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Development of Monocarbonyl Curcumin Analogues Towards Treating Tuberculosis and Cancer

By

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Doctor of Philosophy

Chemistry

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By

Patrick R. Baldwin
B.S., University of South Carolina-Upstate, 2003
M.Sc, Western Carolina University, 2005

Advisor: Dennis C. Liotta, Ph.D.

An abstract of
A dissertation submitted to the Faculty of the James T. Laney School of Graduate Studies of Emory University in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Chemistry

2014

Abstract

Development of Monocarbonyl Curcumin Analogues Toward Treating Tuberculosis and Cancer

By

Patrick Richard Baldwin

While advancements in chemistry and the biological sciences have resulted in the development of therapeutics for previously incurable diseases, their utility has become limited due to drug resistance. One approach toward overcoming this limitation is the development of drugs that target multiple proteins. A potential source for such drugs may be the exploitation of natural products, which have biological activity and are generally safe. A classical example of such a compound is curcumin, which has long been used as a traditional medicine. While curcumin has been identified to have anti-cancer and anti-microbial properties, the usefulness of this compound is limited due to poor bioavailability. To overcome this limitation, synthetic analogues have been developed. The work presented here investigates the use of monocarbonyl curcumin analogues as therapeutics toward treating tuberculosis (TB) and cancer. In chapter 2, we investigated the effect of these monocarbonyl analogues on inhibiting the growth of pathogenic *Mycobacteria* species (*Mycobacteria tuberculosis* and *M. marinum*). Using both disc diffusion and liquid assays, we demonstrated that specific monocarbonyl analogues inhibit the growth of pathogenic *Mycobacteria* species. In addition, we showed that these compounds inhibit both rifampcin-resistant *Mycobacteria* species *in vitro*. In chapter 3, we investigated the non-apoptotic cell death pathways induced by these analogues in order to develop improved anti-cancer therapeutics. Utilizing knockout cell lines and cell death inhibitors, we provided evidence that our lead analogue, UBS-109, induces both necroptosis and autophagy. Using a click chemistry-based target identification strategy, we identified numerous protein targets for these analogues, with several proteins affecting cell death pathways. In chapter 4, we investigated whether UBS-109 binds to heat shock protein 90 (Hsp90), a promising target for anti-cancer therapeutics. Using a competitive fluorescence polarization assay and other methods, we showed that UBS-109 reversely binds to Hsp90, possible by binding to the ATP-binding pocket of Hsp90. Taken together, our finding demonstrate that (i) monocarbonyl analogues could potentially be developed as anti-tuberculosis drugs, (ii) multiple cell death pathways are induced by these analogues, which provide important information for developing new anti-cancer therapeuctics, and (iii) monocarbonyl analogs may represent a new type of Hsp90 inhibitor.

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Chapter 1:

Introduction and Relevant Review of Literature

A. Introduction

During the past century, advances in chemistry and the biological sciences has resulted in the development of therapeutics for previously incurable diseases. As a consequence, the quality of life for countless individuals has been drastically improved. While these drugs have been successful in treating various diseases, their utility has become limited due to the development of drug resistance or because the drugs are too toxic for extended use against chronic ailments. Newer candidate drugs often suffer from the same limitations, with many failing to move past phase 2 and 3 clinical trials [1].

Currently, the dominant concept of drug development is based on the idea of "one disease, one target, one drug, analogous to Paul Ehrlich's "magic bullets" for the treatment of syphilis [1-2]. It has been argued that this view results in safer and more effective drugs. However, clinical data over the last decade has challenged this approach for several reasons. First, this view fails to account for the complex nature of diseases such as cancer, which are caused by dysregulation of multiple cellular pathways [3-4]. Thus, only in limited instances, such as with Gleevec in the treatment of chronic myelogenous leukemia [4], have single-target drugs shown promise in managing cancer. In most cases, multiple drugs or treatment modalities (e.g. chemo- and radiation therapies) are required to contain cancer cells, and in all cases, failure to completely control aberrant cell growth provides a selection for cells resistant to the drugs, which results in relapse and often mortality [4].

The requirement for use of several therapeutic agents to combat complex diseases raises a second important issue of off-target toxicity. With multiple drugs, toxicity issues are often exacerbated. Because diseases, such as cancer, result from dysregulation of normal host signaling molecules, it is virtually impossible to design a drug that specifically targets a cancer cell without also affecting the target in normal cells. Dosing can be adjusted to limit side effects but often at the cost of facilitating the development of resistance. Such off-target effects are even evident for drugs targeting pathogens, as the chemicals targeting pathogen functions specifically, often serendipitously have effects on molecules in the host that have critical functions. In short, no drug is without side effects.

An alternative approach to drug development that takes into account the complex nature of diseases is to develop drugs that target multiple proteins, but which are inherently safe [3]. A potential source for such drugs may be the exploitation of natural products or so called “natraceuticals”, which are compounds with biological activity isolated from natural sources (e.g. plants) [5]. By their very nature, these molecules have activity in biological systems, and if derived from edible plants, for example, have been field tested in humans as part of our diet [6]. Indeed, specific natural products have been used as traditional medicines to treat numerous diseases for millennia [3]. Besides being safe, many of these natural products have been found to affect multiple cellular targets. A classical example of a natural product with multi-targeted properties is a compound known as curcumin.

B. History of Curcumin

Curcumin [1,7-bis-(4-hydroxy-3-methoxyphenyl)-1,6-heptadiene-3,5-dione or diferruloylmethane] is a polyphenol metabolite isolated from the rhizomes of the perennial herb *Curcuma longa* [7-9] (Figure 1A). Besides curcumin, additional curcumin-like metabolites (e.g. demethoxycurcumin and bisdemethoxycurcumin), known as curcuminoids, can also be isolated from these rhizomes [10] (Figure 1A).

The history of curcumin is a complex story dating back over 5000 years and involves a spice known as turmeric [11]. Curcumin is the principle component of this spice and is responsible for its distinct taste and color. Tumeric is commonly known as "Indian saffron" [12]. The specific origin of this curcumin-containing spice is not known. However, it is believed that it was developed in Southeastern Asia, most likely in western India [9], and has been used for thousands of years as in food preparation, a traditional medicine and religious rites [13]. The first record of turmeric was in the holy Hindus treatise 'Atharva Veda' of 1000-1500BCE, in which it is known as 'Haldi' or 'Haridrar'. Since ancient times, this spice has been commonly used in 'Sakthi' worship by Pre-Aryan Indians [13]. Over the next thousand years, the use of tumeric spread throughout all of Southeastern Asia, predominantly as a component of religious ceremonies (in both Hindu and Buddhist ceremonies). For instance, in Malaysia, a paste of turmeric was spread over the umbilical cord of a newborn infant in the belief that it would ward off evil spirits [11]. As a result of the spice trade, turmeric was known to the Greco-Roman, Egyptian and Middle East regions. Around 700CE, the first accounts of turmeric appeared in China, where it was mostly used as a traditional medicine. The

first European explorers to the Asian continent began reporting the use of turmeric around the 14th century. One of the early accounts of the use of turmeric was reported by Marco Polo in his travel memoirs of his voyages along the "Silk Route" in 1280 CE [11 and 14]. In his account, Polo described tumeric as possessing properties akin to saffron but not actually saffron [11].

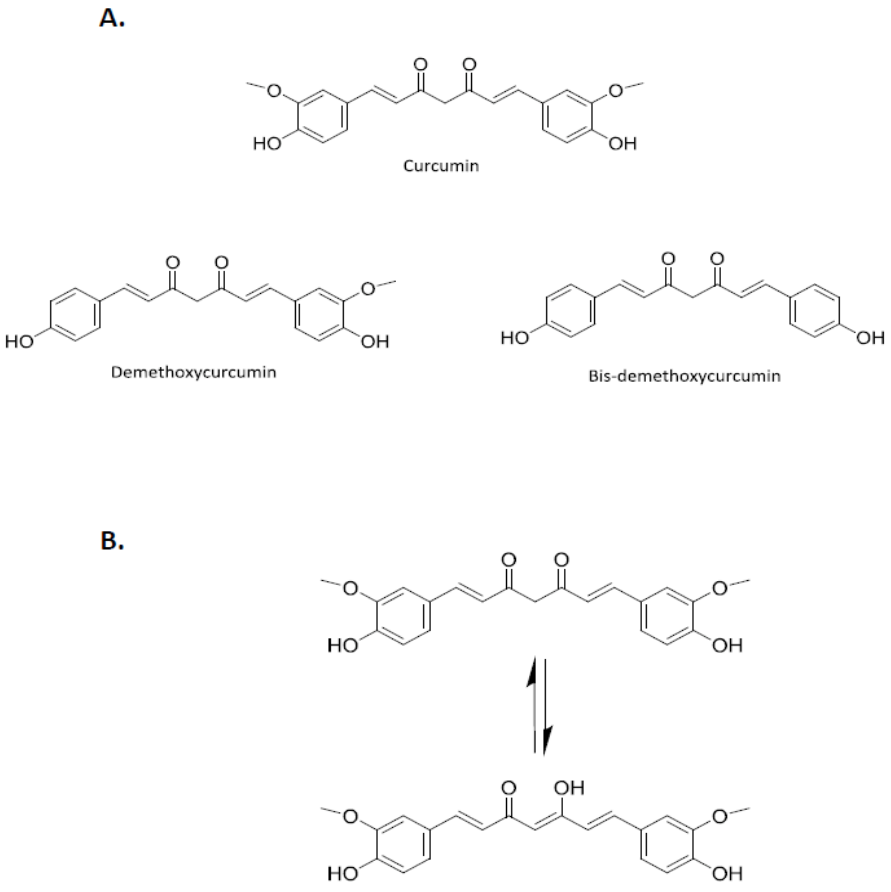

Figure 1. Curcumin occurs as natural product. (A) Curcumin and curcumoids commonly isolated from perennial herb *Curcuma longa***.** (B) Curcumin naturally exists in two isomer forms (enol and β-diketonic).

Around the 13th century, Arabian merchants began selling this spice in the European markets (initially, the source of tuneric was kept secret from European traders) [11]. During the British rule of India in the 15th century, turmeric was combined with other spices to form curry powder. Finally, turmeric was introduced to the American continent by the 1800's. An important bioactive component of turmeric was isolated in 1815 and became known as curcumin [7]. Curcumin was successfully synthesized in 1910 [8]. Even in the 21st century, curcumin is still widely used as a natural dye, a food source, and a traditional medicine throughout the world.

C. Chemical Properties of Curcumin

Curcumin has a molecular formula of $C_{21}H_{20}O_6$, a molecular weight of 368.37g/mol, and a melting point of 183□C [8 and 15]. As shown in Figure 1B, it exists in two isomers, enol and β-diketonic form [16]. While both forms occur, curcumin predominantly exists in the enol form in solution. Curcumin is practically insoluble in water, with predicted values of log P ranging from 2.56 to 3.29 (its water solubility may increase with heat) [7, 17, and 18]. It remains soluble in most organic solvents such as DMSO, ethanol, and methanol. When applied to biological samples, curcumin is dissolved in either DMSO or DMF [7]. In solution, it appears as a brilliant yellow at pH 2.5-7 and red at pH>7.

Generally, curcumin is only chemically unstable under specific conditions [15]. A study by Wang *et al.* compared the effect of various pH and physiological conditions on the stability of curcumin [19]. They found that 90% of curcumin degrades within 30 minutes of being added to a 0.1M phosphate buffer at pH 7.2 (physiological condition *in*

vitro). The degradation products were identified to be trans-6-(4'-hydroxy-3'-methoxyphenyl)-2,4-dioxo-5-hexanal, ferulic acid, feruloylmethane, and vanillin [14 and 19]. However, at acidic conditions (pH 1-6), the degradation of curcumin in buffer solutions was slower. In addition, it was found that curcumin degradation was limited in buffer solutions containing either 10% fetal calf serum or human blood, which had degraded by only 50% at 8 hours.

