA cleavage; however, their combination enhanced DNA cleavage in a dose-
dependent manner. The stable degradation product of curcumin bicyclopentadione itself had little effect on DNA cleavage with or without the presence of potassium ferricyanide. Together, these results indicate that the reactive intermediates formed during curcumin degradation, such as the quinone methide radical intermediates, could contribute to the topoisomerase II-induced DNA cleavage.

If curcumin oxidation products, but not the parent compound, were the cause of curcumin’s biological efficacy, it follows that if curcumin degradation was suppressed, the biological activities of curcumin would be attenuated. However, a recent study did not support this hypothesis. Instead, the results showed that when curcumin degradation was suppressed through co-addition of serum or antioxidants, the biological activities of curcumin were dramatically enhanced. These results suggest that curcumin, but not its degradation products, have potent biological activities. Further studies are needed to better characterize the role of autoxidation in the biological activities of curcumin.

1.6 Conclusion

Although curcumin has been shown to have anti-cancer and anti-inflammatory effects, the compound has been hampered by the lack of metabolic and chemical stability. In alkaline aqueous solutions, curcumin can undergo hydrolysis and oxidation. Through the hydrolysis pathway, curcumin degrades into hydrolysis products such as vanillin, ferulic acid, ferualdehyde, and feruloylmethane. Through autoxidation, curcumin forms a phenolic radical that incorporates oxygen into the reaction; one of the final products is bicyclopentadione. Based on evidence from the current literature, curcumin most likely degrades through oxidation rather than through hydrolysis.
The evidence for the oxidative pathway lies in the evidence from structurally similar curcumin analogues and the stabilizing effects of antioxidants on curcumin. If curcumin degraded primarily through hydrolysis, the functional phenolic group would have little effect on the stability of curcumin, since hydrolysis occurs on the diketone group on the heptadioenone bridge; however, data from curcumin analogue studies show that the phenolic functional groups greatly affect stability. Furthermore, curcumin can be stabilized by other antioxidants in basal media. If hydrolysis was the primary degradation pathway, antioxidants would have little effect on curcumin’s stability since during hydrolysis curcumin does not form radicals.

Because curcumin autoxidation is a recent discovery, the biology of autoxidation products of remains poorly characterized. It has been suggested that curcumin exerted its effects through its oxidative intermediates such as its quinone methides. However, when curcumin is stabilized through co-addition of serum or antioxidants, the biological activities of curcumin were greatly enhanced, suggesting that curcumin, but not its degradation products, has potent biological activities.

In conclusion, emerging research supports the hypothesis that the autoxidation pathway is the major mechanism mediating curcumin degradation, but further studies are needed to characterize the roles of autoxidation in the biological effects of curcumin. In next chapter, the biological activities and mechanisms of the oxidative degradation products of curcumin will be examined.
CHAPTER 2

CURCUMIN, BUT NOT ITS OXIDATIVE DEGRADATION PRODUCTS, ATTENUATES INFLAMMATORY RESPONSES AND NF-kB SIGNALING

2.1 Introduction

Curcumin can autoxidize to bicyclopentadione at physiological pH. To date, there is little literature on the biological activity of curcumin oxidation products. Ketron et al found that curcumin alone did not increase topoisomerase II-mediated DNA cleavage, but when a potassium ferricyanide, a Fe$^{3+}$ source, was added to curcumin, DNA cleavage occurred. The associated DNA cleavage corresponded with a decrease in curcumin's peak at 430nm and an increase in the oxidation products peak at 263nm. Since bicyclopentadione, one of the major oxidation products, had little effect on DNA cleavage regardless of whether Fe$^{3+}$ was present, Ketron et al concluded that the DNA cleavage was caused by curcumin oxidation intermediates. However Nimiya et al reported that curcumin is stabilized by the presence of another antioxidant, leading to enhanced anti-proliferative activities on MC38 colon cancer cells in free media. Antioxidants such as ascorbic acid would stabilize the curcumin radical, which would otherwise undergo autoxidation. In other words, the study suggests that the biology of curcumin does not come from the oxidative degradation products.

Due to the conflicting data on the biology of curcumin oxidative degradation products, further studies must be conducted to elucidate their effects. To date, there have been no studies on the effects of curcumin’s oxidative degradation products on inflammation. Thus, the objective of this study was to compare the effects of these products to curcumin.
in terms of inflammation, and to provide further insight on biology of the degradation products.

