Biological Effects and Action Mechanisms of Dietary Compounds

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BIOLOGICAL EFFECTS AND ACTION MECHANISMS OF

DIETARY COMPOUNDS

A Thesis Presented

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ABSTRACT

BIOLOGICAL EFFECTS AND ACTION MECHANISMS OF DIETARY COMPOUNDS

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The food that we consume contain many dietary compounds which are biologically active. In this thesis we will discuss the biological effects of dietary compounds and the mechanisms behind their activities.

First, we studied on the anti-metastatic effects of curcumin, a dietary compound derived from turmeric, through lymphangiogenesis inhibition. Curcumin inhibited vascular endothelial growth factor-C (VEGF-C)-induced lymphangiogenesis *in vivo* and *in vitro*. Curcumin inhibited lymphangiogenesis, in part through suppression of proliferation, cell cycle progression and migration of lymphatic endothelial cells. Curcumin inhibited expressions of VEGF receptors (VEGFR2 and VEGFR3), as well as down-stream signaling such as phosphorylation of ERK and FAK. Finally, curcumin sulfate and curcumin glucuronide, two major metabolites of curcumin *in vivo*, had little inhibitory effect on proliferation of HMVEC-dLy cells. Our results demonstrate that curcumin inhibits lymphangiogenesis *in vitro* and *in vivo*, which could contribute to the anti-metastatic effects of curcumin.
Next, we investigated the mechanisms underlying the cytotoxic activity of tert-butylhydroquinone (TBHQ), a widely used synthetic food antioxidant. Here we found that the biological effects of TBHQ are mainly mediated by its oxidative conversion to a quinone metabolite tert-butylquinone (TBQ). Co-addition of cupric ion (Cu²⁺) enhanced, whereas ethylenediaminetetraacetic acid (EDTA) suppressed the oxidative conversion of TBHQ to TBQ, and the biological activities of TBHQ in MC38 colon cancer cells. Finally, a structure and activity relationship study was done and together, these results suggest that the biological activities of TBHQ and other para-hydroquinones are mainly mediated by their oxidative metabolism to generate more biologically active quinone metabolites.
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CHAPTER 1
LITERATURE REVIEW

1.1 Introduction

Curcumin is the yellow pigment derived from turmeric, a rhizome from the herb Curcuma longa. Turmeric is commonly used as a main ingredient in curry spices which provides its distinctive color and flavor. Traditionally, turmeric as a spice has been known to be used for centuries as an ingredient for medicinal spice mixture or as part of Ayurveda medicine and Chinese medicine to promote health [1]. Over the few decades, substantial studies have been done on curcumin due to its health beneficial properties, including potent antioxidant properties [2, 3], anti-microbial [4, 5], anti-inflammation [6], anti-cancer effects [7, 8], and cardio-protectiveness [9]. However, despite numerous cell culture studies indicated that treatments with a low dose of curcumin was enough to exhibit the effects of curcumin, many animal and clinical studies showed that in order for the beneficial effects of curcumin can be observed, oral administration of a large dose of curcumin is necessary. This indicated that curcumin efficacy has been limited due to its poor bioavailability.

1.2 Epidemiological studies surrounding curcumin

Curcumin consumption is often associated with curry consumption because turmeric is commonly used as one of the main ingredient. Many epidemiology studies was done on the association between curry consumption and chronic diseases such as cancer to observe the contribution of curcumin on health. One epidemiology study was done by Sinha et. al. regarding the association between cancer risk and diet in India [10]. India is a South Asian country which is known for their high consumption of curry compared to
other countries in the world. In a possible association to this, cancer rates in India compared to more developed country such as United States are considerably lower according to the World Health Organization (WHO) [11].

It is interesting that India has a moderately low incidence in stomach cancer despite having high incidences of oral and esophageal cancers because they are part of the gastrointestinal tract. The high oral and esophageal cancer incidences are significantly due to some lifestyle risk factors including diet. Some case-control studies analyzed by Sinha et al. on consumptions of common Indian foods which may increase the risk of gastrointestinal tract cancers included spicy food (OR=2.3), chili (OR=7.4), and high-temperature food (OR=7.0). On top of that, Indian diets contain a lot of fried foods which also contributed in the gastrointestinal tract cancers due to the cooking process which leads to production of carcinogenic or mutagenic heterocyclic amines (HA). Some animal studies have been done too by feeding the mice with common Indian dishes such as deep-fried vegetables and showed to have 20% increase in gastric carcinoma. However despite all this, stomach cancer incidence rates are moderate to low in India compared to other countries. One of the suggested reason was the high consumption of turmeric which is associated with its protective effect against the carcinogenic bacterium *H. pylori*, a major risk factor for stomach cancer [10].

Another epidemiology study done by Ng et al. focused on the association between curry consumption and the cognitive functions in the elderly [12]. Compared to the first paper done in India, this study was done in Singapore with a more diverse Asian populations. This may be a good data complementation as it has people from a mixture of ethnicity and culture living in the same environment. Also, compared to the first study
done by Sinha et al. where they focused on cancers, here, Ng, et. al. focused on the
cognitive function in elderly, such as Alzheimer’s disease. They are both different
diseases, however they both requires a long period of time to develop, and such, life-style
and diet play big roles in contributing to these diseases.

Alzheimer’s disease is a common disease among elderly due to neurodegenerative
disease where progressive brain cell death occur over a long period of time [13]. Previous
studies have found that anti-inflammatory drugs and antioxidants are promising
neuroprotective agents against Alzheimer’s disease. Indeed, epidemiologic studies have
shown that the use of nonsteroidal anti-inflammatory drugs (NSAIDs) for a long-term
period is associated with reduced risk of Alzheimer’s disease [14]. However, the toxicity
of these drugs on gastrointestinal tract, liver, and kidney, put a heavy toll especially in
elderly bodies and thus, their use is limited.

Curcumin from turmeric, as mentioned in the beginning, has been shown to be potent
anti-inflammatory and antioxidant agent. Curcumin is contained in curry dishes a lot,
however, no epidemiological evidence showed curry lowers dementia risk or cognitive
enhancement in the elderly. And so Ng et al. conducted this epidemiology study to
investigate the relevance between different frequencies of curry consumption with
cognitive performance in elderly.

This cohort study was conducted in Singapore, through Singapore National Mental
Health Survey of the Elderly. Sample of 1010 older adults aging 60-93 years (average
68.9 years) for one year period of time. Singapore population is consisted of multiethnic
population mixture of Chinese, Malaysians, and Indians. The sampling used random
sampling list from the Department of Statistics’ national database in Singapore. To test
the cognitive performance, subjects performed Mini-Mental State Examination (MMSE) which is a widely used instrument that provides global measure of domains of cognitive function that included memory, attention, language, praxis, and visuo-spatial ability [12].

The results showed that subjects who consumed curry often (once a month or more, weekly, or daily) were mainly consisted of Indians due to cultural influence, better educated, socially active, had diabetes, used NSAIDs, or were smokers. The group considered multiple external factors that may influence MMSE performance scores and determine which of the factors had significantly dependent or independent associations. Based on these considerations, subjects with higher levels of curry consumption showed statistically significant higher mean MMSE scores of 25.9 (p=0.004) with no significant difference between “occasional” and “often and very often” curry consumption due to insufficient numbers of subjects. This showed that the association of better cognitive performance and curry consumption in a dose-dependent manner have statistically significant linear trend. Based on the odd ratios of the association between curry consumption and cognitive impairment (MMSE score of ≤ 23) indicated that subjects who “never or rarely” consumed curry have no associations (OR = 1); “occasionally” consumed curry have reduced risk (OR = 0.62); and “often” consumed curry also have reduced risk (OR = 0.51) [12].

These epidemiology studies indicated that people with higher curry consumption is associated with reduced risks of chronic diseases such as cancers and Alzheimer’s disease, suggesting the potent anti-inflammatory and antioxidant properties of curcumin contained in curry.
1.3 Health benefits and biological activities of curcumin

To understand more about the contribution of curcumin on health benefits, many researchers investigated the biological activities of curcumin in molecular cellular, animal, and clinical studies. These studies indicated that the activities of curcumin were involved in multiple pathways and mechanisms, demonstrating its beneficial effects.

1.3.1 Curcumin as antioxidant

In human bodies, the formation of reactive oxygen species (ROS), oxygen containing free radicals, occurs in the cells during normal physiological activities. The accumulation of ROS in cells has been tied to many disease cases, including the damage of the nucleic acids and DNA which can cause mutations, leading to tumor formation [15]. ROS are free radicals including superoxide anion radicals, hydroxyl radicals, hydrogen peroxide, and singlet oxygen. Studies have shown that curcumin has the antioxidant properties such as metal chelating, radical scavenging, and hydrogen peroxide scavenging to protect the body from ROS and other free radicals [16].

1.3.2 Anti-microbial effects of curcumin

In the past decade, there have been an increase in studies on anti-microbial effects of curcumin due to its abundance in nature, low in price, and low in toxicity. Curcumin inhibited the growth of chloroquine-resistant *Plasmodium falciparum*, parasite that cause malaria, *in vitro* in a dose-dependent manner with IC$_{50}$ around 5μM. Oral administration of curcumin to mice that had been infected with the parasite showed a decrease in blood parasitema by 80-90% and increased survival rate by 29% compared to control with 0% survival [17]. Curcumin also showed its antimicrobial activity against *Helicobacter*
*Helicobacter pylori* in vitro and in vivo. This microaerophilic bacterium is one of the major leading cause of gastrointestinal diseases such as gastritis and peptic ulcer. However, the high minimum inhibitory concentration (MIC) of curcumin with oral treatment to mice (25mg/kg) was suggested to be due to the low bioavailability of curcumin [18].