Besides degradation in solution, curcumin also has limited stability upon irradiation [20]. Curcumin rapidly fades in solution, and Cooper *et al*. found that absorbance decreased by 5% if the solution was stored in clear glassware during typical sample preparation [21]. Tonnesen *et al.* investigated the effect of continuous UV/visible light (240-600nm) on curcumin degradation [22], and found that continuous light exposure caused the molecule to decompose into various by-products, with the main one being a cyclization product of curcumin in which two hydrogen atoms are lost. Thus, the stability of curcumin is limited, necessitating preparation of fresh material for each experiment, and potentially limiting its utility as a drug.

The molecular structure of curcumin consists of three important functional regions, a β-diketo group, olefinic linker, and ortho-methoxy phenolic group [23]. Of these functional groups, the β-diketo group plays the principle chemical role in curcumin's pleiotropic biological effects due to it serving as an acceptor in a Michael addition reaction [7]. As seen in Figure 2, this reaction occurs by the addition of a carbon-nucleophile to the conjugated π-electron systems, which, for curcumin, is the α,β-unsaturated ketone [24]. Key features of Michael addition reactions are their

reversibility for their targets and their requirement of only mild reaction conditions (physiological pH, room temperature, etc) [24-25]. In regards to biological systems,

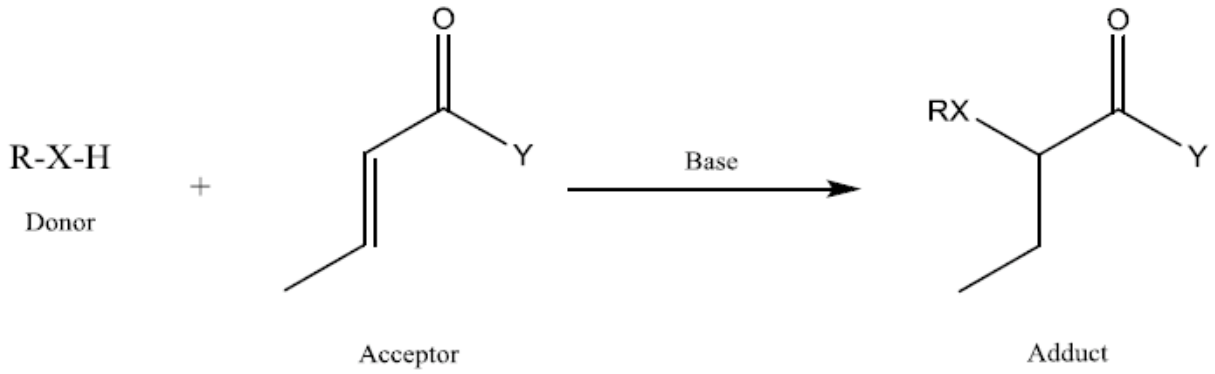

Figure 2. Schematic representation of Michael addition reaction.

the preferred targets for curcumin's Michael acceptor at physiological pH are the sulfhydryl (-SH) groups of peptides [26-27]. An example of this preferrence for sulfhydryl groups can be seen in the conjugation of curcumin to glutathione (GSH). In regard to drug development, compounds like curcumin with reversible binding to protein targets would potentially be beneficial in avoiding many toxic side effects of irreversible binding [28]. However, this reactive moiety may also limit its usefulness as a drug treatment due to potential toxicity caused by reactions with off-target nucleophiles.

The ortho-methoxy phenolic group of curcumin has been identified in playing a role in free radical scavenging activity [29]. The role of the various functional regions of curcumin in this activity has been a point of intensive scientific debate for years.

Initially, it was postulated that only the ortho-methoxy phenolic group was essential for the free radical scavenging activity [14]. Using the stable free radical 2,2'-diphenyl-1-picrylhydrazyl (DPPH) to evaluate the free radical scavenging activity, Priyadarsini *et al* found that the hydroxyl in ortho-methoxy phenolic group was required for rapid neutralization of the DPPH radical [29]. Molecules lacking this phenolic-hydroxyl group (such as dimethoxylcurcumin) had a significantly slower reaction with DPPH, in which the rate constant was ~1800 times slower than with curcumin [29]. In addition, their quantum chemical calculations support that phenolic-hydroxyl group is the main participant in free radical scavenging activity [29-30]. While Priyadarsini *et al.* proposed that ortho-methoxy phenolic group was necessary for free radical scavenging; other researchers have proposed that the β-diketo group may also play a role in this activity [14, 31-32]. Jovanovic *et al.* reported that a curcumin analogue lacking the phenolic-hydroxyl group (methylcurcumin) reacted with dioxygen through the use of the β-diketo group [32]. Recently, Singh *et al*. resolved this point of controversy surrounding the free radical scavenging activity of curcumin by synthesizing three curcumin analogues (bisdemethoxycurcumin, monodemthoxycurcumin, and dimethoxycurcumin) that neutralized various types of free radicals (DPPH, superoxide, singlet oxygen, and peroxyl radicals) [23]. For DPPH neutralizing ability, they found that curcumin had the best IC_{50} values (concentration of compound required to reduce absorbance of DPPH by 50% compared to control DPPH). They found that the rate constants for neutralizing DPPH decreased in the order of curcumin> monodemthoxycurcumin> bisdemethoxycurcumin> dimethoxycurcumin. For singlet oxygen and superoxide

radicals, only a marginal change occurred in quenching rate constants was observed. Finally, rate constants for the neutralization of peroxyl radicals were similar to constants seen with DPPH. From these results, it was concluded that both groups are essential for free radical scavenging activity. As summarized in Figure 3, the ortho-methoxy phenolic group is needed for neutralizing radicals like DPPH and peroxyl radicals (e.g. $CCL_3O_2^{\cdot}$), while the β-diketo group is necessary for radicals like superoxide and singlet oxygen.

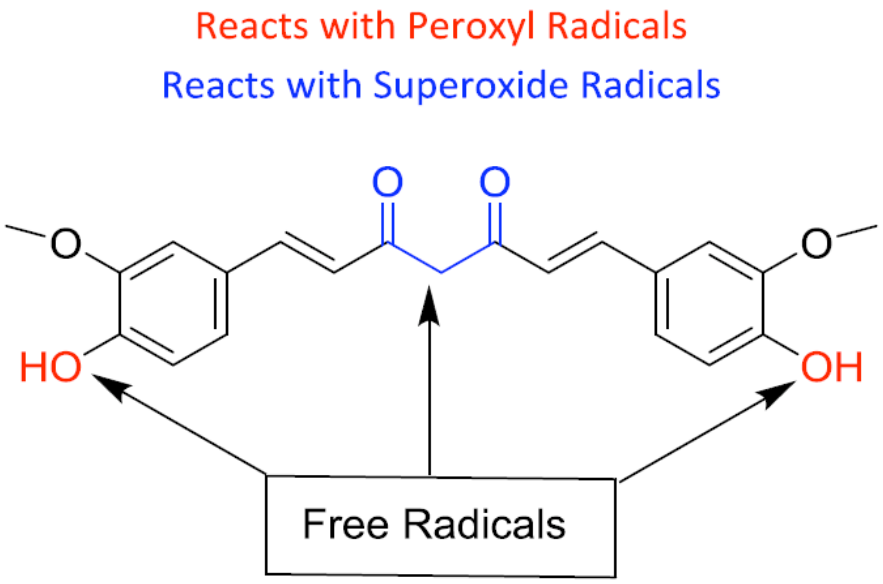

Figure 3. Sectors of curcumin for reaction with free radicals.

While the functions of β-diketo and ortho-methoxy phenolic groups are known, the role of the olefinic linker in biological effects is relatively unknown. However, recent studies using curcumin as a treatment for Alzheimer's disease have identified that the length of the linker plays a role in preventing protein aggregation, which suggest that the olefinic linker may be important for specific biological activity [33-34]. Besides the

previously discussed functions, curcumin also plays a role in metal-chelation in biological environments [7 and 35].

D. Pharmacological Effects of Curcumin

As noted above, curcumin has long been used as a traditional medicine to treat various ailments [11]. Table 1 lists the most common traditional medical applications for curcumin with many applications affecting the respiratory and digestive systems [8]. As a result of these traditional medicinal usages, curcumin has become a molecule of interest for pharmaceutical research. The first published scientific article on the use of curcumin as a medical treatment was in 1937, in which its effect on biliary disorders was reported [14]. Since the 1970s, curcumin has become a compound of interest as a potential drug for a wide-range of diseases [3 and 12]. As of January 2014, Pub Med database lists over 5000 publications on the biological properties of curcumin.

Table 1. The Traditional Medical Applications of Curcumin
Dematological Conditions (e.g. Acne, etc)
Respiratory Conditions (e.g. Asthma, etc)
Gastrointestinal Conditions
Arthritis and Inflamed Joints
Menstrual Difficulties
Diabetes
Eye Diseases
Viral Infections (e.g. Chicken Pox and Small Pox)
Blood Purifier
Liver Disorders
Stimulant

The main interest for developing curcumin as a potential therapeutic is due to its effects (directly or indirectly) on modulating various signaling pathways [18]. Curcumin has been found to affect a wide range of molecular target categories including growth factors, kinases, inflammatory cytokines, cell adhesion molecules, transcription factors and various other enzymes [12 and 18] (Figure 4). In addition, it has been reported that curcumin can specifically target gene expression (affecting epigenetic regulation by altering DNA methylation, histone modification, and alter microRNA expression) [14 and 18]. In addition, the anti-oxidant properties of curcumin (see previous section) play a role in altering the signal pathways of various cells. Because of these effects, curcumin has been strongly investigated as an anti-inflammatory, anti-cancer, anti-microbial, and as a neuroprotective therapeutic (Figure 6). The following sections will discuss each of the effects.

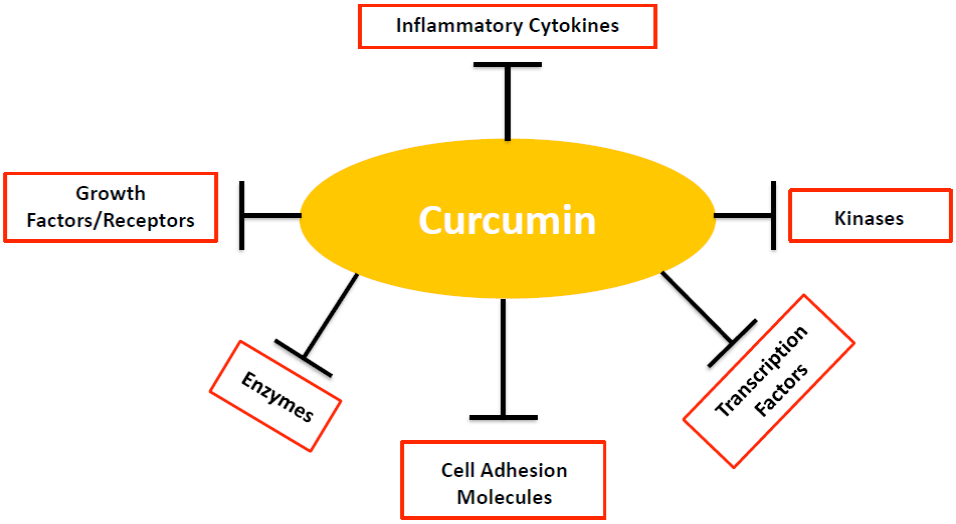

Figure 4. The categories of identified molecular targets of curcumin.