2.2 Materials and Methods

2.2.1 Curcumin degradation products and cell culture

Curcumin was chemically synthesized in the laboratory. Total degradation products of curcumin (TDP) were obtained by incubating curcumin in pH 7.4 phosphate buffer and extracting them with ethyl acetate. Bicyclopentadione (BCPD) was isolated from the total degradation products. RAW 264.7 murine macrophage cells were maintained in Dulbecco’s modified Eagle medium (DMEM, Lonza, Allendale, NJ) supplemented with 10% fetal bovine serum (Corning Inc, Corning, New York) and antibiotics (100 U/mL penicillin and 100 μg/mL streptomycin, Lonza, Allendale, NJ) in a 37°C incubator with 5% CO₂.

2.2.2 Nitric oxide measurement and cell viability

RAW 264.7 cells were seeded into 96-well plates in complete medium (DMEM that containing 10% fetal bovine serum) After 24 hour incubation, cells were treated with test compounds and 0.5 μg/mL LPS. After another 24 hours, cell medium was transferred into another 96-well plate and an equal volume of Griess reagent (0.1% NED, 1% sulfanilamide, 4% phosphoric acid) was added to the cell medium. The absorbance was read at 540 nm. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay.
2.2.3 Sample preparation for iNOS and COX-2 Western blots

RAW 264.7 cells were seeded in 6-well plates for 24 hours in complete medium. After 24 hour incubation, cells were treated with the test compounds and 0.5 μg/mL LPS. After another 24 hours, the medium was decanted and the cells were washed with ice-cold PBS and lysed with RIPA buffer containing phosphatase inhibitors and 1 mM EDTA. The total protein in the lysates was quantified using a BCA kit (Thermo-Scientific, Waltham, MA). Laemli loading buffer (Amresco, Solon, OH) was added into all samples, which were stored at -20°C until ready for use.

2.2.4 Sample preparation for IκBα and p-IKK Western blots

RAW 264.7 cells were seeded in 6 well plates for 24 hours in complete medium. Cells were pre-treated with either the test compounds or DMSO vehicle for 1.5 hours. The cells were then treated with 0.5 μg/mL LPS for 20 minutes. Samples were collected according to the procedure described above.

2.2.5 Sample preparation for NF-κB translocation

RAW 264.7 cells were seeded into 60mm plates for 24 hours in complete medium. Cells were pre-treated with either the test compound or DMSO vehicle for 1.5 hours. The cells were then treated with 2 μg/mL LPS for 30 minutes. The nuclear extracts were collected using nuclear extraction kit (Cayman Chemicals, Ann Arbor, MI) according to the manufacturer’s instructions (a modification was to wash the nuclear pellet with ice-cold phosphate saline buffer 3 times and centrifuging the suspension at 4500 g between each wash).
2.2.6 Western Blotting

Equal amounts of protein were resolved with 8% SDS-PAGE gel and transferred onto nitrocellulose membrane. The membrane was washed with TBS, blocked with 5% bovine serum albumin, incubated with the primary antibodies, and rocked gently at 4°C overnight. After primary incubation, membranes were washed with TBS containing 0.05% TWEEN before probing with LI-COR IRDye 800CW Goat anti-Rabbit and IRDye 680RD Goat anti-Mouse secondary antibodies at room temperature. Membranes were washed again and scanned with the Odyssey imaging system (LICOR, Lincoln, NE).

Western blot signal quantification was performed in ImageJ. Cytoplasmic protein signals were normalized with the β-actin signal, while the nuclear protein signals were normalized with the lamin signals. The following primary antibodies were used: anti-iNOS, anti-COX-2, anti-IκBα, anti-p-IKK, anti-NF-κB p65 (Cell Signaling, Danvers, MA), anti-β-actin (Sigma, St. Louis, MO), and anti-lamin A/C (Santa Cruz, Dallas, TX). Anti-Hsp90 (Santa Cruz, Dallas, TX) was used to check for contamination in the nuclear fraction.