**1.3.3 Anti-inflammatory effects of curcumin**

Inflammation is the reaction of the immune-system of the body associated with many diseases. However, inflammation is a major risk factor for cancer progression, such as tumor development and metastasis [19]. In mammalians, the nuclear factor kappa B (NF-kB) pathway, which plays a big role in the intracellular activities, can be activated by various agents, including pro-inflammatory cytokines such as tumor-necrosis factor-alpha (TNF-α). Once this system has been activated, it will activate the downstream inflammatory pathways such as cyclooxygenase (COX-2), lipoxygenase (LOX), and inducible nitric oxide synthase (iNOS). Research regarding curcumin on inflammation showed that curcumin down-regulated these downstream pathways by down-regulating TNF-α through the suppression of NF-kB, resulting in reduction of inflammation. Curcumin also inhibited the expression of pro-inflammatory cytokines CXCL1 via the NF-kB signaling pathway, and potentially reducing tumor metastasis [20].

**1.3.4 Anti-cancer effects of curcumin**

Many studies have shown that curcumin has potent anti-cancer effects through suppressions of angiogenesis, formation of new blood vessels from the preexisting vessels [21, 22]. There are multiple steps involved in angiogenesis, including activation, proliferation, invasion, and migration of the endothelial cells [23]. Curcumin has shown
to be able to inhibit angiogenesis through multiple suppression of these steps in different types of cancers. Moreover, our recent study has shown that curcumin also inhibited lymphangiogenesis, formation of new lymphatic vessels which plays a critical role in tumor metastasis, in vivo through suppression of VEGF Receptor signaling [24].

- **Colon Cancer**

  Some studies were done on the effects of curcumin on colon cancer. One study showed that curcumin inhibited the proliferation of colorectal cancer cells (HCT116) with IC$_{50}$ of as low as 10 µM of curcumin after being treated for 48 hours. Through cell cycle analysis, curcumin caused G2 arrest in HCT116 cells, which indicates that curcumin inhibited the cell cycle process, disabling them to properly divide and go into the mitotic stage of the cell cycle. This inhibition induced cell apoptosis or program cell death due to the malfunctioning of the cells. In addition, curcumin also inhibited cell migration and invasion of HCT116 cells which contributes to its anti-cancer effects. This shows that curcumin is able to inhibit cancer progression through multi-steps suppression [25].

- **Lung Cancer**

  A study was done by Chen et al., on lung cancer cell CL1-5 to investigate the anticancer therapeutic properties of curcumin. They studied the mechanisms of how curcumin was able to inhibit cancer progression *in vitro* and *in vivo*. Through several preliminary experiments, they found that curcumin inhibition of cancer progression and tumor metastasis in a dose-dependent manner ranging from 1-20 µM/L and time-dependent manner up to 48 hours. Then, further molecular analysis were done to
understand the underlying mechanism. They found that the inhibition of lung cancer cell invasion, migration, and metastasis by curcumin was associated with the up-regulation of a DnaJ-like heat shock protein 40 (HSP40), HLJ1, which is a tumor suppressor and invasion suppressor gene. HLJ1 is normally present, but with increasing dose of curcumin, the expression of this gene also increased, and in terms, cancer progression was inhibited. They proofed the accuracy of this data by doing the same treatment on knockdown of HLJ1 gene both in vitro and in vivo. The result was that there was a significant decrease on the anti-invasive and anti-metastasis effects of curcumin. Next, through luciferase reporter assay, it was discovered that there is an activator protein (AP-1) in HLJ1 enhancer called JunD, which activates HLJ1 expression, by which was transcriptionally regulated by curcumin in a dose-dependent manner. Knockdown of the JunD gene, partially decreased curcumin-induced HLJ1 activation, which decreased curcumin efficacy. In short, in dose- and time-dependent manner, curcumin transcriptionally activated JunD which then activated HLJ1 to inhibit lung cancer cell invasion and metastasis. This study showed the anti-cancer effects of curcumin on lung cancer [26].

- **Prostate Cancer**

Prostatic cancer is the most common malignant cancer among men in the U. S. The malignancy of this cancer is due to its ability to evolve into its hormone refractory stage. Here, Dorai et al., showed that curcumin inhibited prostate cancer progression in vivo by decreasing the proliferation ability and inducing apoptosis through modulation of apoptosis suppression proteins. Here, animal studies were done as the main study. LNCaP prostate cancer cells were used for all of the studies. LNCaP cells were grown,
mixed with Matrigel, and the mixture was then injected into nude mice. This is a common assay for angiogenesis in vivo study, as when the cancer cells containing Matrigel gets injected below the skin of the mice, it would harden and form a gel. Then, the cancer cells would secrete various growth factors to induce the formation of new blood vessels into the gel, thus angiogenesis. After removal of the gels, if there are a lot of blood in it, then angiogenesis occurred. On the other hand, if there is a little to no blood in the gel, then angiogenesis did not occur. Following injection, the mice were fed with diet which contained 2% w/w of curcumin. After 6 weeks, the tumors were excised and various tests were done. Thin sections were made from the tumors, and was immunostained using in situ cell for proliferation analysis and cell death detection. Quantification using BrdU incorporation assay showed that curcumin decreased the proliferation of LNCaP cells, and through the cell death assay, it showed that curcumin significantly increased apoptosis. Microvessel density was also measured by using the CD31 antigen staining, and it also showed that it was significantly decreased by curcumin. These in vivo results demonstrated the anti-cancer therapeutic potential of curcumin to significantly inhibit prostatic carcinoma by preventing the cancer to progress to its hormone refractory stage [27].

- **Pancreatic cancer**

A Phase II clinical trial was done by Dhillon et. al. where they found oral administration of curcumin decreased NF-kB (p65), COX-2, and pSTAT3 expressions in patients with advanced pancreatic cancer. The study was done by administering 8 grams of curcumin per day to 25 patients for 8 weeks. The result based on 21 evaluable patients showed that one patient had an overall 73% decrease of hepatic lesions after 2 months of
curcumin administration. Another patient also showed decreased expression of NF-kB (p65), COX-2, and pSTAT3 in peripheral blood mononuclear cells (PBMC) based on immunohistochemical staining after 8 days of oral treatment. It is important to observe the expressions of these cytokines because NF-kB is a transcriptional factor for inflammation which promotes cancer progression, COX-2 is the enzyme that promotes inflammation, and pSTAT3 is regulated by epidermal growth factor which plays a role in tumorigenesis. However at 24 hours, only 1.8-117 ng/mL free curcumin observed in plasma, indicating the effects of curcumin was attenuated by the low bioavailability of curcumin when administered orally [28].

### 1.4 Factors limiting curcumin bioavailability

Numerous studies have shown how the bioactivities of curcumin are very effective against multiple chronic diseases and to promote health. Not only that, curcumin was found to be generally safe up to high dose through oral administration. Several previous toxicology studies have been done with different doses of curcumin through oral administration, and no adverse effects were observed. A study was done by Soni and Kuttan where participants were orally administered with 0.5 g of curcumin daily for 7 days, and no clinical toxicity was observed [29]. Another two clinical studies done to evaluate curcumin efficacy against arthritis or postoperative inflammation. It was found that oral administration of curcumin with 1.2 – 2.1 g dose per day for up to 6 weeks showed no adverse effects [30, 31].

Finally, another study was done by Lao, et al. to determine the toxicology dose level of curcumin in human. Analysis was done on twenty-four participants where they were administered with curcumin dose ranging from 0.5 to 12 g orally and safety assessment
was done for 72 hours. This result showed that a single oral dose of curcumin up to 12 g and still, no toxicity was observed. So far, 12 g of curcumin is the highest dose tolerated and accepted by patients due to the bulky volume of the tablets [32]. Further studies needed to be done to identify the maximum tolerated dose of curcumin in humans.

Despite the numerous health benefits provided by curcumin, it is necessary to intake curcumin in a very high dose orally, up to 8-12 grams per day, in order to observe its beneficial effects on health. Many studies suggested that it is due to the low bioavailability of curcumin. A phase 1 clinical trial observed peak curcumin concentration in the serum at 1 to 2 hours after oral administration. The average peak serum concentration of 0.51 ± 0.11 μM, 0.63 ± 0.06 μM, and 1.77 ± 1.87 μM were observed after curcumin intake of 4 grams, 6 grams, and 8 grams respectively [33]. Furthermore, based on our previous in vitro experiment, we found that curcumin treatment with a low dose of 15 μM for 24 hours inhibited around 50% HMVEC-dLy cell proliferation and around 70% inhibition with a low dose of 10 μM for 48 hours [24]. This indicated the importance of increasing curcumin bioavailability, to lower the necessary curcumin intake while increasing its efficacy. In order to be able to discuss that, it is important to investigate various factors limiting the bioavailability of curcumin.