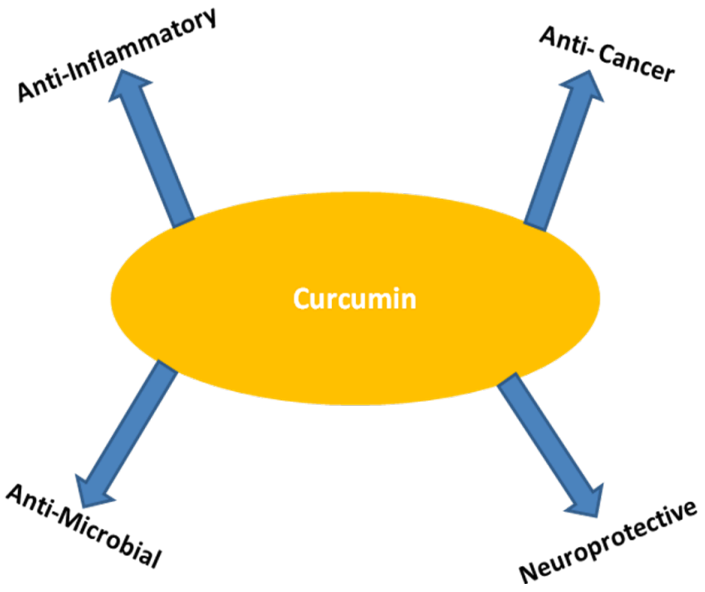

Figure 5. Potential pharmacological profile of curcumin.

E. Anti-Inflammatory Properties of Curcumin

Inflammation is an adaptive physiological response triggered by the immune system due to infection or tissue injury [36]. Inflammation is associated with alterations in signal pathways, which cause increased levels of inflammatory markers, lipid peroxides, or free radicals that control wound healing. While inflammatory responses are beneficial in protecting all living organisms, inflammation without limits can cause damage to the host tissues and organs, which in turn exacerbates inflammatory responses [37 and 38]. It has been shown that chronic inflammation will stimulate the progression of various diseases such as Crohn's disease [36]. These chronic inflammatory diseases include metabolic diseases, cardiovascular diseases, rheumatoid arthritis, and other diseases.

Curcumin has been shown to regulate a diverse range of molecular targets involved in inflammation [36]. It has been shown that curcumin will inhibit inflammatory cytokines (signaling molecules that aid in cell-to-cell communication in immune responses) such as Tumor necrosis factor alpha (TNF-α), interleukins (IL-1 -2, 6, 8, and -12), mitogen-activated protein kinase (MAPK), and c-Jun N-terminal kinase (JNK). Curcumin will also block TNF-α-induced secretion of intercellular adhesion molecule-1 (ICAM-1) and vascular cell adhesion molecule-1 (VCAM-1), which are important for stabilizing cell-to-cell communication. Curcumin also prevents TNF-α-induced secretion of monocyte chemotactic protein-1 (MCP-1), which results in the reduced recruitment of immune cells to sites of tissue injury or infection.

The key mechanism for curcumin's properties of combating inflammation is due to its affect on inhibiting the activation of the protein complex known as nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB), which is a major player in regulating the immune response to infection and other cellular stressors [39 and 40]. NF-κB is normally inactivated in the cytoplasm due to inhibitory kappa B alpha (IκBα). During activation, IκBα becomes phosphorylated by IκBα kinase kinase (IKK) and is subsequently degraded. The activated NF-κB will now translocate to the nucleus and mediate transcription of various inflammatory and catabolic gene products [40]. Curcumin prevents this activation by promoting the degradation of IKK. The resulting inhibition of NF-κB suppresses activation of several inflammatory gene products, including cyclo-oxygenase 2 (COX-2) and matrix metalloproteinase, as well as proteins controlling cell survival (e.g. Bcl-2).

Because curcumin suppresses inflammation through multiple signaling pathways, it has been investigated as a treatment for various chronic inflammatory diseases [36], including metabolic diseases, such as obesity and diabetes [41]. Obesity is a serious disease that results from an imbalance between energy intake, energy storage (e.g. triglycerides), and energy expenditure [41]. A major component of this disease is inflammation, in which excess pro-inflammatory cytokines secreted from activated macrophages and adipocytes contribute to systemic metabolic dysfunction [42]. The effects of this secretion include increased insulin-resistance, macrophage infiltration, and altered of the metabolism. Because of the key identified role of inflammation in obesity, various studies of curcumin's effect on obesity have been conducted. Woo *et al.* studied the effect of curcumin on suppressing the expression of obesity-induced inflammatory responses and showed that curcumin prevents macrophage migration into the adipose tissue by blocking the secretion of TNF-α and MCP-1. Weisberg *et al.* expanded the role of curcumin in affecting obesity-associated inflammation using genetically obese $Lep^{ob/ob}$ mice and obese mice consuming high-fat diets (HFD) [44]. They showed that 3% dietary curcumin significantly decreases insulin-resistance, reduced macrophage infiltration in adipose tissue, decreased hepatic NF-κB activation, and caused weight loss in the obese mice. Besides affecting obesity-induced inflammatory responses, curcumin was found to affect differentiation and genesis of adipocytes, and lowers triglyceride levels [42 and 45].

Another metabolic disease being investigated for the application of curcumin is diabetes [41]. This disease is a hyperglycemic disorder that affects various organs [46].

Diabetes occurs in two types: Type I, in which the body does not produce insulin, and Type II, in which the body does not produce enough insulin or becomes resistant to insulin. Chronic inflammation plays a key role in Type II diabetes, in which various inflammatory cytokines, transcription factors, and enzymes promote both disease initiation and progression. Because of its anti-inflammatory properties, curcumin has gained attention as a potential therapeutic in treating diabetic patients [47]. The effect of curcumin on diabetes was first reported in 1972, in which curcumin was found to reduce the blood glucose levels in a diabetic patient [47]. Since this initial report, it has been extensively shown that curcumin regulates blood glucose. Curcumin has been found to reduce high blood glucose levels in Type II diabetic KK-A^y mice [48]. Weisberg *et al.* found that dietary curcumin improved insulin sensitivity [44]. Using streptozotocin-induced Swiss diabetic mice, intraperitoneal (IP) administration of curcumin (10mM) for 28 days following onset of diabetes was shown to significantly reverse hyperglycemia, glucose intolerance, and hypoinsulinemia[49]. The most likely mechanism for these effects is due to the attenuation of the immune response, which has been shown to contribute to the dysfunction of β-cells during the onset of diabetes [49]. Curcumin prevents this dysfunction and improve glycemia by inhibiting the secretion of the inflammatory cytokines and NF-κB activation.

A second category of chronic inflammatory diseases treated with curcumin is cardiovascular diseases (CVD) [36]. Numerous reports support that inflammation plays a role in most CVDs, such as atherosclerosis [46]. It has been observed that curcumin to affect CVDs through diverse mechanisms. Curcumin has been shown to induce heme

oxygenase-1 (HO-1) expression by activating Nrf2-dependent antioxidant response elements (ARE) to inhibit the excessive proliferation of vascular smooth muscle cells, which is critical for the development of atherosclerosis lesions [50]. Curcumin has been found to inhibit the cellular proliferation triggered by TNF-α in human aortic smooth muscle cells by a H0-1-dependent manner [50]. Curcumin has also been found to reduce cardiomyocte hypertrophy by inhibiting p300/GATA4 complex [51]. Furthermore, oral administration of curcumin was found to disrupt the development of abdominal aortic aneurysms (AAAs) in mice by preventing NF-κB activation and lowering the concentrations of IL-1β, IL-6, and MCP-1 in the aortic tissue [52]. Finally, curcumin has been shown to reduce cardiac ischemia-reperfusion injury by decreasing the expression of the innate immunity mediator toll-like receptor 2 (TLR2) [53].

A third category of chronic inflammation diseases is arthritide diseases, such as rheumatoid arthritis (RA) [46]. RA is a progressive inflammatory disease that causes body joint distortion, loss of function, and demolition of cartilage and bone [36]. The long-term prognosis of RA is poor with most patients being disabled. Because of the role of inflammatory cytokines and inflammatory enzymes (products of NF-κB activation) play a role in pathogenesis of this disease, curcumin was investigated as potential therapeutic for RA. Park *et. al.* investigated the effect of curcumin on human synovial fibroblasts (cells responsible for pathogenesis of this disease) isolated from RA patients [54]. They found that curcumin reduced the viability of fibroblasts in a dose-dependent manner due to its effect on promoting programmed cell death through down-regulation of cell survival proteins (e.g. Bcl-2) and up-regulation of pro-death

proteins (e.g. Bax). In addition, they found that curcumin suppressed the inflammatory response of these cells through inhibiting COX-2. Curcumin was also found to inhibit the inflammatory responses of activated human articular chondrocytes, which also plays a role in RA pathogenesis [55]. Shakibaei *et al.* reported that curcumin inhibited the inflammatory response of these cells by blocking NF-κB activation and degrading COX-2 and MMP-9 proteins. Because of the promising *in vitro* data on the effects of curcumin in treating RA, a pilot clinical trial was conducted to evaluate the effectiveness of curcumin *in vivo* [56]. For this clinical trial, 45 patients with active RA were randomly assigned to three groups: curcumin-only (500mg), the anti-inflammatory compound diclofenac sodium-only (50mg), and a combination of curcumin (500mg) and diclofenac sodium (50mg). The groups received oral dosages twice daily for 8 weeks. The results of this trial found that curcumin was more efficient in alleviating pain and inflammation compared to diclofenac sodium (no synergistic or additive efficacy was observed for the combination).

Besides previously discussed inflammatory diseases, curcumin has been proposed as treatment for digestive, respiratory and skin disorders [36]. The role of curcumin as a therapeutic has been extensively characterized for inflammatory bowel disease (IBD), a disorder involving the chronic inflammation of the digestive tract. It has been shown that curcumin reduces colon inflammation in various *in vitro* and *in vivo* IBD models due to the inhibition of NF-κB products [57-58]. Recently, a limited clinical trial using curcumin to treat IBD patients was conducted. The results of this trial showed that curcumin decreased relapse rate and suppressed disease-associated clinical activity [59].

Respiratory disorders, such as allergies and asthma, are another potential target for curcumin treatment. Curcumin has been found to block allergic responses by repressing mast cell histamine release and blocking inflammatory cytokines [36 and 46]. Finally, curcumin has been applied toward various skin disorders, such as psoriasis and scleroderma, in which NF-κB activation is heavily involved [36].

F. Anti-Cancer Properties of Curcumin-

Cancer is a diverse cluster of diseases characterized by an abnormal increase in cell proliferation driven by the accumulation of genetic mutations in the cell [60]. Cancer cells differ from normal cells in 5 key ways that promote cell proliferation: (i) insensitivity or hypersensitivty to signals regulating growth, (ii) evasion of cell death signals, (iii) unlimited replicative potential, (iv) control of angiogenesis (for solid tumors), (v) capacity to extravagate or metastasize, and (vi) the capacity to evade immune surveillance [61]. While chemotherapeutics and radiotherapies have been developed, most approaches fail to completely eradicate cancer cells. For chemotherapeutics, development of resistance to the chemotherapeutic agent, such as the up-regulation of efflux pumps within cancer cells, is responsible for preventing the complete eradication of these cells [63].

Over the past few decades, extensive research has focused on the use of curcumin to prevent and treat cancer [64]. It has been shown that curcumin will inhibit cell proliferation and survival in multiple types of cancer cells [61]. These anti-cancer properties are due to the accumulation of curcumin-induced modulation of multiple signaling pathways involved in cell proliferation and death. The advantage of these anti-

cancer properties of curcumin, compared to other therapeutics, is that the likelihood of developing drug resistance is very low.