2.2.7 Statistical Analysis

All results are expressed as mean ± standard deviation (S.D.). All statistical calculations were performed using ANOVA followed by Tukey’s test with the assistance of the R statistical software. If a significant difference among the interaction between treatment and dose was found, the interaction was separated by either treatment or dose and analyzed again with ANOVA followed by Tukey’s. Statistical significance was considered significant at p<0.05. Results are from 3 independent experiments.
2.3 Results

2.3.1 Curcumin, not its degradation products, attenuates LPS-induced nitric oxide production

Under inflammatory conditions, nitric oxide is synthesized, and curcumin is a known nitric oxide inhibitor. In this experiment, the comparative effects of curcumin, TDP, and BCPD on LPS-induced nitric oxide production in RAW 264.7 were examined. The cells were treated with these test compounds at several different doses in combination with LPS for 24 hours. Since there was a significant difference among the interaction of treatment and dose, the interaction was separated by dose to determine the effect of the treatment. Curcumin significantly decreased LPS-induced nitric oxide production, which is consistent with previous reports. TDP also showed a slight dose-dependent decrease in nitric oxide production. On the other hand, treatment with BCPD did not decrease nitric oxide production (Fig. 2).

2.3.2 Curcumin, but not its degradation products, inhibits LPS-induced iNOS expression

Because nitric oxide is produced from iNOS under inflammatory conditions, the comparative effects of curcumin, TDP and BCPD on iNOS expression were examined (Figure 3). Since there was a significant difference among the interaction of treatment and dose, the interaction was partitioned by treatment to determine the effect of the treatment dose on iNOS expression. Starting from 3.68 μg/mL, curcumin significantly inhibits iNOS expression in RAW 264.7 cells stimulated with LPS, consistent with previous reports. TDP appeared to have a small dose-dependent effect on iNOS inhibition, while BCPD failed to significantly inhibit iNOS expression regardless of dose.
Figure 2: The effects of curcumin, TDP, and BCPD on LPS-induced nitric oxide production.

Concentration of nitric oxide in medium was calculated as the fraction of the absorbance of the treatment over the absorbance of the LPS control. Results are represented as means±S.D from three independent experiments. A significant difference among interaction of treatment and dose concentration was found, so the interaction was separated by dose to examine the effect of the treatment at given certain dose. * indicates significance from the LPS only control at p<0.05. Different letters indicate significant difference between the treatments at the given concentration (p<0.05).

2.3.3 Curcumin, but not its degradation products, inhibits LPS-induced COX-2 expression

Like iNOS, COX-2 is another protein that is produced under inflammatory conditions. To examine the comparative effects of curcumin and the oxidative degradation products on COX-2 expression, RAW-264.7 cells were treated with 0.5 μg/mL LPS and 9.2 μg/mL of curcumin, TDP, or BCPD for 24 hours. COX-2 expression was then examined with Western blotting (Figure 4). Curcumin greatly inhibited COX-2 expression, consistent with previous reports, however, neither the TDP nor BCPD significantly inhibited COX-2 expression.
Figure 3: The effects of curcumin, TDP, and BCPD on iNOS expression.

RAW 264.7 cells were treated with test compound and 0.5 μg/mL LPS for 24 hours, iNOS expression was analyzed by Western blotting (A). iNOS signals were normalized with the β-actin signals and the intensity was calculated as the fraction of the averaged normalized LPS signal. Results are represented as means±S.D from three independent experiments (B). Since there was a significant difference in the interaction of treatment and dose, the interaction was separated by dose to examine the effect of treatment at a given dose. * indicates significance from the LPS-only control at p<0.05. Different letters indicate significant differences between the groups at the given dose at p<0.05.

2.3.4 Curcumin, but not its degradation products, inhibits LPS-induced p65 translocation

Both iNOS and COX-2 are produced when the NF-κB pathway is activated. Under inflammatory conditions, the NF-κB dimer translocates into the nucleus and initiates the transcription of many inflammatory mediators such as iNOS and COX-2. Curcumin is known to inhibit translocation of NF-κB. To assess the ability of curcumin’s degradation products to inhibit NF-κB translocation, RAW 264.7 cells were pre-treated with 36.2 μg/mL of test compound for 1.5 hours, followed by a 30 minute induction of inflammation with 2 μg/mL LPS. The nuclear fraction was collected and subjected to
Western blotting. Curcumin inhibited the translocation of p65 into the nucleus, which is consistent with previous reports \(^79\); neither total degradation products nor bicyclopentadione significantly inhibited the protein’s translocation.