1.4.1 Bioaccessibility

In order for nutraceuticals, in this case curcumin, to exert its biological activities, it needs to be bioaccessible to be absorbed into the gastrointestinal tract epithelium and then transported into the blood circulation. For that, solubilization of the compounds in the aqueous gastrointestinal fluids is crucial. Curcumin is a hydrophobic compound, and therefore it has poor solubility in the aqueous fluids leading to poor bioaccessibility [34].
Co-ingestion of curcumin with lipid can help increase curcumin solubility and bioaccessibility through encapsulation of curcumin into mixed micelles formed due to hydrolysis of the lipid in the GIT. This will not only help protect curcumin from degradation, but also increases its chance to be absorbed into the GIT epithelium lining. The length of the fatty acid chains and degree of saturation will determine the solubility of nutraceuticals in the mixed micelles. In this case, medium chain triglycerides (MCT) and long chain triglycerides (LCT) have been reported to be suitable for curcumin to increase its solubility and biaccessibility [35, 36].

1.4.2 Absorption

One of the reason for the low bioavailability of curcumin is due to its poor absorption. The main absorption site of curcumin in the gastrointestinal tract is in the small intestine [37]. However, curcumin is a lipophilic phenolic compound, which cause oral administrated curcumin hard to be absorbed by the small intestine epithelium cells [38]. Even if it were to be absorbed into the epithelium, its hydrophobic property would cause it to be flushed back into the lumen through efflux system.

Animal studies have shown that oral administrated curcumin undergoes rapid metabolic reduction and conjugation, which result in poor systemic bioavailability. A study on mice with oral administration of 0.1 g/kg curcumin yielded a peak plasma concentration of free curcumin only 2.25 μg/mL [39]. Another study on rats, free form curcumin was completely undetected from plasma within 1 hour after 40 mg/kg intravenous dose. Major metabolites of curcumin observed were curcumin glucuronide and curcumin sulfate based on enzymatic hydrolysis studies [40]. This observation of
extremely low serum levels showed how curcumin was poorly absorbed which supported the poor bioavailability of curcumin.

Piperine, an active compound derived from peppers, had been reported to be able to reduce efflux transport back to the lumen by modulating the cell membrane dynamics to altering the lipid fluidity due to its apolar nature [41]. Another study reported piperine inhibition of cytochrome P-450 and glucuronidation, further supporting its potent ability as an inhibitor for metabolism [42]. Indeed co-ingestion of piperine with curcumin to rats, increased bioavailability of curcumin by 154% and 2000% to humans [43].

1.4.3 Tissue Distribution

After being absorbed and circulated, how curcumin is distributed throughout the body tissues is an important factor for its biological activities. However, limited amount of studies have been done on this. A study done by Ravindranath et al showed that only traces of free-formed curcumin were found in the liver and kidney after oral administration of 400 mg of curcumin to rats [37]. Another study was done using tritium-labeled curcumin to evaluate, and they found that radioactivity was detectable in blood, liver, and kidney with treatment doses of 400, 80, and 10 mg of [3H]curcumin [44]. More studies are needed to assess the tissue distribution of curcumin, comparing different administration processes.

1.4.4 Transformation

A major factor that currently limits the bioavailability of curcumin is its high susceptibility to chemical and biochemical transformation into products that are less active than the parent compound. In this section, we therefore provide an overview of the
major chemical and biochemical pathways that have been shown to lead to transformation of curcumin in foods and the GIT.

1.4.4.1 Chemical Transformation

The chemical transformation of curcumin may occur during the processing, storage, or preparation of food products through a variety of mechanisms including oxidation, pH induced instability, and photodegradation.

- Oxidation

Curcumin in phosphate buffer undergoes rapid transformation into bicyclopentadione, a major curcumin degradation product, with the presence of oxygen. It was reported that this oxidation process was pH-dependent, with peak transformation at pH=8, and catalyzed by cyclooxygenase-2 (COX-2) [45]. Despite the rapid oxidation of curcumin into its degradation products, curcumin still exhibits its biological activities strongly. This resulted in a debate whether the activities observed are due to the parent compound or degradation products. However, a recent study comparing the anti-proliferative abilities of curcumin and bicyclopentadione indicated that bicyclopentadione to be significantly less active compared to curcumin [46]. Furthermore, co-addition of redox active antioxidants (such as ascorbate, TBHQ, and Trolox) dramatically increased curcumin stability, which in term also increased its activities. With increasing chemical stability, curcumin efficacy in MC38 colon cancer cell proliferation inhibition significantly increased. These results suggest that the biological activities of curcumin are due to the parent compound, not the degradation products [47].
Furthermore in animal model, co-administration of antioxidant increased the level of curcumin concentration in plasma by around 6 folds [47]. These results further support the importance of stabilizing curcumin to increase its efficacies.

- **pH induced instability**

Curcumin has poor stability in aqueous buffer at physiological pH. A study showed that when added to 0.1 M phosphate buffer at pH 7.2 and incubated in 37 °C, more than 90% curcumin rapidly degraded within 1 h, producing vanillin, ferulic acid, and feruloyl methane as its degradation products [48]. Unlike the autoxidation product bicyclopentadione, the biological activities of vanillin and ferulic acid have been well studied. Both of these compounds have indeed shown to have anti-cancer effects. However, the biological activities of both of them are much less potent compared to curcumin. For example, ferulic acid required a dose range of 100 – 1000 μM did not have any effect on cell proliferation of MDA-MB-231 breast cancer cells, whereas curcumin at a dose as low as 30 μM inhibited around 50% of MDA-MB-231 proliferation. Similar to ferulic acid, vanillin was unable to inhibit proliferation of the same cell line even at a dose as high as 1mM [49]. This indicates how the activities of curcumin are not mediated by its degradation product, but instead, its activities are deactivated because the degradation products possess much less activity compared to its parent compound.

Similarly, HPLC analysis done by Nimiya, et. al, showed that 80-90% of curcumin in phosphate buffer (pH 7.4) degraded after ~12 minute incubation [47]. Another stability study by Kharat, et. al., at different pH indicated that at alkaline pH values (pH ≥7.0) curcumin chemically degraded into its respective degradation products, whereas at acidic
pH values (pH < 7.0) curcumin crystallized, leading to limitation of curcumin bioactivities [50].

- **Photodegradation**

  As curcumin undergoes degradation, the yellow color intensity lowers due to the production of colorless products such as vanillin, ferulic acid, and other small phenolic compounds. With exposure to sunlight, curcumin undergoes much faster degradation [51]. High performance thin layer chromatography (HPTLC) analysis showed that exposure to UV (254 nm) caused curcumin to degrade producing 3 degradation products, whereas exposure to sunlight caused curcumin to degrade producing 5 degradation products [52]. However, a few studies reported that singlet oxygen and superoxide were photogenerated by curumin in biological systems, which may participate in curcumin phototoxicity [53]. More studies on the mechanisms underlying curcumin photodegradation are necessary to further understand possible detrimental effects of curcumin phototoxicity.

### 1.4.4.2 Biochemical Transformation

Our body is designed to metabolize and deactivate any foreign compounds that enter the body, including curcumin. When curcumin enters the body, it will be metabolized, degraded, and deactivated to prevent it from reacting with the bodily systems.

A pharmacokinetic study of curcumin was done where healthy human volunteers were given a single oral dose of 10 g and 12 g of curcumin, which then serum samples were tested for free curcumin, glucuronide conjugate, and sulfate conjugate. The results showed free curcumin level peaked in the plasma for 10 g and 12 g doses at time 3.29 ±
0.43 hr and 6.77 ± 0.83 hr with peak concentrations of 2.3 ± 0.26 μg/mL and 1.73 ± 0.19 μg/mL respectively. Most of the curcumin found in the plasma were in its conjugate forms, where the ratio of glucuronide:sulfate was 1.92:1 [54]. This indicated how curcumin has poor metabolic stability.

Inside our body, after compounds managed to be absorbed by the gastrointestinal tract, there are two types of metabolism processes that occur to deactivate foreign compounds trying to enter our body. The absorbed compounds will first undergo phase 1 metabolism in the gut epithelium cells. After passing through the epithelium cells, some compounds will be pumped back into the lumen due to efflux, and the remaining compounds get transported through the blood circulation to the liver where they undergo phase 2 metabolism.

- **Phase 1 Metabolism**

  During phase 1 metabolism process in the epithelium cells, curcumin undergoes reduction catalyzed by NADPH-reductase yielding dihydrocurcumin, tetrahydrocurcumin, hexahydrocurcumin, and octahydrocurcumin (phase 1 metabolites) [39, 55]; autoxidation process yielding bicyclopentadione [48, 56]; and alkaline hydrolysis reaction yielding minor hydrolysis products ferulic acid, vanillin, ferulaldehyde, and feruloyl methane [49].

- **Phase 2 Metabolism**

  This metabolism process includes conjugation reactions by conjugative enzymes primarily found in liver, kidney, and intestinal mucosa. Through this process, curcumin and its phase 1 metabolites are further metabolized to form glucuronide and sulphate...
conjugates, making it bulkier and more water soluble to be excreted out of the body [57]. A study was done and found that these conjugated products had much less activities in HMVEC-dLy cells than curcumin due to its hydrophilicity [24]. Numerous animal and clinical studies have also found these metabolites to be the major curcumin form in serum, and are largely excreted out through feces and urine [32, 33, 37-39]. This not only indicated the deactivation of curcumin bioactivities through phase 2 metabolism, but also indicated the rapid excretion of curcumin, leading to poor bioavailability of curcumin.