The most common anti-cancer property of curcumin is its induction of cell death pathways [65]. There are three major pathways responsible for cell death, which are apoptosis, necrosis, and autophagy. Apoptosis is a highly regulated pathway of cell death that occurs in a series of complex cellular events, which include chromatin condensation, DNA fragmentation, cell shrinkage, and the breakage of the cell into membrane-associated apoptotic bodies [61]. Generally, the loss of a cell to apoptosis is not harmful to the surrounding cells. The key proteins responsible for this cellular breakdown are caspases (cysteine-aspartic acid proteases), whose activation can occurs by two distinct pathways, called extrinsic and intrinsic [65]. The extrinsic pathway, as shown in Figure 6, is initiated by the interactions of specific ligands (e.g.TNF-α and Fas ligand) and surface receptors (e.g. TNF receptor 1 and death receptors 3), which results in the assembly of the death-induced signaling complex (DISC) that activates caspases 8 and 10. In turn, these caspases subsequently activates caspases 3 and 7, which are responsible for promoting cell death. However, as shown in Figure 6, the intrinsic pathway is initiated by various intracellular stimuli (i.e. DNA damage and cytotoxic drugs) that converge on the mitochondria to promote outer membrane permeabilization and the release of cytochrome c into the cytosol. In turn, cytochrome c binds with Apaf-1 and caspase 9 to form the apoptosome, which then activates caspase 3 and 7. Because of the destructive nature of caspase activation, cells have a network of pro-apoptotic (e.g. Bid, Bad, and Bim) and anti-apoptotic (e.g Bcl-2 and Bcl-

xl) regulatory proteins. In cancer cells, this regulation is often compromised due to up-regulation of anti-apoptotic protein, down-regulation of pro-apoptotic protein, or both. Necrosis is the second major pathway of cell death, which until recently was considered to be an unscheduled and unregulated death due to mechanical or toxic cell injury [61 and 66]. The stages of necrotic cell death consist of an increase in cell volume, plasma membrane rupture, and finally leakage of intracellular contents, which induces an inflammatory response in surrounding tissue. However, recent evidence has established the occurrence of a regulated form of necrotic cell death, known as "necroptosis" or "programmed necrosis" (Figure 7) [67]. While many aspects of this form of necrotic cell death need to be elucidated, it has been shown that stimulation by TNFα, FasL, and TRAIL will trigger activation of necroptosis [68]. It should be noted that each of these ligands will also induce apoptosis by the extrinsic pathway, which has complicated the identification of molecular mechanisms involved in necroptosis. Through the use of caspase inhibitors and knockout cell lines, receptor-interacting protein 1 and 3 [RIP1 and RIP3] have been identified as being required for necroptosis [67]. As shown in Figure 7, RIP1 has been found to play a role in both activating cell survival (NF-κB) and cell death pathways (apoptosis and necroptosis), whereas the only identified role for RIP3 is in triggering necroptosis.

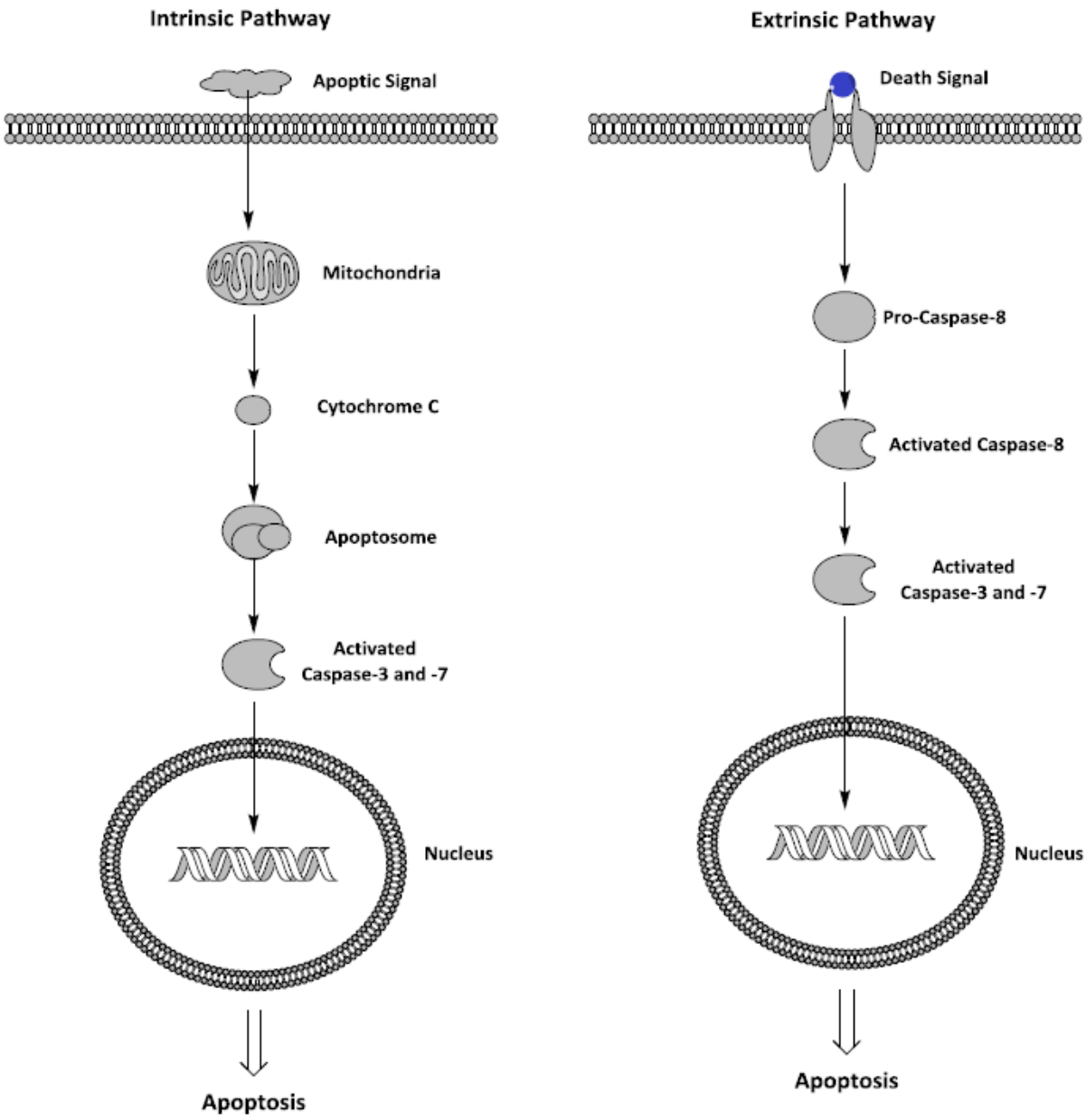

Figure 6. The intrinsic and extrinsic of apoptosis.

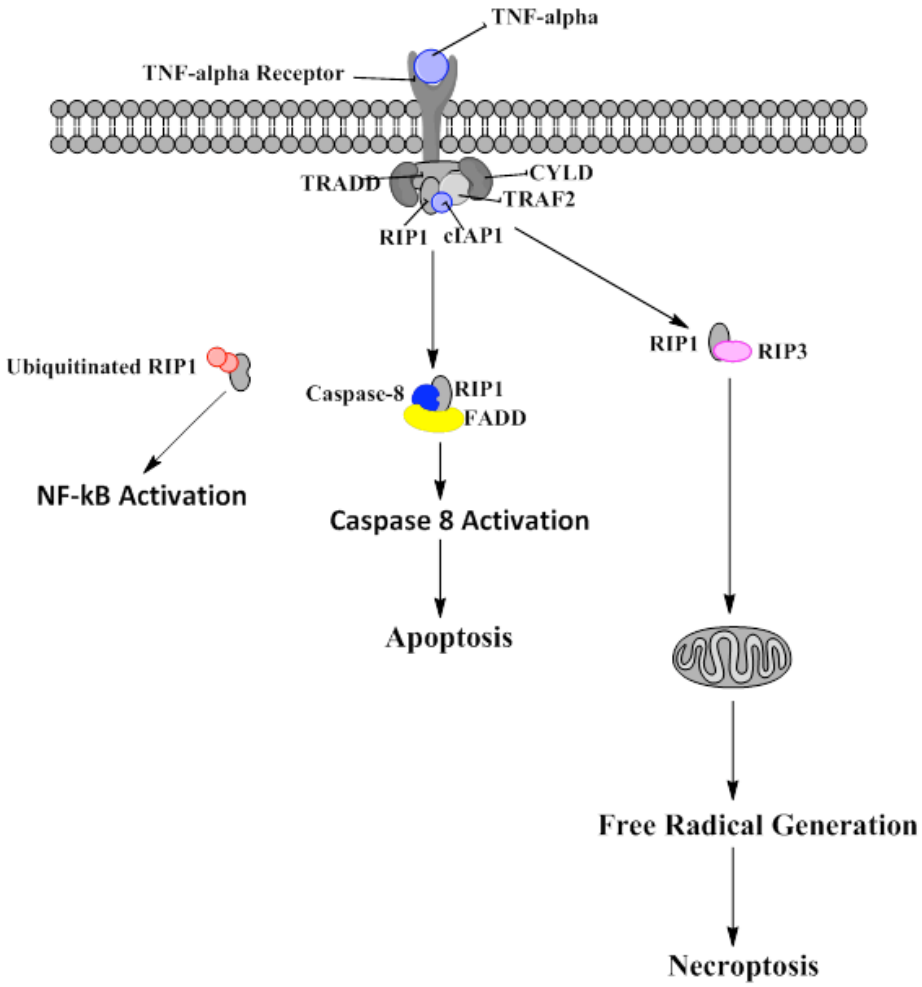

Figure 7. The death receptor pathway of necroptosis.

Experimentally, the activation of necroptosis by the death receptors has only been shown to occur if apoptosis is inhibited. Under these conditions, ligands such as TNFα stimulate formation of a protein complex (includes TRADD, TRAF2, RIP1, and cIAP1). Depending on cellular factors, the complex will result in either NF-κB-dependent transcription or apoptosis. For example, the ubiquitination of RIP1 at K377 by cIAP1 will result in activation of NF-κB [68]. Alternatively, RIP1 and FADD dissociate from the complex and interact with caspase 8, which subsequently triggers apoptosis. However,

all of the factors involved in determining whether a cell survival or death pathway becomes activated remain unclear. Under conditions that inhibit apoptosis (e.g. addition of inhibitor zVAD), RIP1 interacts with RIP3 via the RIP homotypic interaction motif domain and induces necroptosis. Additionally, necroptosis may also be induced by mechanisms independent of death receptors [69-71]. Two different studies have reported that a cytosolic complex of FADD, caspase 8, and RIP1 ("ripoptosome") will form independently of death receptors due to a low concentration of cIAPs [70 and 71]. Depending on the occurrence of cFLIP modulation, this complex will induce either apoptosis or necroptosis. Regardless of the method of inducing necroptosis, its activation has many health consequences. It has been proposed that necroptosis may serve as a second line of defense against infection [68]. It may also serve as a method to stimulate the immune system. Currently, it is unknown if necroptosis has a beneficial role in cancer elimination.

The third major pathway of cell death is autophagy, which is a multistep process involved in vesicular sequestration of cytoplasmic protein and organelles (e.g. mitochondria) [66]. As shown in Figure 8, the resulting double-membrane vesicles, autophagosomes, will then fuse with lysosomes to degrade their content. This process is highly regulated and plays a role in maintaining a balance of cellular products for

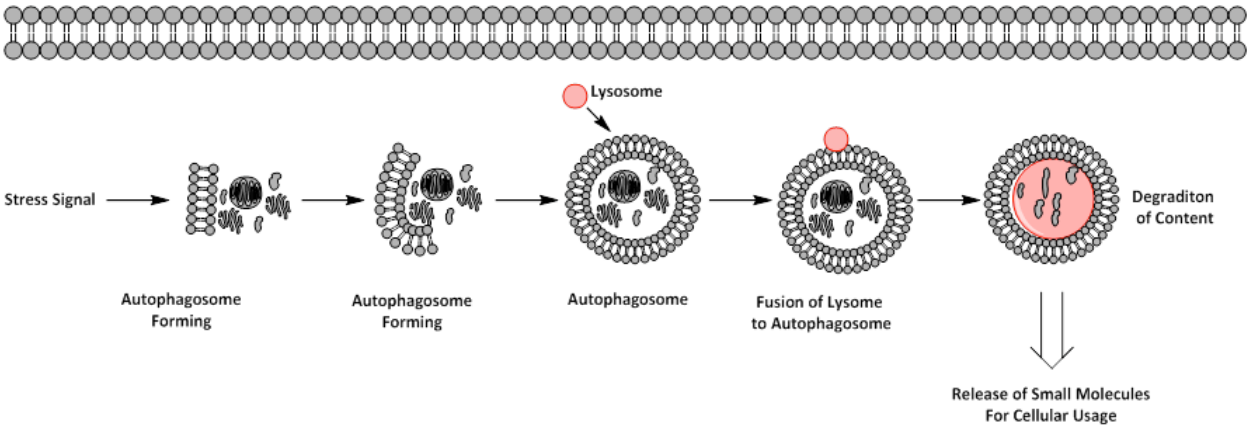

Figure 8. The pathway of autophagy.

normal cell growth and development. The induction of autophagy has been found to occur in response to both metabolic (e.g interruption of growth factor signaling pathways, and inhibition of proteasome degradation) and therapeutic stresses (e.g. rapamycin). The execution of autophagy involves a set of evolutionarily conserved gene products, ATG proteins, which are required for the formation of the autophagic machinery (isolation membrane and autophagosome) [72]. While limited autophagy is beneficial for cell survival, persistent activation results in cell death (autophagic cell death) due to the depletion of critical proteins and organelles. The autophagic cell death has been shown to have severe consequences for various diseases such as cancer and inflammatory bowel disease [73]. For cancer, autophagy has been reported to have a paradoxical relationship in which the stage of disease determines whether activation is beneficial or harmful. During early stages of carcinogenesis, the activation of autophagy is beneficial in suppressing cancer. However, during late stages of carcinogenesis,

activation of autophagy is beneficial for cancer cell survival due to the protection of the cell against various cellular stresses.