**Figure 4:** The effects of curcumin, TDP, and BCPD on COX-2 expression

RAW 264.7 cells were incubated with 0.5 \(\mu\)g/mL LPS and 9.2 \(\mu\)g/mL of each test compound for 24 hours, COX-2 expression was analyzed by Western blotting (A). COX-2 signals were normalized with the \(\beta\)-actin signals and the intensity was calculated as the fraction of the averaged LPS signal. Results are represented as means±S.D from three independent experiments (B). Different letters indicate significant difference between the groups at \(p<0.05\).

2.3.5 Curcumin, but not its degradation products, inhibits LPS-induced I\(\kappa\)B\(\alpha\)

degradation and IKK phosphorylation

Upstream of NF-\(\kappa\)B nuclear translocation are the proteins I\(\kappa\)B\(\alpha\) and I\(\kappa\)B kinase (IKK). I\(\kappa\)B\(\alpha\) is responsible for sequestering NF-\(\kappa\)B in the cytoplasm; under inflammatory conditions, I\(\kappa\)B\(\alpha\) is phosphorylated by IKK and is sent for degradation. IKK itself is
Figure 5: The effects of curcumin, TDP, and BCPD on NF-κB (p65) nuclear translocation.

RAW 264.7 cells were pre-treated with 36.2 μg/mL test compound or DMSO vehicle for 1.5 hours, and then stimulated with 2 μg/mL LPS for 30 min. The nuclear fraction was collected and subjected to Western blotting (A). p65 signals were normalized with the lamin signals and the intensity was calculated as the fraction of the LPS only signal. Results are represented as means±S.D from three independent experiments (B). Different letters indicate significant difference between the groups at p<0.05.

also activated by phosphorylation. Curcumin is known to modulate the NF-κB pathway by inhibiting degradation of IκBα and phosphorylation of IKK \(^{79}\). To assess the effects of curcumin’s oxidative degradation products on these two proteins, RAW 264.7 cells were pre-treated with 36.2 μg/mL of curcumin, TDP, or BCPD for 1.5 hours, followed by 20 minute stimulation of 0.5 μg/mL LPS; the cell lysate was collected and subjected to Western blotting (Figures 6-7). Curcumin rescued IκBα from degradation during LPS-
induced inflammation, whereas neither TDP nor BCPD significantly inhibited its degradation. Additionally, curcumin inhibited phosphorylation of IKK, whereas neither TDP nor BCPD significantly inhibited IKK phosphorylation.

![Figure 6: The effects of curcumin, TDP, and BCPD on IκBα degradation.](image)

RAW 264.7 cells were pre-treated with incubated 36.8 μg/mL test compound for 1.5 hours followed by 0.5 μg/mL LPS stimulation for 30 minutes. IκBα signals were normalized with the β-actin signals. Relative intensity was calculated as the fraction of the DMSO (No LPS) control. Results are represented as means±S.D from three independent experiments (B). Different letters indicate significant difference between the groups at p<0.05.

2.4 Discussion

The results of this study further support that curcumin, but not its oxidative degradation products, is biologically active. Curcumin is known for its anti-inflammatory
Figure 7: The effects of curcumin, TDP, and BCPD on IKK phosphorylation.

RAW 264.7 cells were pre-treated with incubated 36.8 μg/mL of each treatment for 1.5 hours followed by 0.5 μg/mL LPS stimulation for 30 minutes (A). IKK signals were normalized with the actin signal. IKK phosphorylation intensity was calculated as the fraction of the LPS control. Results are represented as means±S.D from three independent experiments (B). Different letters indicate significant difference between the groups at p<0.05.

Effects via the NF-κB pathway, and the results for curcumin are consistent with previous studies. On the other hand, neither TDP nor BCPD was as effective in inhibiting nitric oxide production, iNOS and COX-2 expression, translocation of p65 into the nucleus, and NF-κB pathway proteins upstream of p65 translocation. Total degradation products showed a slight dose-dependent decrease in both nitric oxide production and iNOS expression, but did not inhibit expression of COX-2, degradation of IκBα, phosphorylation of IKK, and translocation of p65. The slight dose-dependent decrease
may be attributed to the residual curcumin that may have been present in the degradation mixture. BCPD showed no significant effect in all the experiments performed. Although high doses of each test compound were used for the NF-κB experiments, the results nevertheless suggest curcumin is more biologically active than the oxidation products.