- **Metabolism by Gut Microbiota**

  Until now, curcumin metabolism pathways in the intestinal cells and liver have been intensively studied. However, as recent studies showed the high importance of gut microorganisms in health, the fate of curcumin by intestinal microorganisms is largely unknown.

  One study done by Hassaninasab, et al. discovered a new metabolic pathway of curcumin which involved a unique enzyme produced by the gut microorganism named CurA (NADPH-dependent curcumin or dihydrocurcumin reductase). They were able to isolate the curcumin-metabolizing microorganism from human feces and identified it as E. coli. Through 16S rRNA sequence, the E. coli H10407 strain, O55:H7 strain CB9615, BW2952 strain, and K-12 strain DH10B substrain were determined to have curcumin-converting ability. CurA was responsible for converting curcumin through two reaction steps into dihydrocurcumin and tetrahydrocurcumin. The lack of further reduction products than THC suggested the catalytic reduction by CurA only effective for compounds with C = C double bonds. [58]. Further studies are necessary to understand the importance of gut microbiota in curcumin metabolic fate in the colon.
2.1 Introduction

Tumor metastasis, the process by which tumor cells spread from the primary tumor site to other organs, causes approximate 90% of human cancer deaths [59]. Therefore, compounds which inhibit tumor metastasis are important in cancer therapy. Curcumin, a dietary compound from turmeric, has been shown to have potent anti-metastatic effects. Indeed, several animal studies have shown that curcumin inhibited tumor metastasis of prostate cancer [60-62], breast cancer [63-66], colorectal cancer [67], and pancreatic cancer [68]. In addition, two recent Phase II human clinical trials demonstrated that curcumin is efficacious to reduce the risks of colorectal and pancreatic cancer in some patients [69, 70]. Together, these results support that curcumin is a highly promising dietary compound for cancer prevention and/or treatment.

Curcumin has been shown to suppress tumor metastasis through multiple possible mechanisms. Previous studies demonstrated that curcumin inhibits migration and invasion of cancer cells, which are critical cellular process involved in tumor metastasis [71]. Curcumin has also been shown to modulate the expressions of inflammatory cytokines and microRNAs in cancer cells, leading to reduced metastasis [61, 62, 67, 72]. Besides directly targeting cancer cells, curcumin could also target tumor-associated endothelial cells, which results in reduced tumor angiogenesis and associated tumor progression and metastasis [73]. However, until now the mechanisms by which curcumin inhibits tumor metastasis are not well understood. A better understanding of the
underlying mechanisms could facilitate the development of better therapeutic paradigms, leading to effective implementation of curcumin to reduce cancer risks.

Lymphatic vessels are known to serve as a major route for cancer cells to escape from the primary tumor site and spread to other organs in the body. Recent developments in lymphatic biology, in particular the discoveries of molecular markers specific for the lymphatic endothelial cells, such as lymphatic vessel endothelial hyaluronan receptor (LYVE-1), have demonstrated that de novo lymphatic formation (lymphangiogenesis) plays a critical role in tumor metastasis [74]. Cancer cells can secrete various lymphangiogenesis inducers, which act on lymphatic endothelial cells to enhance formation of lymphatic vessels and accelerate tumor metastasis. Vascular endothelial growth factor-C (VEGF-C) is the most important lymphangiogenesis inducer involved in tumor metastasis. Indeed, overexpression of VEGF-C in breast cancer cells stimulates tumor lymphangiogenesis and enhances metastasis to regional lymph nodes and lungs [75]. Blocking VEGF-C signaling, through abolishing its receptor VEGF receptor 3 (VEGFR3), suppresses tumor lymphangiogenesis and associated tumor metastasis [76]. Together, these studies support a critical role of lymphangiogenesis, in particular VEGF-C-induced lymphangiogenesis, in promoting tumor metastasis.

Until now the actions of curcumin on lymphangiogenesis are largely unknown. To this end, we investigated the effects and mechanisms of curcumin on lymphangiogenesis, using primary human lymphatic endothelial cells and animal models.
2.2 Materials and methods

2.2.1 Materials and cell culture

Curcumin was purchased from Thermal Fisher Scientific (Waltham, MA). Two curcumin metabolites, curcumin sulfate and curcumin glucuronide, were purchased from TLC PharmaChem (Vaughan, Ontario, Canada). Human dermal microvascular lymphatic endothelial cells (HMVEC-dLy, purchased from Lonza, Allendale, NJ) were cultured in EBM-2 medium with supplements according to the manufacturer’s instructions. Assays with HMVEC-dLy cells were conducted with cells from passages 2–6.

2.2.2 Cell proliferation assay

HMVEC-dLy cells were seeded in 96-well plates at a cell density of 4,000 cells per well and were allowed to attach overnight. The cells were treated with curcumin or DMSO vehicle (0.1%) for 24-48 h, cell viability was measured using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich, St. Louis, MO).

2.2.3 Tube formation assay

The 96-well plates were coated with 35 μL per well of growth-factor reduced Matrigel (BD Biosciences, San Jose, CA), after the gel was solidified at 37°C for 30 min, 2.5 × 10^4 HMVEC-dLy cells in 100 μL basal medium containing 100 ng/mL VEGF-C (R&D Systems, Minneapolis, MN) were added to each well. After a 6-h treatment, microscope images were recorded and tube formation was quantified using Wimasis Image Analysis (http://www.wimasis.com).
2.2.4 Boyden chamber cell migration assay

Cell culture inserts (for 24-well plate) containing membrane with 8-μm pores were coated with 15 μg/mL collagen (Thermal Fisher Scientific, Waltham, MA) at 37°C for 1 h. HMVEC-dLy cells (10^5 cells per well) were seeded onto the upper chamber of the inserts in 200 μL basal medium; the bottom chamber was filled with 500 μL complete EBM-2 medium as chemoattractant. Curcumin or DMSO vehicle was added into both top and bottom chambers. After a 12-h incubation, the inserts were washed by PBS, fixed by 5% glutaraldehyde for 15 min, the uninvaded cells were removed by cotton swabs, the invaded cells were stained with crystal violet and microscope images were recorded. Cell counting was carried out using ImageJ software.

2.2.5 Gelatin zymography

HMVEC-dLy cells were seeded in 96-well plates and allowed to attach overnight, the cells were treated with curcumin or DMSO vehicle, then matrix metalloproteinase-2 (MMP-2) and MMP-9 activities in the cell culture medium were analyzed using gelatin zymography, as we described previously [77, 78].

2.2.6 Cell cycle analysis

HMVEC-dLy cells were treated with curcumin or DMSO vehicle for 48 h, then the cells were collected, washed by ice-cold PBS and fixed in 70% ethanol at -20°C overnight. The fixed cells were centrifuged, washed by PBS and stained with propidium iodide solution (10 μg/mL propidium iodide, 100 μg/mL RNAse) for 30 min at room temperature. After staining, the cells were analyzed using BD LSRFortessa™ cell analyzer (BD Biosciences) and data were processed using FlowJo software.
2.2.7 Immunoblotting

HMVEC-dLy cells were treated with curcumin or DMSO vehicle in complete medium for 48 h, then the medium was decanted, the cells were washed with cold PBS and lysed, the cell lysates were resolved using SDS/PAGE and transferred onto a nitrocellulose membrane. The membranes were blocked in Odyssey Blocking Buffer (LI-COR, Lincoln, NE) for 1 h at room temperature and probed with antibodies against VEGFR2, VEGFR3, phosphor-ERK1/2 (Thr202/Tyr204), phosphor FAK (Tyr925), and total ERK1/2 and FAK from Cell Signaling Technology (Danvers, MA), cyclin D1 from Santa Cruz Biotechnology (Santa Cruz, CA), and β-actin from Sigma-Aldrich (St. Louis, MO). The membranes were then probed with LI-COR IRDye® 800CW Goat anti-Rabbit and IRDye® 680RD Goat anti-Mouse secondary antibodies, and then detected using Odyssey imaging system (LI-COR). Western blot quantification was performed using Image Studio™ Lite Software (LI-COR).

2.2.8 Matrigel plug assay of lymphangiogenesis

The animal experiment was conducted in accordance with the protocols approved by the Institutional Animal Care and Use Committee (IACUC) of University of Massachusetts Amherst. Briefly, 0.25 mL growth factor-reduced Matrigel (BD Biosciences), which was pre-mixed with 1000 ng VEGF-C (R&D Systems) and curcumin or DMSO vehicle, was subcutaneously injected into 6-week-old C57BL/6 male mice in the abdominal area. After 7 days, the mice were euthanized to dissect the implanted Matrigel plugs. The plugs were digested using Corning® cell recovery solution (Corning, NY), filtered through 70 μm cell sorters (BD Biosciences) to obtain single cell suspension, which were stained with rat anti-mouse LYVE-1 phycoerythrin-conjugated
monoclonal antibody or isotype control antibody (R&D Systems). The stained cells were analyzed using BD LSRFortessa™ cell analyzer (BD Biosciences) and data were processed using FlowJo software.

2.2.9 Statistics

Group comparisons were carried out using one-way analysis of variance or Student t test. P values less than 0.05 were considered statistically significant.