Curcumin has extensively been reported to affect various cellular proteins and pathways involved in cell death [61]. It has been shown that both extrinsic and intrinsic apoptotic pathways are activated by curcumin by promoting the activation of pro-apoptotic proteins (Bax, Bim, and Bak) and suppressing anti-apoptotic proteins (e.g. Bcl-2, Bcl-xL, survivin, and XIAP) [74]. Similarly, we have also observed that curcumin analogues will also induce the extrinsic and intrinsic apoptotic pathways, as discussed in Chapter 3.

While the role of curcumin on activation of apoptosis is well defined, its effects on necrosis (necroptosis) and autophagy is less well characterized. Currently, no information is available on whether curcumin induces or inhibits necroptosis. In addition, the ability of curcumin to regualtate RIP1 or RIP3 activity has not been reported. However, our data, described in Chapter 3, suggest that an analogue of curcumin, called UBS-109, may induce the necroptosis pathway.

Curcumin has also been found to induce the autophagy pathway [75]. Cells treated with curcumin have shown increases in autophagic biomarkers (LC3-II), which promotes autophagosome formation. The mechanism of curcumin activation of autophagy has been proposed to involve ROS generation and up-regulation of beclin-1, an initial component in forming the autophagosome [75]. Our data described below suggest UBS109 may directly target a key component in the autophagy pathway (see Chapter 3).

Besides targeting cell death pathways, curcumin will also affect proliferation pathways. Curcumin has been found to inhibit proliferation pathways (e.g. cyclin D1), protein kinases pathways (e.g. Akt and JNK), and various growth factors (e.g. EGF), and molecular chaperones (e.g. heat shock proteins) [74]. Curcumin also limits cell proliferation by inhibiting the activity of NF-κB (see above). Finally, curcumin will bind to microtubules and disrupt their assembly to prevent cell growth [61]. Similarly to curcumin, our target identification data identified several proteins (e.g. heat shock protein) involved in proliferation as binding targets for UBS-109 (see chapter 3 and 4).

While curcumin has been shown to kill cancer cells, it has little effect on non-transformed cells [61]. Three reasons have been proposed for these differential phenotypes. First, cellular uptake of curcumin may be higher in cancer cells compared to non-transformed cells, which may result in increased toxicity [76]. Second, GSH levels are generally lower in cancer cells compared to normal cells [61]. GSH has been shown to counteract the inhibitory effects of curcumin on NF-κB activation. Thus, lower levels of GSH in cancer cells may allow for increased sensitivity to curcumin. Third, non-transformed often have inducible Nf-κB, whereas cancer cells have constitutively active NF-κB, which may facilitate survival.

Besides the *in vitro* studies, the use of curcumin as a potential anti-cancer therapeutic has been evaluated in human clinical trials. The first reported clinical trial was conducted in 1987 on 62 patients with external cancerous lesions [77]. In this trial, topical curcumin was applied to the lesions. The results found that curcumin relieved certain symptoms (reductions in itching, lesion size, and pain) over a period of several

months. Since this trial, curcumin has been applied toward the treatment of various cancers (e.g. pancreatic, colorectal and breast cancers). An example of a clinical trial evaluating the effect of curcumin on pancreatic cancer had 25 patients with pancreatic adenocarcinoma receiving oral dosages of curcumin (8g) daily until disease progression occurred [78]. The effect of curcumin in these patients was evaluated by monitoring NF-κB and cyclooxygenase-2 activity. At the end of the trial, two patients showed a biological response to curcumin. One patient had no disease progression for >18 months in response to curcumin, while a second patient showed a brief tumor regression. Curcumin was found to have down-regulated expression of NF-κB and cyclooxygenase-2 in these patients. In addition, no toxicities were observed. Besides pancreatic cancer, a similar outcome was observed for a phase I trial investigating the effect of curcumin on colorectal carcinoma [79]. In this trial, patients with advanced colorectal cancer refractory to standard chemotherapies received daily doses of curcumin (36-180 mg/day) for four months. The results of this trial found that one-third of these patients experienced no disease progression for 3-4 months, with one patient showing a decline in cancer biomarker carcinoembryonic antigen [79]. Curcumin affect on multiple myeloma, skin cancer, and head and neck squamous cell carcinoma (HNSCC) has also been evaluated clinically [77 and 80]. In each of these studies, curcumin has been found to delay disease progression. Beside its use as a monotherapy, the effect of curcumin in combination with other chemotherapeutics has been clinically examined. For instance, a clinical trial involving 14 patients with advanced and metastatic breast cancer received a combination therapy of curcumin and a microtubule inhibitor

(docetaxel), which had previously shown good efficacy in treating ~60% of metastatic breast cancer patients [81]. By the end of this trial, there was no disease progression detected in any of the patients. Further, most of these patients showed a decrease in tumor biomarkers and regression of cancer lesions. The benefit of using curcumin in a combination therapy was also seen in a trial involving 100 patients having high prostate-specific antigen (PSA), which indicates a high risk of developing prostate cancer [82]. For this trial, these patients were administered either a supplement containing isoflavones and curcumin or placebo daily for 6 months and then serum PSA levels were quantified. Patients receiving the supplement were found to have significantly lower PSA levels, and thereby a reduced chance of developing prostate cancer. In summary, all of the various trials demonstrated that curcumin, either alone or in combination with other chemotherapeutics, had potential effects in treating these various cancers [77].

G. Anti-Microbial Properties of Curcumin

Curcumin has been found to inhibit the growth of both bacterial and viral pathogens [83]. For example, curcumin has been found to affect *Helicobacter pylori*, a gram-negative bacterium that is a leading cause of peptic ulcers and gastric cancer. The pathogenesis of an *H. pylori* infection requires the development of a gastric inflammatory response, particularly NF-κB activation. Foryst-Ludwig *et al.* determined that curcumin would inhibit the *H. pylori*-activation of NF-κB through the degradation IKK in a dose-dependent manner in *H. pylori*-infected human epithelial AGS cells [84]. Curcumin will also decrease pro-inflammatory responses during *H. pylori* infection by down-regulating the expression of matrix metalloproteinase-3 and -9, which are critical

in degrading the gastric extracellular matrix during infection [85]. Besides these effects on *H. pylori*-infected cells, curcumin has been found to directly inhibit bacterial growth [86] *in vitro* (MIC= 16μg/mL) by targeting shikimate dehydrogenase (SDH), an enzyme critical for survival of the bacteria [86]. However, curcumin may also directly inhibit bacterial growth by other mechanisms. A study of 65 clinical isolates of *H. pylori* found differential curcumin MICs among the different isolates [87]. An examination of the SDH-encoding *aroE* genes from the various isolates show no significant difference at the nucleotide or amino acid level, which supports that curcumin may directly affect *H. pylori* by various mechanisms.

Curcumin's ability to inhibit *H. pylori* was also investigated in both mouse and human studies. Daily oral doses of curcumin were found to be highly effective in eradication of *H. pylori* from the stomachs of infected mice (C57BL/6) [87]. However, this positive *in vivo* effect was not seen in a human clinical trial [88]. In a trial involving 25 *H. pylori*-positive patients, curcumin was co-administered as part of a drug cocktail (curcumin, lactoferrin, N-acetylcysteine, and pantoprazole) for seven days. Following treatment, *H. pylori* status and upper gastrointestinal symptoms (e.g. nausea, vomiting, heartburn, etc) were measured two months after treatment and compared to the baseline. Only 3 patients (12%) were cured of the infection, however, a significant decrease in the symptoms severity was observed. In addition, a reduction in the gastric inflammation was also observed post-treatment.

Besides *H. pylori*, curcumin has also been reported to have inhibitory effects against various groups of bacteria [83]. Curcumin has been shown to inhibit many

dangerous pathogenic species such as *Salmonella*, *Staphylococcus aureus*, and *Escherichia coli* [83]. Our data, described in Chapter 2, demonstrate that monocarbonyl curcumin analogues, such as UBS-109, inhibit the growth of pathogenic *Mycobacteria* species.

Curcumin has also been found to be effective against various viruses [83]. The molecule disrupts the pathogenesis of HIV by inhibiting the transactivator of transcription (Tat)-induced long terminal repeat (LTR) transactivation that is critical for transcriptional initiation and by inhibiting HIV integrase [89-90]. Curcumin will also affect hepatitis C virus (HCV) by targeting Akt-SREBP-1 activation [91-92].

H. Neuroprotective Properties of Curcumin

While the occurrence of neurodegenerative diseases has increased globally, the pathophysiological processes involved remain poorly understood [93]. Two factors that have been established in mediating the pathogenesis of neurodegenerative diseases are oxidative stress and neuroinflammation. Currently, therapeutic inventions for these diseases are basically limited to treating disease symptoms and do not offer neuroprotection or modification of causative mechanisms. Because of its anti-oxidant and anti-inflammatory properties, curcumin has become a compound of interest in treating neurodegenerative diseases.

Curcumin's role as a neuroprotective compound has mostly been studied for Alzheimer's disease (AD), which causes the deposition β-amyloid aggregates to form senile plaques and results in progressive neuron loss [94]. Numerous studies have shown the utility of curcumin in treating AD. Kuner *et al.* observed that curcumin

prevents β-amyloid cell death in human neuroblastoma cells by inhibiting the NF-κB activation [95]. Kim *et al.* found that curcumin and its natural analogues will protect various cell lines from β-amyloid cell death [96]. Furthermore, curcumin has been shown to inhibit the formation of β-amyloid fibrils in a dose-dependent manner. Notably, the Michael acceptor within the molecule was required for this effect [97-98]. These protective effects of curcumin have also been observed using *in vivo* models. Fratschy *et al.* found that dietary curcumin (2000ppm) protected aged rats (22 months) from Aβ-induced damages by reducing oxidative stress, and microgliosis in cortical layers [99]. Yang *et al.* found a similar result using the AD mice model (β-amyloid-overexpressing mouse Tg2576). In these experiments, a diet supplemented with curcumin prevented β-amyloid aggregations and plaque formation in the brain [100].

Based on these *in vitro* and *in vivo* results, effect of curcumin has also been evaluated in humans. Epidemiologic evidence for curcumin effects on AD was observed in studies on rural communities in India [101], where the prevalence of AD in India was 4-fold less than an equivalent population in the United States. One of the major factors cited for this effect was the regular intake of curcumin in the curry spice. Likewise, elderly Asians were found to have improved cognitive function if they consumed curry [101]. The therapeutic potential of curcumin in treating AD has so far been evaluated in two clinical trials. The first trial consisted of a 6-month randomized, placebo-controlled, double-blind study of the effect of curcumin on 34 AD patients [103]. These patients received daily doses of 0-4g of curcumin for 6-months. The results of this study indicated no protective effect of curcumin regardless of dose. The second trial consisted

of a double-blind study on mild to moderate AD patients in California, who received daily doses (2-6mg) of curcumin for 6 months [104]. This study likewise found no significant improvement in cognitive function nor in changes in Aβ levels. Thus, curcumin has not proven to be effective *in vivo* despite promising *in vitro* data.