In conclusion, curcumin’s oxidative degradation products, particularly bicyclopentadione, have little anti-inflammatory effect compared to curcumin. Further research should be conducted to examine the role of curcumin oxidative degradation products in other biological models.
APPENDIX A

EFFECT OF TRICLOCARBAN ON ANIMAL BODY WEIGHT

The consumption of polyunsaturated omega-6 fatty acid is high in the Western world due to the use of vegetable oil, which contains linoleic acid. On the other hand, omega-3 fatty acid intake is low in the Western world, and some studies have shown a correlation between high omega-6 to omega-3 ratio and obesity. In obese mothers, the ratio of omega-6 to omega-3 fatty acid in breast milk was greater than in normal-weight mothers. Omega-6 fatty acids can be metabolized through the cytochrome P450 epoxygenase pathway. First, the omega-6 arachidonic acid is metabolized by epoxynogenase to form epoxyeicosatrienoic acids (EET), which are quickly metabolized by soluble epoxide hydrolase (sEH). Blocking this enzyme increases tissue levels of EET, which may have pro-obesity effects (unpublished results).

Triclocarban is an antibacterial agent used in personal care products such as hand soaps. It has been found in human urine, raising concerns about the potential hazards of continuous exposure to this bacteriocide. Schebb et al found that after a regular shower using triclocarban-containing soaps, micromolar amounts of the compound could be detected in the urine; furthermore, topical application of triclocarban was sufficient to block sEH activity in mice. In other words, triclocarban may promote obesity by increasing the concentration of EET, which is derived from omega-6 fatty acids. Based on the available results, it is hypothesized that triclocarban promotes obesity by increasing concentration of EET.
The first objective was to compare the weights of mice fed with 0.08% triclocarban together with 10% corn oil compared to mice fed with 10% corn oil alone. Mice weight was recorded twice a week for 6 weeks. Food intake was monitored and food was changed twice per week. Student t-test was utilized to determine significant difference between the two groups for each day the weights of the mice were recorded. However, mice that were fed 0.08% triclocarban together with 10% corn oil gained weight more slowly than mice fed with corn oil alone, thus contradicting our hypothesis (Figure 8). It is likely that the dose of triclocarban used in this experiment induced toxicity effects in the mice, leading to the loss in weight.

![Figure 8](image_url)

**Figure 8:** Weights of mice fed with 10% corn oil alone (Control) or with 10% corn oil supplemented with 0.08% triclocarban (TCC). Student t-test was performed between the groups for each pair of data point (p<0.05).
APPENDIX B

COMPARATIVE STABILITY OF CURCUMIN, DIDEMETHYL CURCUMIN, AND DIMETHOXY CURCUMIN, AND THEIR EFFECTS ON NITRIC OXIDE PRODUCTION

Although curcumin has been reported for its numerous health benefits, it is also metabolically unstable. Furthermore, in buffers at physiological pH, curcumin degrades rapidly\(^\text{29}\). Due to its instability, there is a need for a stable curcumin analogue that is as effective as curcumin. Recent data have suggested that methoxylating or hydroxylating the functional groups on curcumin’s phenolic rings may affect curcumin activity. For example, didemethylcurcumin (a curcumin analogue where all methoxy groups were replaced with hydroxyl groups) showed higher antioxidant activity than curcumin, whereas dimethoxycurcumin (an analogue where all hydroxyl groups were replaced with hydroxyl groups) showed no antioxidant effect\(^\text{84}\). The result suggests a possible structure relationship between the functional groups found on the curcumin’s benzene rings and the activity of the curcumin or its analogue. While the effects of curcumin have been compared with either didemethylcurcumin or dimethoxycurcumin in several biological studies\(^\text{67-69,85-88}\), no systematic biological study has been done on the structural relationship activity of both analogues compared to curcumin. Here, the comparative effects of curcumin and the two analogues on nitric oxide production were examined. Additionally, HPLC analysis of intracellular curcumin and its analogues was also conducted to examine the stability of each compound within the cells after treatment.
RAW 264.7 cells were seeded in complete media and were treated with varying doses of curcumin, didemethylcurcumin, or dimethoxycurcumin, with or without 0.5 μg/mL LPS. Nitric oxide measurement was performed as described in Materials and Methods in Chapter 2. For HPLC analysis, cells were treated with 20 μM of each treatment compound without LPS stimulation; after 24 or 48 hours of incubation, the medium was removed. Plates were rinsed with ice-cold PBS, and cells were lysed with RIPA buffer to release intracellular treatment compounds.