2.3 Results

2.3.1 Curcumin inhibits VEGF-C-induced lymphangiogenesis in vivo

To evaluate the effect of curcumin on lymphangiogenesis, we studied lymphangiogenesis using a Matrigel plug assay in C57BL/6 mice. VEGF-C is the most important lymphangiogenesis inducer [75]. Implantation of Matrigel plugs containing 1000 ng VEGF-C in mice triggered a robust lymphangiogenic response, with an approximate 4-fold increase of infiltration of LYVE-1-positive lymphatic endothelial cells into the implanted plugs. Co-addition of curcumin in the Matrigel abolished VEGF-C-induced lymphangiogenesis, demonstrating the anti-lymphangiogenic action of curcumin in vivo (Figure 1).
Figure 1: Curcumin inhibits VEGF-C-induced lymphangiogenesis in a Matrigel plug assay in mice.

Matrigel plugs containing DMSO vehicle, 1000 ng VEGF-C, or 1000 ng VEGF-C plus 9 μg curcumin, were implanted in C57BL/6 mice; after 1-wk treatment, the plugs were digested and the liberated cells were subjected to flow cytometry analysis. (Top) Representative flow cytometry analysis of liberated cells from Matrigel plugs. (Bottom) Quantification of lymphangiogenesis in the Matrigel plugs. Y-axis is expressed as percentage of LYVE-1-positive lymphatic endothelial cells to the total cells in the Matrigel plugs. n = 4-8 Matrigel plugs per group, * P<0.05, the results are mean ± SD.
2.3.2 Curcumin inhibits proliferation and cell cycle progression of lymphatic endothelial cells

We studied whether curcumin had direct anti-lymphangiogenic effect in lymphatic endothelial cells. Curcumin inhibited proliferation of HMVEC-dLy cells in a dose- and time-dependent manner. At a dose of ~10 µM, curcumin had little effect on cell proliferation after a 24-h treatment in complete medium (Figure 2A), while it inhibited ~70% of cell proliferation after a 48-h treatment (Figure 2B). Curcumin also inhibited VEGF-C-induced cell proliferation in HMVEC-dLy cells: addition of 300 ng/mL VEGF-C increased ~50% of HMVEC-dLy proliferation after a 48-h treatment, which was suppressed by curcumin in a dose-dependent manner (Figure 3).

Since curcumin potently inhibited proliferation of lymphatic endothelial cells, we investigated the effect of curcumin on cell cycle progression. Flow cytometry analysis shows that curcumin caused a G2 arrest in HMVEC-dLy cells, increasing cell population in G2 stage from 6.3±1% to 16.4±2.5% after a 48-h treatment with 10 µM curcumin (Figure 2C). This is consistent with previous studies which showed that curcumin causes G2 arrest in multiple cell lines [79-81].
Figure 2: Curcumin inhibits proliferation and cell cycle progression of HMVEC-dLy cells.

(A) Curcumin inhibits cell proliferation of HMVEC-dLy cells in a time- and dose-dependent manner. The cells were treated with curcumin or DMSO vehicle (0.1%) in complete EBM-2 medium for 24-48 h, cell proliferation was assessed by MTT assay. (B) Representative microscope images of HMVEC-dLy cells which were treated with curcumin or DMSO vehicle in complete medium for 48 h, scale bar: 200 µm. (C) Curcumin causes a G2 cell cycle arrest in HMVEC-dLy cells, HMVEC-dLy cells were treated with 10 μM curcumin in complete medium for 48 h. (Left) Representative images of flow cytometry analysis. (Right) Quantification of cell population at different stages of cell cycle. * P<0.05, the results are mean ± SD.
Figure 3: Curcumin inhibits VEGF-C-induced cell proliferation of HMVEC-dLy cells.

The cells were treated with curcumin in basal medium containing 300 ng/mL VEGF-C for 48 h, then cell proliferation was assessed by MTT assay.
2.3.3 Curcumin inhibits tube formation of lymphatic endothelial cells

To test whether curcumin inhibited lymphangiogenesis through mechanisms other than its inhibitory effect on cell proliferation, we further characterized the action of curcumin on lymphangiogenic responses. After a 6-h treatment with 3-10 μM curcumin (during this treatment period, curcumin has no effect on cell proliferation, see Figure 2A), curcumin inhibited VEGF-C-induced tube formation of HMVEC-dLy cells in a dose-dependent manner. At a dose of 3 and 10 μM, curcumin reduced total tube length by ~20% and ~50% respectively, and reduced branching points by ~40% and ~90% respectively, demonstrating its anti-lymphangiogenic effect in vitro (Figure 4).
Figure 4: Curcumin inhibits tube formation in HMVEC-dLy cells.

(A) Representative microscope images of HMVEC-dLy cells. The cells (plated on Matrigel) were treated with curcumin or DMSO vehicle (0.1%) in basal EBM-2 medium containing 100 ng/mL VEGF-C for 6 h, scale bar: 100 µm. (B) Quantification of total tube length. (C) Quantification of number of branching points. * P<0.05, the results are mean ± SD.
2.3.4 Curcumin inhibits migration of lymphatic endothelial cells

In a Boyden chamber cell migration assay, curcumin at 10 µM inhibited ~57% of complete EBM-2 endothelial medium-induced cell migration in HMVEC-dLy cells after a 12-h treatment (Figure 5). During this treatment period (12 h), curcumin at 10 µM had no effect on proliferation of HMVEC-dLy cells (see Figure 2A), suggesting the inhibitory effect of curcumin on cell migration was independent of its inhibition on cell proliferation.
Figure 5: Curcumin inhibits HMVEC-dLy cell migration.

HMVEC-dLy cells were treated with curcumin or vehicle DMSO in a Boyden chamber assay for 12 h, using complete EBM-2 medium as the chemoattractant. (Top) Representative microscope images of migrated HMVEC-dLy cells which were stained with crystal violet. (Bottom) Quantification of migrated HMVEC-dLy cells. * P<0.05, the results are mean ± SD.
2.3.5 Curcumin has little effect on MMP activities of lymphatic endothelial cells

In terms of MMP activities, gelatin zymography showed that curcumin (0.78-25 μM) had little effect on MMP-2 or -9 activity after 24-48 h treatment in complete or basal medium (Figure 6A-B). We further analyzed whether curcumin directly inhibited enzymatic activities of MMPs, by adding curcumin (10 μM) into the developing buffer of zymography; and zymography analysis also showed little effect of curcumin on MMP-2 and -9 activity (Figure 6C-D).
Figure 6: Curcumin has little effect on MMP-2 or MMP-9 activity in HMVEC-dLy cells.

(A) A simplified scheme of zymography protocol 1: HMVEC-dLy cells were treated with DMSO vehicle or curcumin in complete or basal medium for 24-48 h, then MMP-2 and MMP-9 activities in the cell culture medium were analyzed by gelatin zymography. (B) Representative images of stained zymography gels from protocol 1, which showed that curcumin had little effect on MMP-2 or MMP-9 activity. (C) A simplified scheme of zymography protocol 2: HMVEC-dLy cells were maintained in complete medium, cell culture medium was subjected to a standard zymography assay procedure, except in the zymography developing stage, the gels were incubated in zymography developing buffer containing DMSO vehicle (0.1%) or curcumin overnight. (D) Representative images of stained zymography gels from protocol 2, which showed that curcumin also had little effect on MMP-2 or MMP-9 activities.
2.3.6 Curcumin inhibits expressions of VEGF receptors (VEGFR2 and VEGFR3) and down-stream signaling pathways in lymphatic endothelial cells

Because VEGF receptor 2 (VEGFR2) and receptor 3 (VEGFR3) play critical roles in lymphangiogenesis, we studied the actions of curcumin on VEGFR2 and VEGFR3. After a 12-h (Figure 7A) or 48-h (Figure 7B) treatment of 3-10 μM curcumin in HMVEC-dLy cells, curcumin (10 μM) dramatically inhibited expressions of VEGFR2 and VEGFR3, as well as down-stream signaling proteins such as phosphorylation of ERK and FAK, while it did not have any effect on expressions of total ERK and FAK. In Figure 7A, during the 12-h treatment period, curcumin at the tested doses had no effect on proliferation of HMVEC-dLy cells (see Figure 2A), suggesting the inhibitory effects of curcumin on VEGF receptor and signaling proteins were not due to its cytotoxic effect. We conducted a time-course study to further investigate the effect of curcumin on VEGFR2 expression. The result showed that curcumin dramatically inhibited VEGFR2 expression after a treatment period as short as 1 h (Figure 7C). Curcumin also inhibited expression of cyclin D1 (Figure 7A-B), consistent with its inhibitory effect on cell cycle progression.
Figure 7: Curcumin inhibits expressions of VEGFR2 and VEGFR3, and down-stream signaling proteins (phosphorylation of ERK and FAK), as well as cyclin D1, in HMVEC-dLy cells.