Besides AD, the effect of curcumin on other neurodegenerative disease has also been investigated. Curcumin has been proposed as a potential therapeutic for Parkinson's disease (PD) due to its effect on inhibiting the formation and aggregation of α-synuclein fibril, the major component of PD pathogenesis [105]. In addition, curcumin has been found to protect dopaminergic cells in the substantia nigra in 6-OHDA mouse model of PD [106]. Currently, no clinical trials of curcumin treatment of PD have been conducted [93]. Curcumin has also been proposed as a treatment for multiple sclerosis (MS) due to its effects on neuroinflammation [107].

I. Toxicity and Bioavailability of Curcumin

Because of the ancient application of curcumin as both a dietary component and a traditional medicine, it has generally been considered to be non-toxic. Recent epidemiologic studies in countries such as Nepal support this idea, where large amounts of curcumin (2-2.5 g per day of turmeric spice) are consumed daily with no indication of toxicity or adverse effects [24 and 108]. Currently, the United States Food and Drug Administration (FDA) has classified curcumin to be generally recognized as safe (GRAS) [24]. The World Health Organization (WHO) Expert Committee on Food Additive has also granted curcumin an acceptable daily intake (ADI) level of 0.1-3 mg/kg body weight.

Besides these epidemiologic findings, extensive toxicity studies using animals have been conducted. Wahlstrom and Blennow found that daily oral doses of up to 5g/kg of curcumin administered to rats showed no significant toxicity [109]. In addition, preclinical studies conducted by the Chemoprevention Branch of the U.S. National Cancer Institute found no adverse toxicity in rats, dogs, and monkeys given daily doses up to 4g/kg body weight for 3 months [110]. The potential reproductive toxicity of curcumin was studied in Wistar rats that received daily oral doses up to 1g/kg body weight in two successive generations [111]. No toxicological effects were observed in either parental animals or successive generations. Similar to the animal studies, clinical trials showed no toxicity on human patients. In a phase I clinical trial conducted in Taiwan, patients with pre-invasive malignant or high pre-malignant conditions received daily oral doses of curcumin (up to 8 g) for 3 months [112]. The outcome of this trial reported no toxicity for any patient. This lack of toxicity was also observed in a United Kingdom clinical trial that administered daily doses up to 3.6 g of curcumin for 4 months to patients with advanced colorectal cancer (2 patients did report experiencing diarrhea) [113]. Currently, there has been only one reported account of curcumin-induced toxicity [114]. In South Korea, a 38-year-old healthy man was found to have developed a complete atrioventricular blockage after in-taking curcumin pills for 1 month. After the examination of the patient's history, curcumin was determined to be the cause of the blockage. To validate this conclusion, the patient was given curcumin-containing pills at the same dosage and the blockage was found to have occurred for a

second time, which supports that curcumin caused the blockage. After cessation of pills, no further blockages occurred.

Despite its established relative safety and efficacy, use of curcumin as a therapeutic has been hindered by its short biological half-life and low bioavailability [24]. Extensive studies in both animals and humans have been conducted to determine the bioavailability of curcumin. Wahlstrom and Blennow found that 75% of an oral dose of curcumin (1g/kg) administered to rodents was excreted into the feces and with only negligible amounts in the plasma and urine [109]. Similarly, Ravindranath and Chandrasekhara discovered 60% of an oral dose of curcumin was excreted as glucuronide and sulphate conjugates [115-116]. Beside oral administration, the poor bioavailability of curcumin has also been observed using other delivery methods (intravenous [IV] and IP). Holder *et al*. showed that curcumin delivered by IV or IP will result in large quantities being excreted into the bile as metabolites, which was mostly tetrahydrocurcumin and hexahydrocurcumin glucuronides [117- 118]. The rate of the metabolic reduction of curcumin was determined by HPLC and mass spectrometry analysis of subcellular fractions of both human and rat intestinal tissue that were treated with curcumin [119]. It was determined that metabolic reduction occurred very rapidly in human intestinal tissue, which was several fold higher than rat intestinal tissue. These results support the concept that curcumin undergoes transformation during intestinal absorption, perhaps explaining its low oral bioavailability.

The bioavailability of curcumin in humans has not been as extensively studied as in animals [24]. In a study using 12 health volunteers, oral doses of curcumin (10g or

12g) were administrated and blood samples were collected over 72 hours [120]. It was determined that only one subject displayed free curcumin in their plasma at each time point. However, all subjects were found to have curcumin metabolites (curcumin gucuronides and sulfates) in their plasma at all time points. In a phase I clinical trial conducted in Taiwan, serum curcumin concentrations were found to have peaked around 1-2 hours after oral intake and declined over the next 12 hours [112]. Patients receiving 8g of curcumin daily were found to have a peak concentration of 1.75±0.80 µM. In addition to plasma, the curcumin concentration in liver tissue has been studied. A study of 12 patients with hepatic metastases from colorectal cancer was given oral doses (0.45 to 3.6g) of curcumin for one week and then underwent hepatic surgery (6 hours after the last dose) [121], the molecule was detected in nanomolar concentrations in peripheral blood, bile and liver tissue. The concentration of curcumin in malignant colorectal tissues of patients consuming 3.6g daily was found to be 7.7±1.8 nmol/g tissue [122] but the blood was found to only contain trace levels of curcumin [122]. The tissues and blood were also found to contain sulphate and glucuronide metabolites. This study concluded that while curcumin was available in colorectal tissue, it was probably at too low a level to be efficacious, and metabolic processes hindered its systematic dispersal.

In summarizing these findings from both the animal and human studies, it can be concluded that the low systematic bioavailability of curcumin is due to rapid metabolism and elimination. This low bioavailability is the major limiting factor in using curcumin as a therapeutic compound. To improve the therapeutic potential of curcumin, the

molecule must be modified for improved delivery to various tissues throughout the body. However, improving bioavailability may present additional problems [123]. While curcumin is generally considered to be safe, the lack of toxicity may actually be the result of the poor bioavailability. It has been reported that curcumin may cause under special conditions DNA damage, impair the drug-metabolizing enzymes cytochrome P450, iron deficiency, and increased free radical generation in normal cells [123]. While these toxic side effects rarely occur, improvements in the bioavailability of curcumin may increase their occurrence. As a result, the modification of curcumin for improved bioavailability may in fact increase its toxicity.

J. Curcumin Analogues

Currently, several approaches have been developed to improve the bioavailability of curcumin, with most of these methods focused on modifying the delivery system of curcumin to various tissues [124]. These methods include dosing formulations (e.g. nanoparticles), prodrugs, and combination with dietary components (e.g. piperine). So far, these methods have been largely unsuccessful in improving the bioavailability [124].

An alternative approach to modifying the delivery system is the development of novel synthetic analogues, which will provide an advantage in that these curcumin-like compounds could have an enhanced pharmacological profile (i.e. bioavailability, selectivity, toxicity, and stability). As shown in Figure 9A, there are three sectors of curcumin that have been targeted for modification in attempts to produce an "improved" curcumin: aromatic rings (highlighted with green circles in figure), β-

diketone moiety (highlighted with blue box in figure) and the olefin double bonds conjugated to the β-diketone moiety (highlighted with red circles in figure) [20].

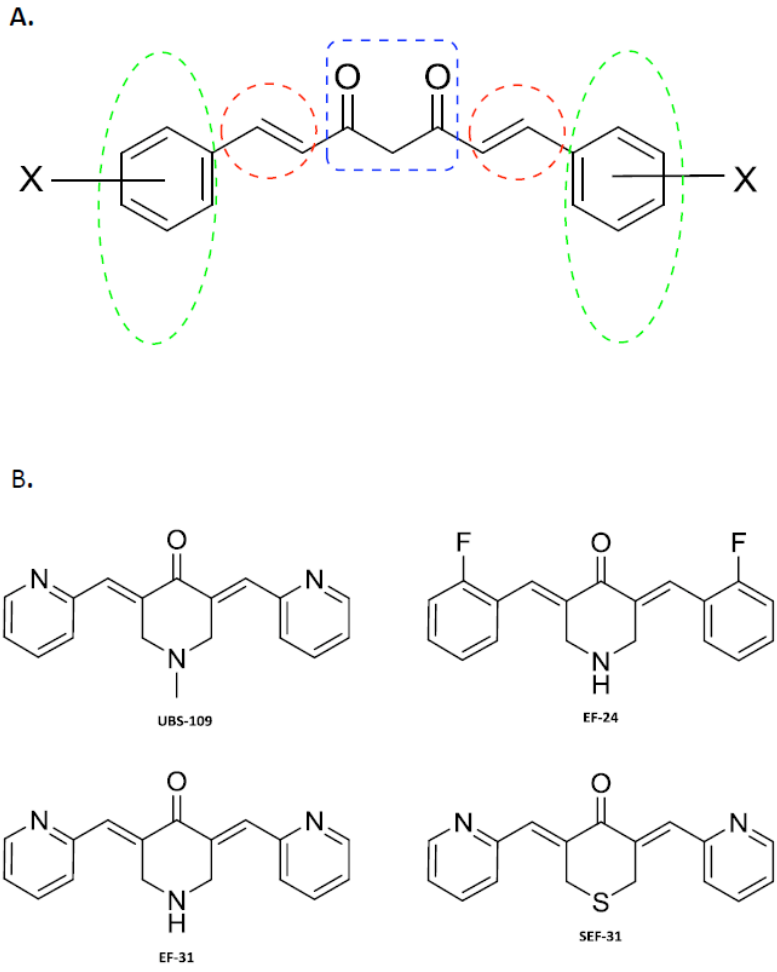

Figure 9. The development of curcumin analogues improves bioavailable and pharmacological profile. (A) The three sectors of curcumin used for modification (green= aromatic rings, red= olefin double bonds, and blue= β-diketone). (B) Examples of monoketone curcumin analogues synthesized by the Liotta-Synder Group.

While important for biological activity, the olefin double bonds have generally not been the focus of modification [125]. Instead, synthetic efforts have focused typically on the modification of the aromatic rings and β-diketone moiety. Over the years, extensive

synthesis projects have developed numerous analogues, with many being found to have improved on the biological activity [124-132].

Currently, hundreds of monocarbonyl analogues of curcumin have been synthesized by Dennis Liotta and James Synder at Emory University (Figure 9B) [129]. This series of compounds have been extensively evaluated for anti-cancer properties, which has successfully identified specific analogues (e.g. EF-24) with a significant inhibitory effect on tumor cell growth *in vitro.* This inhibitory effect was also observed *in vivo* using human breast tumors grown in athymic nude mice [129]. In a later study, the analogue EF-24 was identified in causing cell cycle arrest in human breast cancer (MDA-MB-231) and prostate cancer (DU-145) cells, which resulted in apoptosis induction [130]. It was also determined that EF-24, like curcumin, will reduce intercellular GSH and thioredoxin [130-131]. A recent study of EF-24 and other analogues (UBS-109, EF-31, and SEF-31) has also identified these analogues as pleiotropic kinase inhibitors, which predominantly inhibits activity by binding into the ATP-binding pockets [132]. While these studies have provided insight to various cellular effects of these analogues, no significant investigation into identifying cellular targets or pathway affected by these analogues has been conducted.

K. Objective of Research

The work extensively detailed in the following chapters seeks to expand the application of the analogue UBS-109 as a therapeutic compound for potentially treating tuberculosis and cancer. In Chapter 2, we present data that support the development of UBS-109 and related molecules as a therapeutics for the treatment of pathogenic

Mycobacteria species. In Chapter 3, we identified the cell death pathways and the cell targets affected by UBS-109 in order to gain insight into using this compound as the basis for developing improved anti-cancer therapeutics. In Chapter 4, we present data that supports the development of curcumin-based heat shock inhibitors for the treatment of cancer.