**Figure 9:** Nitric oxide production dose response at 24 hours (A) and 48 hours (B) for all treatments. At 48 hours, there was a significant difference among the treatments at 6.25μM and 12.5μM (C). Different letters indicated significance (p<0.05).
Ethyl acetate was used to extract the treatments from the buffer and was evaporated in a vacuum centrifuge. Before HPLC analysis, samples were dissolved in methanol, and reverse phase chromatography with a C-18 column was used. Prior to ethyl acetate extraction, RIPA buffer was aliquoted to perform the BCA assay (see Materials and Methods, Chapter 2). HPLC results were expressed as the weight of each treatment compound remaining intracellularly over the total protein weight. Statistical calculations were performed using ANOVA followed by the Tukey test with the assistance of the R statistical software.

![Figure 10: HPLC analysis of didemethylcurcumin (A), curcumin (B), and dimethoxycurcumin (C).](image)

For each compound, the top chart shows the peak of their respective standards, while the bottom chart shows the peaks of the compound after cells were treated for 24 or 48 hours with the compounds. (D) shows the amount of intracellular compound found in the RAW 264.7 cells at each time point; didemethylcurcumin was below the range of detection at both times. Different letters indicate significant differences (p<0.05)
The difference in nitric oxide inhibition between each compound was more apparent after 48 hours; no significant differences among the compounds were found at each dose in the 24 hour treatment (Figure 9). At 6.25 μM, dimethoxycurcumin was more effective at inhibiting nitric oxide production than either curcumin or didemethylcurcumin, suggesting the critical role of the methoxy functional groups in stabilizing the curcumin analogue. Indeed, dimethoxycurcumin was found to be significantly more stable than either curcumin or didemethylcurcumin after 24 and 48 hour treatments (Figure 10). On the other hand, didemethylcurcumin was below the detection range was at both time points, and curcumin's small peak at 24 hours decreased even further after 48 hours.
APPENDIX C

CURCUMIN STABILIZED BY ANTIOXIDANTS CAN ATTENUATE LPS-INDUCED NITRIC OXIDE PRODUCTION AND iNOS EXPRESSION

Curcumin is unstable in serum-free media at physiological pH; under these conditions, curcumin can undergo oxidative degradation. Recently, Nimiya et al discovered that curcumin can be stabilized by the addition of antioxidants. Details on curcumin oxidative degradation can be found in Chapter 1. The objective of this study was to compare the anti-inflammatory effects of curcumin dissolved in free media versus curcumin co-dissolved with antioxidants in the same media.

RAW 264.7 cells were seeded in complete media and were treated with 6.25 μM curcumin and/or 2.9 μM of a given antioxidant in serum-free basal medium. Inflammation was stimulated with 0.5 μg/mL LPS. Nitric oxide measurement, cell viability, and Western blotting were performed as described in the Materials and Methods section of chapter 2. For statistical analysis, one-way ANOVA was performed for all groups followed by Dunnett’s test.

Treatment with curcumin alone or antioxidant alone did not significantly decrease LPS-induced nitric oxide production. However, their combination significantly induced LPS-induced nitric oxide production (Figure 11). Furthermore, curcumin combined with antioxidant significantly decreased LPS-induced iNOS expression (Figure 12). These results suggest that the antioxidants increase curcumin’s efficacy by stabilizing curcumin, which would otherwise undergo oxidative degradation.
Figure 11: Nitric oxide production of LPS-induced RAW 264.7 cells treated with curcumin and/or Trolox, sodium ascorbate (SA), caffeic acid (CA), or gallic acid (GA).

Neither curcumin nor antioxidant alone inhibited nitric oxide production, but both compounds synergistically decreased nitric oxide production. Nitrite concentration was calculated as the fraction of the absorbance of the LPS only control. Results are expressed as mean±S.D.

Figure 12: iNOS expression of LPS-induced RAW 264.7 cells treated with curcumin and/or Trolox, sodium ascorbate (SA), caffeic acid (CA), or gallic acid (GA) (B).

Neither curcumin nor antioxidant alone inhibited iNOS expression, but both compounds synergistically decreased nitric oxide production (B). Results are expressed as mean±S.D.
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43