(A) HMVEC-dLy cells were treated with DMSO vehicle or curcumin in complete medium for 12 h, then protein expressions were analyzed. During this treatment period (12 h), curcumin at 3-10 µM had no effect on proliferation of HMVEC-dLy cells. (B) The cells were treated with DMSO or curcumin in complete medium for 48 h and protein expressions were analyzed. (C) Time-course effect of curcumin on VEGFR2 expression in HMVEC-dLy cells. The immunoblotting analysis was repeated in at least three independent experiments. The relative expressions of VEGFR2, VEGFR3 and cyclin D1 are normalized to β-actin, and the relative expressions of phosphor-ERK and phosphor-FAK are normalized to total ERK and FAK, respectively. * P<0.05, the results are mean ± SEM.
2.3.7 Two major metabolites of curcumin, curcumin sulfate and curcumin glucuronide, have little inhibitory effect on proliferation of lymphatic endothelial cells

Curcumin sulfate and curcumin glucuronide are the two major metabolites of curcumin (Figure 8A) [82, 83]. We studied the effects of these two metabolites on lymphangiogenesis in HMVEC-dLy cells. At a dose range of 10-30 μM, curcumin sulfate and curcumin glucuronide had little inhibitory effect on proliferation of HMVEC-dLy cells, while curcumin potently inhibited cell proliferation (Figure 8).
Figure 8: Curcumin sulfate and curcumin glucuronide have little inhibitory effect on proliferation of lymphatic endothelial cells.

(A) A simplified scheme for the metabolism of curcumin to form curcumin sulfate and curcumin glucuronide. (B) Curcumin sulfate and glucuronide have little inhibitory effect on proliferation of HMVEC-dLy cells. (Left) Representative microscope images of HMVEC-dLy cells which were treated with 10 μM test compound in complete EBM-2 medium for 48 h, scale bar: 100 μm. Curcumin caused cell morphology change, while curcumin sulfate and glucuronide had little effect. (Right) Quantification of HMVEC-dLy cell proliferation after 48-h treatment using MTT assay. * P<0.05, the results are mean ± SD.
2.4 Discussion

The central finding of this study is that curcumin inhibits lymphangiogenesis. The process of lymphangiogenesis involves several cellular steps, including cellular proliferation, migration, and production of MMPs in lymphatic endothelial cells. Here our data shows that curcumin inhibited lymphangiogenesis, in part through suppression of proliferation, cell cycle progression and migration of lymphatic endothelial cells, while it had little effect on MMP activities.

VEGF-C is the first identified lymphangiogenesis inducer, and is among the most important inducers involved in tumor lymphangiogenesis and metastasis. Indeed, overexpression of VEGF-C in cancer cells stimulates lymphangiogenesis and tumor metastasis [75], while blocking VEGF-C signaling inhibits these processes [76]. Human studies have also shown that serum level of VEGF-C is a promising biomarker for cancer diagnosis [84]. Currently VGX-100, a monoclonal antibody of VEGF-C, is in Phase I human clinical trial for cancer therapy [85]. Here our results showed that curcumin suppressed VEGF-C-induced proliferation and tube formation in HMVEC-dLy cells, and VEGF-C-induced infiltration of lymphatic endothelial cells in a Matrigel plug assay in mice. These results demonstrate that curcumin inhibited VEGF-C-induced lymphangiogenesis in vitro and in vivo.

VEGF-C acts though binding to its cellular receptors VEGFR2 and VEGFR3, which leads to phosphorylation of VEGFR2 and VEGFR3 and activation of down-stream signaling pathways. Many lymphangiogenesis or angiogenesis inhibitors are VEGF receptor Tyrosine kinase inhibitors, which inhibit phosphorylation of VEGFR3 or VEGFR2 [86]. Examples of these kinase inhibitors include some widely used anti-cancer
drugs such as sorafenib (Nexavar®) and regorafenib (Stivarga®) [87-90]. Here our study showed a novel mechanism by which curcumin modulates VEGFR2 and VEGFR3 signaling, through inhibiting expressions of these two critical receptors. Consistent with our findings, a recent animal study showed that curcumin significantly inhibited protein and mRNA expressions of VEGFR3 in gastric tumor tissues [91]. Since VEGFR3 is a biomarker of lymphatic endothelial cells [74], this study suggest that curcumin treatment could also reduce tumor lymphangiogenesis. The degradation of VEGF receptors was mainly mediated by the ubiquitin-proteasome system [92]. A recent study showed that curcumin can induce ubiquitin-activating enzyme, which is the enzyme to catalyze the first step of the ubiquitination reaction, leading to down-regulation of epidermal growth factor receptor [93]. Therefore, it is likely that curcumin could also inhibit the expressions of VEGF receptors through an ubiquitin-dependent mechanism. Since curcumin inhibits VEGFR2/VEGFR3 expressions and VEGF receptor Tyrosine kinase inhibitors inhibit phosphorylation of VEGFR2/VEGFR3, their combination could synergistically inhibit tumor angiogenesis and lymphangiogenesis. Many of these kinase inhibitors, such as sorafenib and regorafenib, have severe adverse effects, limiting their clinical applications [87-90]. This synergistic interaction could be utilized to develop novel strategies to increase the efficacy and/or reduce the toxicity of these important anti-cancer drugs.

Consistent with the reduced inhibitions of VEGFR2 and VEGFR3, curcumin also inhibited VEGF receptor down-stream signaling pathways, such as phosphorylation of FAK and ERK (Figure 7). We need to point out phosphorylation of these signaling proteins (FAK and ERK) are controlled by multiple pathways, curcumin could inhibit
these signaling proteins through VEGFR2- and VEGFR3-independent mechanisms. FAK has been shown to play a critical role in cell migration [94], and ERK has been shown to regulate cell proliferation [95, 96]. Therefore, the inhibitory effects of curcumin on these signaling proteins are consistent with its suppression of cell migration and proliferation of lymphatic endothelial cells.

A major barrier to use curcumin for cancer prevention and treatment is its poor bioavailability in vivo. After oral intake of curcumin, free-form curcumin was barely detected in circulation, curcumin sulfate and curcumin glucuronide are the two major metabolites of curcumin [82, 83]. Because of this limitation, high doses of curcumin were used in animal and human studies. In a recent Phase II human clinical trial of colorectal cancer, a dose of 4 grams of curcumin per day was used, which caused ~40% reduction of aberrant crypt foci [69]. In another Phase II human trial, daily intake of 8 grams of curcumin was used and curcumin was efficacious in some patients with advanced pancreatic cancer [70]. Here our results showed that curcumin sulfate and curcumin glucuronide, which are the two major water-soluble metabolites of curcumin in vivo, had little inhibitory effect on proliferation of HMVEC-dLy cells (Figure 8), suggesting that these two metabolites don’t contribute to the anti-lymphangiogenic effect of curcumin. Our results are consistent with previous studies which showed that these two metabolites were less-active or inactive in many assays [97-99]. It remains to determine the biological activities of other curcumin metabolites on lymphangiogenesis; however, many studies suggest that curcumin, instead of its metabolites, plays a major role in the observed health-promoting biological activities [97-99]. Novel strategies which enhance delivery of curcumin to its target tissues (such as lymphatic vessels) and/or bypass un-wanted
metabolism could be important to effectively implement curcumin for cancer prevention and treatment.

In conclusion, our study demonstrates that curcumin inhibited lymphangiogenesis in vitro and in vivo, through a novel mechanism to inhibit expressions of VEGFR2 and VEGFR3. Lymphangiogenesis has been established to play a critical role in tumor metastasis [74]; therefore, our study suggests that curcumin could suppress tumor metastasis through inhibition of lymphangiogenesis.

In the future, it would be interesting to study whether curcumin can suppress VEGF-C-mediated tumor metastasis, which could increase our understanding for the effects and mechanisms of curcumin on tumor metastasis.
CHAPTER 3

OXIDATIVE CONVERSION MEDIATES ANTI-PROLIFERATIVE EFFECTS OF TERT-BUTYLHYDROQUINONE (TBHQ):

STRUCTURE AND ACTIVITY RELATIONSHIP STUDY

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3.1 Introduction

Tert-butylhydroquinone (TBHQ) is an antioxidant widely used in many food products such as vegetable oils, animal fats, and meat products; as well as some cosmetic products [100, 101]. TBHQ could also be formed metabolically from another food antioxidant 3-tert-butyl-hydroxyanisole (BHA) [102]. However, emerging studies showed that TBHQ could have adverse effects on human health. Indeed, TBHQ has been shown to be cytotoxic to many types of cells such as V79 Chinese hamster lung cells, U937 human monocytic leukemia cells, human lymphocytes, A549 lung cancer cells, human endothelial cells, and MDCK cells [103-107]. TBHQ has also been shown to be genotoxic through its ability to intercalate between DNA base pairs and generate reactive oxygen species (ROS), leading to mutagenesis, cleavage and fragmentation of DNA [102-104, 106, 108, 109].

However, the effects of TBHQ on cancer are controversial: many studies showed that TBHQ promoted carcinogenesis in cell culture and animal models, which could be due to its cytotoxic and genotoxic properties [110-113]; while other studies showed that it inhibited carcinogenesis, likely due to induction of Phase II detoxification enzymes [111, 114].
The underlying mechanisms of the biological activities of TBHQ are not well understood. Previous studies have shown that TBHQ induces Nrf2-mediated phase II detoxification enzymes through oxidative conversion to a quinone metabolite termed tert-butylquinone (TBQ) [114]. TBQ, instead of TBHQ, directly interacts with the cysteine residues of Keap1 protein, which is a negative regulator of Nrf2 signaling; therefore, the oxidative conversion of TBHQ to TBQ leads to activation of Nrf2 signaling [114]. It remains to determine whether TBHQ exerts its other biological activities through oxidative conversion to TBQ. In terms of the cytotoxic and genotoxic effects of TBHQ, previous studies showed that TBQ has more potent actions compared with TBHQ [103-105], suggesting a potential role of TBQ formation in the cytotoxic and genotoxic effects of TBHQ. Here we modulated the oxidative conversion of TBHQ in MC38 colon cancer cells, in order to study the roles of TBHQ oxidation and TBQ formation in the effects of TBHQ on cell proliferation, cell cycle progression and apoptosis. In addition, we conducted a structure and activity relationship (SAR) study to better understand the oxidative conversion of TBHQ and other para-hydroquinone compounds.