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Chapter 2:

Monocarbonyl analogues of curcumin inhibit growth of antibiotic sensitive and resistant strains of *Mycobacterium*

Monocarbonyl analogues of curcumin inhibit growth of antibiotic sensitive and resistant strains of *Mycobacterium*

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Abstract:

Tuberculosis is a major public health concern, with over 2 billion people infected and >1.8 million deaths per year. The rise of strains of *Mycobacterium tuberculosis* (*Mtb*) that are resistant to some or all first and second line antibiotics, including multidrug-resistant (MDR-TB), extensively drug resistant (XDR-TB) and totally drug resistant (TDR-TB), are of particular concern and there is an urgent need for new anti-TB drugs that are effective against such strains. Curcumin, a natural product used in traditional Indian medicine, has been identified to have anti-microbial activity, including against *Mtb*. While curcumin does have useful properties, it also has extremely poor bioavailability. To improve activity and bioavailability, mono-carbonyl analogues of curcumin were synthesized and screened for their capacity to inhibit the growth of *Mycobacterium* species, including *Mtb* and related *Mycobacterium marinum* (*Mm*). Using disc diffusion and liquid culture assays, we identified several analogues that inhibit growth *in vitro* of *Mm* and *Mtb.* Notably, these analogues also inhibited the growth of rifampcin-resistant *Mtb* and *Mm in vitro*.

Introduction:

Tuberculosis is a major public health issue with 2 billion infected and causing >1.8 million deaths annually [1-2]. The current drug regimen for tuberculosis consists of a cocktail of four drugs (isoniazid, rifampicin, ethambutol, and pyrazinamide) administered over six months and has a high success rate. However, the utility of this regimen has been limited by compliance issues, which has resulted in the rise of strains that are resistant to some or all of the first and second line antibiotics [3]. These strains, called MDR, XDR, and TDR *Mtb*, have proven particularly robust [4]. Recent efforts in TB

drug development have resulted in the discovery of newer therapeutics such as rifapentine, which have fewer toxic side effects and retain activity against antibiotic resistant strains [4]. However, additional drugs are urgently needed.

Whereas re-engineering old antibiotics has proven a rich source of new drugs, natural products, particularly from plants, may represent a new source of drugs with limited toxicity, which may facilitate compliance during long periods of administration. One natural product candidate of interest is a compound known as curcumin [1,7-bis(4-hydroxy-3-methoxy phenyl)-1,6-heptadiene-3,5-dione], a phenolic compound originally extracted from the plant *Curcuma longa* and the primary component of the spice turmeric [5-6] (Figure1). For centuries, various Asian cultures have used curcumin as a traditional medicine to treat various disorders, particularly associated with the skin and digestive tract. Recently, curcumin has been found to have anti-cancer and anti-inflammatory properties [6]. Moreover, curcumin has been found to inhibit the growth of various microorganisms including *Escherichia coli*, *Bacillus subtilis*, *Helicobacter pylori*, and *Mtb* [7-9]. In addition, curcumin acts synergistically with co-administered antibiotics to suppress growth of *Staphylococcus aureus in vitro* [10].

While curcumin inhibits growth of various bacterial species, this effect requires a high minimum inhibitory concentration [MIC] compared to other antimicrobial agents, which is not achievable *in vivo* due to poor bioavailability and chemical instability. One approach to overcome these limitations and improve the inhibitory activity of curcumin is to develop structural analogues [11-12]. There are three sectors of curcumin that can be targeted for modification: the β-diketone moiety, the aromatic rings, and the flanking

double bonds conjugated to the β-diketone moiety. In developing new analogues of curcumin, we have developed a series of monocarbonyl analogues (Figure 1) and evaluated their capacity to inhibit growth of pathogenic mycobacteria including Mm and *Mtb, in vitro*.

Experimental Procedures:

Chemicals. All compounds were prepared at Emory University (Atlanta, GA), as previously described [13]. Briefly, the monocarbonyl analogues were generated by allowing the ketones to react with a variety of aromatic aldehydes under basic aldol condensation conditions. Curcumin (94% curcuminoid content), Rifampicin and dimethyl sulfoxide ≥99.9% A.C.S. spectrophometic grade (DMSO) were purchased from Sigma (St. Louis, MI).

Bacterial Strains and Growth Conditions. *M. tuberculosis* (H37Rv, H37Rv-RifR, and Beijing F2 strains) and *M. marinum* strain (I218R) were grown in Difco Middlebrook 7H9 broth (Becton, Dickinson, and Company, Sparks, MD) supplemented with BBL Middlebrook ADC Enrichment (Becton, Dickinson, and Company, Sparks, MD) and 0. 5% Tween 80 (*Mtb*) or 0.025% Tween 80 (*Mm*) (7H9-ADC broth). Difco Middlebrook 7H10 agar (Becton, Dickinson, and Company, Sparks, MD), supplemented with 10% oleic acid-albumin-dextrose-catalase (7H10-OADC) was used for *Mm*. Stocks of *Mm* were grown at 30□C in 5% CO_2 until OD_{600} of 0.8, centrifuged to remove supernatant, resuspended in fresh 7H9 broth, aliquoted, and stored at -80□C.

Generation of a Rifampicin-Resistant Mutant of *M. marinum*. As described in Napier *et al.*[14], *M. marinum* strain 1218R was grown on 7H10 plates with low concentration of rifampicin (1ug/mL) for 7 days. Colonies from this plate were isolated and restreaked on 7H10 plates containing rifampicin to confirm resistance. The mutation was by amplifying and then sequencing the rifampicin resistance-determining region of rpoB using primers 5□GACGACATCGACCACTTC3□ and 3□TAGTCCACCTCTGACGAG5□.

Generation of a Rifampicin-Resistant Mutant of *M. tuberculosis* H37Rv. *M. tuberculosis* strain H37Rv was grown in 7H9-ADC broth. Electrocompetant H37Rv cells were prepared from a culture grown to an OD_{600} of 1.0 and cell pellets from this culture was washed in warm 10% glycerol (4x). The cells were electroporated with plasmid pJV75-Amber and allowed to stabilize overnight in 7H9-ADC broth. The cells were then plated on 7H10 agar supplemented with 10% OADC and 35 µg/mL of Kanamycin (KAN). Next, the H37Rv::PJV75-Amber cells were grown to mid-log phase in 7H9-ADC containing 25 µg/mL KAN and then subcultured into 7H9 medium containing 0.2% succinate, 0.05% Tween-80, and 25 µg/mL KAN. At mid-log phase, expression of mycobacteriophage recombinase protein was induced with 0.2% acetamide for 24h at 37□C. Electrocompetant cells were prepared by washes (4x) in 10% glycerol, transformed with 100ng of oligo JVC198 and 500ng of a *rpoB* H526Y R oligo, and then allowed to recover for 3 days at 37□C in 7H9-ADC. In addition the cells were plated on 7H10-OADC agar containing 25 µg/mL KAN and 50 µg/mL hygromycin. Integration of the *rpoB* mutation in individual were synthesized and purified by Integrated DNA

Technologies. The sequence of the *rpoB* H526Y R oligo was GCCGGGCCCCAGCGCCGACAGTCGGCGCTTGTGGGTCAACCCCGACAGCGGGTTGTTCTGGTC.

Disc Diffusion Assay Screening of Analogues for *M. marinum* Growth Inhibition. Frozen stocks of *Mm* were thawed, centrifuged, and resuspended in fresh 7H9 broth, and incubated until OD_{600} of 0.35. After reaching this OD_{600} of 0.35, stock was diluted to OD_{600} of 0.04. 100µL of the diluted stock was applied to 7H10 plates and spread over the plates. The plates were allowed to air dry for 10 minutes. Next, the sterilized paper 6mm diameter BBL discs (Becton, Dickinson, and Company, Sparks, MD) were placed in the center of each plate. 10µL of each curcumin analogue (100mM) was added to discs (done in triplicate for each analogue). The plates were then incubated at 30□C in 5% CO_2 for 7 days. After 7 days, the plates were removed from incubation and the extent of the zone of inhibition measured.

Determination of Minimum Inhibition Concentratory (MIC) for Analogues in *M. marinum* Growth Inhibition. *Mm* were grown in 7H9-ADC broth until OD_{600} of 0.35 and then diluted to an OD_{600} of 0.04. Cultures were grown in 7H9-ADC broth containing no drug, 1% DMSO (vehicle control), or curcumin analogues (UBS-109, EF-24, EF-31, and ECMN-909) in concentrations of 0.780 µM, 1.560 µM, 3.125 µM, 6.250 µM, 12.5 µM, 25 µM, 50 µM, or 100 µM in multiple repeats. Growth was determined by measuring the OD_{600} of cultures after 72h using a spectrophotometer. The data was normalized to the vehicle control.

Determination of Minimum Inhibitory Concentration (MIC) for Analogues in *M. tuberculosis* Growth Inhibition. *Mtb* strains H37Rv, H37Rv-Rif^R, and Beijing F2 were

grown to mid-log in 7H9-ADC broth and then diluted to an OD_{600} of 0.05. Cultures were grown in 7H9-ADC broth containing no drug, 1% DMSO, or curcumin analogues (UBS-109) in concentrations of 1 µM, 5 µM, 10 µM, 20 µM, or 50 µM in triplicate. Growth was determined by measuring the OD_{600} of cultures for 14 days using a spectrophotometer.

Determination of Synergic Effects of Moncarbonyl Analogues and Rifampicin on *M. marinum.* *Mm* were grown in 7H9-ADC broth until OD_{600} of 0.35 and then diluted to an OD_{600} of 0.04. Cultures were grown in 7H9-ADC broth containing no drug, 1% DMSO (vehicle control), rifampicin-only or in combination with curcumin UBS-109 (6.250 µM, 12.5 µM, 25 µM, 50 µM, or 100 µM) at various concentrations (4 µg/mL, 2 µg/mL, 1 µg/mL, 0.5 µg/mL, or 0.250 µg/mL) in multiple repeats. Growth was determined by measuring the OD_{600} of cultures after 72h using a spectrophotometer. The data was normalized to the vehicle control.

Results:

Evaluation of Monocarbonyl Analogues on Mm.

A series of monocarbonyl analogues were synthesized and screened for anti-mycobacterial properties using a disc diffusion assay (Figure 1). To do this, curcumin or monocarbonyl analogues were added to filter discs on 7H10 plates that had been seeded with *M. marinum,* and the plates incubated for 7 days. As a control, the vehicle solution was added to the disc (DMSO or H_2O). After 7 days, the zone of inhibition was measured for each analogue as (Figure 2A). Monocarbonyl analogues displayed a range of effects, with some showing little inhibition of growth (e.g. U2-260) whereas others inhibited growth (e.g. UBS-109) much more substantially than curcumin. By comparison, the vehicle control had no effect. To further characterize the growth inhibitory effect of these analogues, *Mm* was cultured in liquid media in the presence of various concentrations of UBS-109 or EF-24 for 72hr and the OD_{600} measured at various time points (Figure 2B). The MIC was determined from these data to be 10 μM for UBS-109 and 25 μM for EF-24.