3.2 Materials and Methods

3.2.1 Chemicals

The compounds used in this study, including tert-butylhydroquinone (TBHQ) and its analogs, tert-butylquinone (TBQ), copper sulfate (CuSO₄), and ethylenediaminetetraacetic acid (EDTA), were purchased from Thermal Fisher Scientific (Waltham, MA) or Sigma-Aldrich (St. Louis, MO).
3.2.2 Cell Culture

MC38 colon cancer cells (a kind gift from Prof. Ajit Varki at the University of California San Diego) were routinely cultured in Dulbecco's Modified Eagle Medium (DMEM, Lonza, Allendale, NJ) supplemented with 10% Fetal Bovine Serum (FBS, Corning Inc., Corning, NY) in a 37 °C incubator under an atmosphere with 5% CO₂.

3.2.3 Cell proliferation assay

MC38 cells were plated into 96-well plates (8,000 cells per well) in 100 µL DMEM complete medium and allowed to attach overnight. The cells were then treated with the test compounds in complete medium or basal medium (without FBS) for 24 h. Cell proliferation was assessed by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay, as we reported [115].

3.2.4 Flow cytometry analysis of cell cycle and apoptosis

MC38 colon cancer cells were plated in 6-well plates (200,000 cells per well) in 1.5 mL DMEM complete medium and allowed to attach overnight. The cells were then treated with the test compounds in complete medium or basal medium (without FBS) for 24 h. For cell cycle analysis, the cells were collected, washed by ice-cold PBS, and fixed in 70% ethanol at -20°C overnight. The fixed cells were centrifuged, washed by PBS, and stained with propidium iodide solution (10 µg/mL propidium iodide, 100 µg/mL RNAse) for 30 min at room temperature. For apoptosis analysis, the cells were collected, washed by ice-cold DMEM fortified with 2% FBS medium, centrifuged, and stained with propidium iodide and Annexin V solution (10 µg/mL propidium iodide, 0.5 µg/mL Annexin V) for 10 min at room temperature. After staining, the cells were analyzed using
BD LSRFortessa™ cell analyzer (BD Biosciences) and data were processed using FlowJo software.

### 3.2.5 HPLC analysis of TBHQ oxidation

Solutions of 50 μM TBHQ, with or without Cu$^{2+}$ or EDTA, were freshly prepared in basal DMEM medium. At different time points, the formed TBQ was analyzed by HPLC. The HPLC analysis was conducted on an Agilent 1100 HPLC system, using an Agilent TC-C18(2) column (4.6 x 250 mm, 5 μm) eluted with a mobile phase of 65% phase B (methanol with 0.1% acetic acid) and 35% phase A (water with 0.1% acetic acid), flow rate = 1 mL/min, detection wavelength at 254 nm. The concentration of TBQ was calculated against the calibration curve with standard (TBQ could be detected at a dose as low as 0.12 μM).

### 3.2.6 UV absorbance analysis of oxidation of TBHQ and its analogs

Solutions of 100 μM of TBHQ and other hydroquinones were freshly prepared in phosphate buffer (pH = 7.5) and placed into micro quartz cuvettes (Thor Labs, Newton NJ). For kinetics studies, the UV absorbance of the solutions were analyzed in 2 min intervals for 1 h at the detection wavelength 250 nm, using a Spectra Max M2 plate reader (Molecular Devices). For UV spectrum analysis, absorbance of the solutions were analyzed at different time points at the detection wavelengths ranging from 200-600 nm.

### 3.2.7 Statistics

Group comparisons were carried out using one-way analysis of variance or Student t test. P values less than 0.05 were considered statistically significant. The results are mean ± SD, derived from at least three independent experiments.
3.3 Results

3.3.1 Co-addition of Cu\textsuperscript{2+} and EDTA modulates oxidative conversion of TBHQ to TBQ

Previous studies have shown that Cu\textsuperscript{2+} accelerates TBHQ oxidation through a Fenton reaction, while EDTA suppresses this process by quenching transition metal ions [114]. Here we used HPLC to validate the roles of Cu\textsuperscript{2+} and EDTA on TBHQ oxidation (Figure 9). HPLC analysis of a freshly prepared solution of 50 µM TBHQ in basal DMEM medium at room temperature showed that TBHQ was rapidly converted to its quinone metabolite TBQ: after 2 hours of incubation, ~26% of TBHQ was converted to TBQ. Co-addition of Cu\textsuperscript{2+} (50 µM) significantly accelerated this process: after 2 hours of incubation, ~47% of TBHQ was converted to TBQ (~80% increase compared with TBHQ alone). In contrast, co-addition of EDTA (50 µM) inhibited TBHQ oxidation, with ~19.7% of TBHQ converted to TBQ after 2-h incubation (~25% inhibition compared with TBHQ alone). This result showed that co-addition of Cu\textsuperscript{2+} and EDTA modulates the oxidation of TBHQ to generate TBQ.
**Figure 9:** Co-addition of Cu$^{2+}$ enhances, while EDTA suppresses, the oxidative conversion of TBHQ to TBQ.

(A) Representative HPLC chromatography: a solution of 50 μM TBHQ was freshly prepared in basal DMEM medium and was subjected to HPLC analysis, detection wavelength is 254 nm (TBHQ had weak absorbance at 254 nm, and its peak was not observed on HPLC at this detection wavelength). (B) Quantification of TBHQ oxidation to generate TBQ. The results are mean ± SD, * P<0.05.
3.3.2 Co-addition of Cu\(^{2+}\) and EDTA modulates the effect of TBHQ on cell proliferation

After we demonstrated that Cu\(^{2+}\) and EDTA modulated TBHQ oxidation, we used these two compounds to study the roles of TBHQ oxidation in its biological activities. When the cellular treatments were conducted in basal DMEM medium, co-addition of Cu\(^{2+}\) enhanced, while EDTA attenuated, the anti-proliferative effects of TBHQ in MC38 cells. Treatment with low-dose TBHQ (1.88-7.5 µM) had little effect on cell proliferation, while co-addition of Cu\(^{2+}\) (50 µM) significantly enhanced its anti-proliferative effect, with 30-70% inhibition of cell proliferation. As a control, treatment with Cu\(^{2+}\) alone had no effect on MC38 proliferation (Figure 10A). In contrast to the enhancing effect of Cu\(^{2+}\), co-addition of EDTA (50 µM) suppressed the anti-proliferative effect of TBHQ: treatment with high-dose TBHQ (15 µM) inhibited 50±4% of MC38 proliferation, which was abolished by co-addition of EDTA (Figure 10A). A similar trend was also observed when the cellular treatments were conducted in complete medium (Figure 10B). Together, these results suggest that TBHQ oxidation plays a major role in mediating the anti-proliferative effect of TBHQ.
**Figure 10:** Co-addition of Cu$^{2+}$ and EDTA modulates the anti-proliferative effect of TBHQ in MC38 colon cancer cells.

The cellular treatments are conducted in basal medium (A) and complete medium (B). The results are mean ± SD, * P<0.05.
3.3.3 Co-addition of Cu$^{2+}$ modulates the effect of TBHQ on cell cycle and apoptosis

Consistent with the modulating effects of Cu$^{2+}$ on anti-proliferative effect of TBHQ, we found that Cu$^{2+}$ also enhanced the effects of TBHQ on cell cycle progression and apoptosis, which are two critical processes involved in regulating cell proliferation. After 24 h incubation in basal medium, treatment with 15 µM TBHQ alone or 50 µM Cu$^{2+}$ alone had little effect on cell cycle progression in MC38 cells, while their combination caused a ~2-fold increase of the cell population in the G2 stage (Figure 11A). A similar result was also observed when the cellular treatments were performed in complete medium: treatment with 30 µM TBHQ alone or 50 µM Cu$^{2+}$ alone had little effect on cell cycle progression, while their combination caused a ~70% increase of the cell population in the G2 stage (Figure 11B).

In terms of apoptosis, after 24 h incubation in basal medium, treatment with 15 µM TBHQ alone or 50 µM Cu$^{2+}$ alone had little effect on apoptosis, while their combination significantly increased early-stage, late-stage and total apoptosis (Figure 11C). A similar result was also observed when the cellular treatments were performed in complete medium (Figure 11D). Together, these results further support that TBHQ oxidation plays a critical role in mediating its biological activities.
Figure 11: Co-addition of Cu\(^{2+}\) enhances the effects of TBHQ on cell cycle and apoptosis.

(A-B) Co-addition of Cu\(^{2+}\) enhances the effects of TBHQ on cell cycle. MC38 cells were treated with 15 µM TBHQ, 50 µM Cu\(^{2+}\), or a combination of TBHQ and Cu\(^{2+}\) in basal medium (A) and 30 µM TBHQ, or 50 µM Cu\(^{2+}\), or a combination of TBHQ and Cu\(^{2+}\) in complete medium (B) for 24 h, then cell cycle was analyzed. (C-D) Co-addition of Cu\(^{2+}\) enhances the effects of TBHQ on apoptosis. MC38 cells were treated with 15 µM TBHQ, 50 µM Cu\(^{2+}\), or a combination of TBHQ and Cu\(^{2+}\) in basal medium (C) and 15 µM TBHQ, 50 µM Cu\(^{2+}\), or a combination of TBHQ and Cu\(^{2+}\) in complete medium (D) for 24 h, then apoptosis was analyzed. The results are mean ± SD, * P<0.05.
3.3.4 Comparative biological activities of TBHQ and TBQ

To further study the role of TBHQ oxidation in its biological activities, we compared the biological activities of TBHQ with its oxidation product TBQ. In both basal and complete medium, TBQ showed more potent effects on cell proliferation, cell cycle progression, and apoptosis. After 24 h treatment in basal medium, TBQ and TBHQ at a dose of 15 µM suppressed 90.5±2.8% and 34.4±7.7% of MC38 cell proliferation, respectively (Figure 12A). TBHQ at 15 µM had little effect on cell cycle progression, while TBQ caused ~80% increase of the cell population in the G2 stage (Figure 12B), which is well consistent with the G2 cell cycle arrest caused by the combination of TBHQ and Cu²⁺ (see Figure 10B). TBHQ at 15 µM had little effect on apoptosis, while TBQ significantly increased early-stage, late-stage, and total apoptotic cells (Figure 12C). A similar result was also observed when the cellular treatments were performed in complete medium (Figure 12D-F). Together, these results further support a critical role of TBHQ oxidation in its biological activities: oxidative metabolism of TBHQ leads to formation of TBQ with enhanced biological activities.
Figure 12: Compared to TBHQ, TBQ has more potent effects on cell proliferation, cell cycle progression, and apoptosis in MC38 cells.

(A-C) TBQ has more potent effects on cell proliferation (A), cell cycle progression (B), and apoptosis (C) after 24 h treatment in basal medium. (D-F) TBQ has more potent effects on cell proliferation (D), cell cycle progression (E), and apoptosis (F) after 24 h treatment in complete medium. The results are mean ± SD, * P<0.05.
3.3.5 Structure and activity relationship (SAR) of TBHQ for oxidative conversion and biological activity

To better understand the oxidative mechanism of TBHQ, we studied the oxidative conversion and biological activities of several TBHQ analogs. Compared with TBHQ, these analogs have the same para-hydroquinone core structure, but have substitution groups with different electron donating properties (-CH$_3$, -CH$_3$, -C$_6$H$_5$, -H, and -Br groups, see chemical structures in Figure 13A).

First, we used UV spectrum to study spontaneous oxidation of these compounds in aqueous buffer, as previous studies showed that when hydroquinones are oxidized to quinones, there is a significant increase of absorbance at 250 nm (see example of TBHQ in Figure 13B) [114]. A kinetics study using UV spectrum showed that the rate of spontaneous oxidation was in the order of: methoxy-hydroquinone > methyl-hydroquinone > TBHQ > phenyl-hydroquinone > bromo-hydroquinone ≈ hydroquinone, which was highly correlated with electron donating properties of the substitution groups (Figure 13C) [116]. Next, we studied the anti-proliferative effects of these compounds in MC38 cells. In both basal and complete medium, the fast-oxidized compounds, such as methoxy-hydroquinone and methyl-hydroquinone, showed potent inhibitory effects on cell proliferation; while the slow-oxidized compounds, such as bromo-hydroquinone and hydroquinone, showed weak or little activities (Figure 13D-E). Together, the SAR study further supported that the biological activities of hydroquinones are mediated by their oxidative conversions.
Figure 13: Structure and activity relationship of TBHQ on oxidative conversion and biological activities.

(A) Chemical structures of TBHQ and its analogs. (B) UV spectrum of TBHQ oxidation: a solution of 100 µM TBHQ was freshly prepared in phosphate buffer (pH = 7.5) and the UV spectrum was recorded at t = 0 and t = 1 h. (C) Comparative oxidation of TBHQ and its analogs: solutions of 100 µM hydroquinones were freshly prepared in phosphate buffer (pH = 7.5) and the absorbance at 250 nm was recorded for 1 h. (D-E) Comparative anti-proliferative effects of TBHQ and its analogs (dose = 15 µM) in MC38 cells after 24 h treatment in basal medium (D) and in complete medium (E). The results are mean ± SD, * P<0.05.
3.4 Discussion

Here our central findings are that the biological effects of TBHQ on cell proliferation, cell cycle progression, and apoptosis are mainly mediated by its oxidative conversion to a quinone metabolite TBQ. The SAR study of TBHQ further showed that the biological activities of hydroquinones are mediated by their oxidative conversions. Previous studies have shown that TBHQ at high doses have cytotoxic effects in multiple cell lines including several cancer cell lines [103-107]; therefore, our study used a similar cell line, MC38 colon cancer cells, to study the mechanisms involved in the cytotoxic effects of TBHQ. We chose the doses of TBHQ at which TBHQ can cause reduction of cell proliferation, allowing us to study the mechanisms involved. In a similar manner, the doses of Cu^{2+} and EDTA were chosen, because previous studies used similar doses [113, 116], and our data showed that the chosen doses of Cu^{2+} and EDTA can modulate TBHQ oxidation (see Figure 9).

Our results showed that the biological activities of TBHQ are mainly mediated by its oxidation to generate TBQ. In complete medium, there is little oxidation of TBHQ (data not shown), which is likely due to quenching of transition metal ions by the serum proteins [117]. With little oxidative conversion, TBHQ at a dose up to 60 μM had no effect on cell proliferation in complete medium. In basal medium, TBHQ was rapidly converted to TBQ and showed more potent inhibitory effects on cell proliferation, cell cycle progression, and apoptosis. In addition, our results showed that Cu^{2+} and EDTA, which modulated the oxidative conversion of TBHQ to TBQ, mediated the biological effects of TBHQ. Together, these results suggest that the biological activities of TBHQ on cell proliferation are mainly mediated by its oxidative conversion to generate TBQ.
which is consistent with previous studies of TBHQ on other biological responses [114, 118]. Several possible factors, such as concentrations of TBHQ or oxygen, could be involved in the oxidative conversion of TBHQ to TBQ. Indeed, previous studies have shown that the biological activities of TBHQ are attenuated under hypoxia conditions [114]. It is likely that other reactive products could be formed during the TBHQ oxidation process, such as hydrogen peroxide, could contribute to the biological activities of TBHQ; however, further studies are needed to further characterize the mechanism involved. Also, since TBQ is a reactive quinone which can modify many protein and non-protein thiols, the effects of TBHQ and TBQ on cell proliferation seem to be cytotoxic.

The quinone metabolite TBQ has more potent biological activities than TBHQ, most likely because the α,β-unsaturated carbonyl structural moiety in TBQ makes TBQ highly reactive toward thiol groups (-SH) through a Michael reaction [114]. Upon cellular treatment, TBQ, instead of TBHQ, can directly react with the thiol groups of proteins or glutathione, leading to covalent modification of proteins and reduced levels of intracellular glutathione, which have been shown to play critical roles in regulating cell proliferation, cell cycle, and apoptosis [119]. Previous studies have also shown that TBQ, instead of TBHQ, reacts with cysteine residues of Keap1 to activate the Nrf2 signaling [114]. However, excessive formation of quinone compounds such as TBQ in tissues could cause some detrimental effects through covalent modification of proteins and DNA, leading to mutations and carcinogenesis [120]. We expect that TBHQ oxidation to TBQ will be a minor process in vivo, since serum proteins could quench transition metals [117]. This may explain some of the previous studies which showed that TBHQ at high doses could cause DNA damage and carcinogenesis [110, 111].
To better understand the roles of TBHQ oxidation in its biological activities, we studied the SAR of TBHQ for oxidative conversion and biological activity. Previous research has shown that a phenyl radical (Ph-O\textsuperscript{*}) was formed as an intermediate during the oxidation of TBHQ to TBQ [114]. The rate of phenyl 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 [116].

Therefore, here we studied a series of TBHQ analogs which have substitution groups with different electron donating abilities (the substitution groups include -CH\textsubscript{3}O, -CH\textsubscript{3}, -C\textsubscript{6}H\textsubscript{5}, -H, and -Br groups). Our data showed that the compounds with highly electron donating substitution groups have higher rates of spontaneous oxidation in aqueous buffer, which is well correlated with the phenyl radical mechanism for the oxidation of hydroquinones to form quinones. The fast-oxidizing compounds showed potent anti-proliferative effects in MC38 cells, while the slow-oxidizing compounds have weak actions, further supporting that the biological activities of hydroquinones are mediated by oxidative metabolisms.

In summary, here our results showed that the biological activities of TBHQ on cell proliferation, cell cycle progression, and apoptosis are mainly mediated by its oxidative conversion to TBQ which has enhanced biological activities. Other substituted para-hydroquinones may also exert their biological activities through the same mechanism.
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