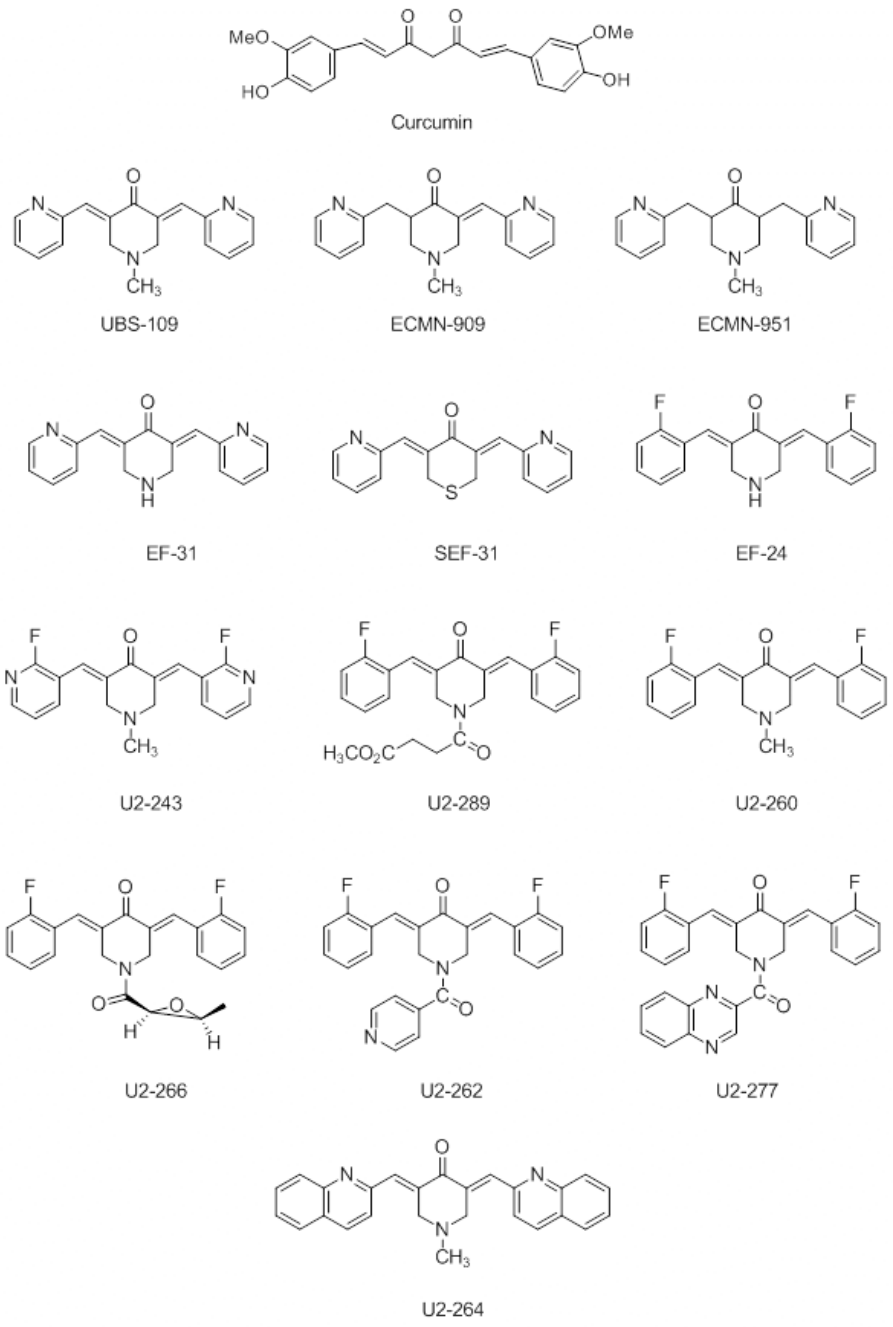
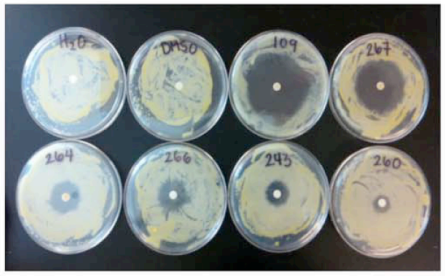


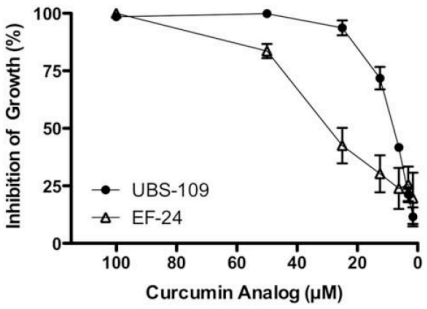
Figure 1. The monoketone curcumin analogues synthesized and evaluated for anti-mycobacteria properties.

A.



Curcum Analog	Average Diameter of Zone of Inhibition (mm)
DMSO	0
Ultra Purified Water	0
Curcumin	1.1±0
UBS-109	5.7±0.3
EF-31	4.7±0.2
SEF-31	2.7±0.4
EF-24	2.6±0.1
U2-243	2.7±0.1
U2-260	1.4±0
U2-262	1.8±0
U2-264	2.6±0.1
U2-266	2.5±0.1
U2-267	4.5±0
U2-277	2.1±0.1
U2-289	1.8±0.3

B.



C.

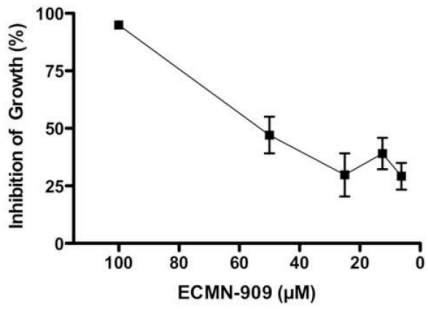


Figure 2. Monocarbonyl curcumin analogues inhibit the growth of *Mycobacterium marinum*. (A) Initial screening of inhibition properties was analyzed by disk diffusion assay (100mM analogues was added to *Mm containing* plate, incubated for 7 days at 32°C, and zone of inhibition measured); (B) Liquid culture assay validating specific analogues inhibit Mm (different concentration of analogues were added to *Mm* culture, incubated for 72hr, then the OD_{600} was measured); (C) Liquid culture assay validating that the UBS-109-metabolite, ECMN-909, inhibit *Mm* growth.

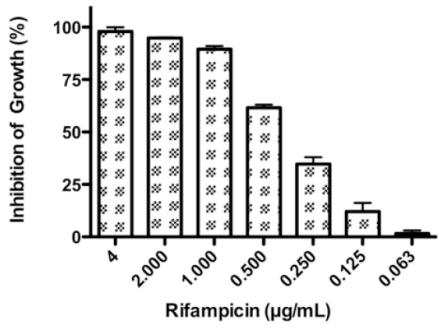
The Michael Acceptor Is Required For Anti-Mycobacterial Activity.

Previously, these monocarbonyl analogues were shown to display pleiotropic effects in other biological systems (13). Moreover, the comparison of the structural features of various analogues in relation to their capacity to affect bacterial growth suggested that three distinct domains within the molecules control antimicrobial activity: the aromatic rings, the Michael acceptor, and the central ring. When differences in solubility are accounted for, alterations at either the central ring (e.g. by addition of large side chains) or the aromatic rings (e.g. by addition of fluorine groups in EF-24) reduced antimicrobial activity by two fold (Figure 2A). To further elaborate the relationship between the Michael acceptor and antimicrobial activity, we evaluated two additional compounds, ECMN-909 and ECMN-951, in which Michael additions to the conjugated unsaturated ketone are reduced or eliminated. ECMN-909 contains only one double bond flanking the carbonyl, which reduces the capacity of the molecule to undergo Michael additions. Likewise, ECMN-951 contains no flanking double bonds (Figure 1), and cannot undergo Michael addition reactions at all. In the disc diffusion assay, indicated that ECMN-909 has the second largest zone of inhibition amongst all analogues (4.6$\pm$0 mm compared to 5.7mm for UBS-109). By contrast, no zone of inhibition was evident with ECMN-951. Liquid culture assays confirmed these results, and showed that ECMN-909 was somewhat less effective in inhibiting bacterial growth (MIC=~50 µM) compared to UBS-109 (MIC= 10 µM) (Figure 2C). Together, these data suggest that several structural domains contribute to the antimicrobial activity of UBS-109, but that the Michael acceptor is most important.

Efficacy of curcumin analogues is diminished in the presence of antibiotics.

To determine whether UBS-109 had synergistic effect when co-administrated with front-line anti-tuberculosis antibiotics, drug-sensitive *Mm* was cultured in the presence of various combinations of rifampicin and UBS-109 concentrations. As a control, *Mm* was also cultured in presence of rifampicin alone (Figure 3A). No synergistic interactions were evident between rifampicin and UBS-109. Instead, UBS-109 exhibited greater inhibition when cultured alone compared to co-administration with rifampcin (Figure 3B). Similar effects were evident when UBS-109 was cultured with isoniazid (not shown). Together, these data suggest that antibiotics reduced the efficacy of UBS-109 on *Mycobacteria*.

A.



B.

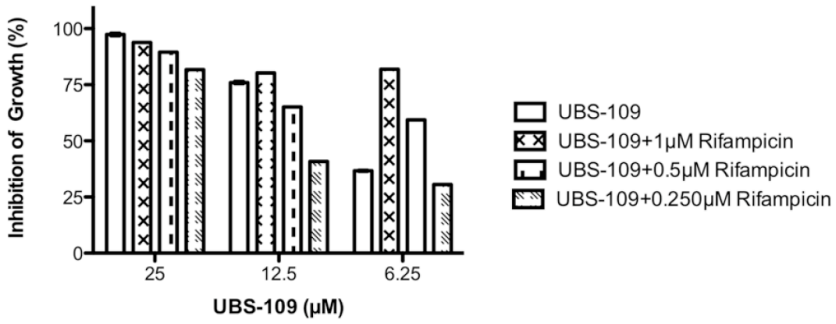


Figure 3. Rifampicin induces an antagonist effect on growth inhibition of *Mycobacterium marinum* by UBS-109. (A) The effect of rifampicin on the growth of *Mm* (different concentration of rifampicin were added to *Mm* culture, incubated for 72hr, then the OD_{600} was measured); (B) Rifampicin reduces the effectiviness of UBS-109 to inhibit *Mm* growth.

Evaluation of curcumin analogues on rifampicin-resistant Mm.

To determine whether curcumin analogues can be used to treat drug-resistant *Mycobacterial* species, we evaluated the ability of UBS-109 to inhibit the growth of rifampcin-resistance stain of *Mm* (*Mm*^{rif}). Using liquid culture assays, we grew *Mm*^{rif} in the presence of DMSO, rifampicin or UBS-109. As a control, rifampicin was found to

have no effect at any concentration on inhibiting the growth of *Mm*^{rif}. As shown in Figure 4, UBS-109 inhibited bacterial growth to the same extent as the rifampicin-sensitive strain, as indicated by identical MICs. Thus, UBS-109 can inhibit antibiotic resistant Mycobacteria.

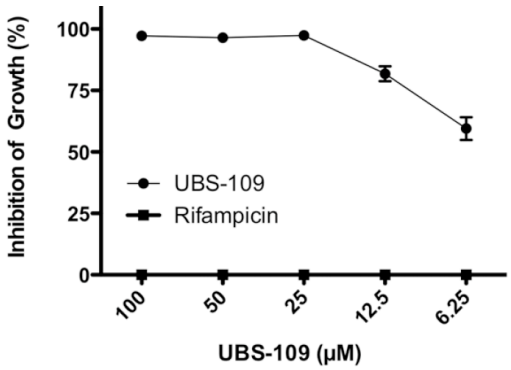


Figure 4. UBS-109 inhibits the growth of rifampicin-resistant *Mycobacterium marinum*. Liquid culture assay validating UBS-109 inhibits rifampicin-resistant *Mm* (different concentration of analogues were added to *Mm* culture, incubated for 72hr, then the OD_{600} was measured).

Evaluation of curcumin analogues on *Mycobacteria tuberculosis*.

To validate the effects of UBS-109 on other clinically relevant Mycobacteria, we next tested the effects of UBS-109 and EF-24 on several *M. tuberculosis* strains using the disc diffusion or liquid assays (Figure 5). Two different *Mtb* strains, H37Rv and Beijing F2, were cultured in the presence of various concentrations of UBS-109 or EF-24 for two weeks and the OD_{600} measured daily. UBS-109 inhibited the H37Rv strain with an MIC of ~10 μM, and the

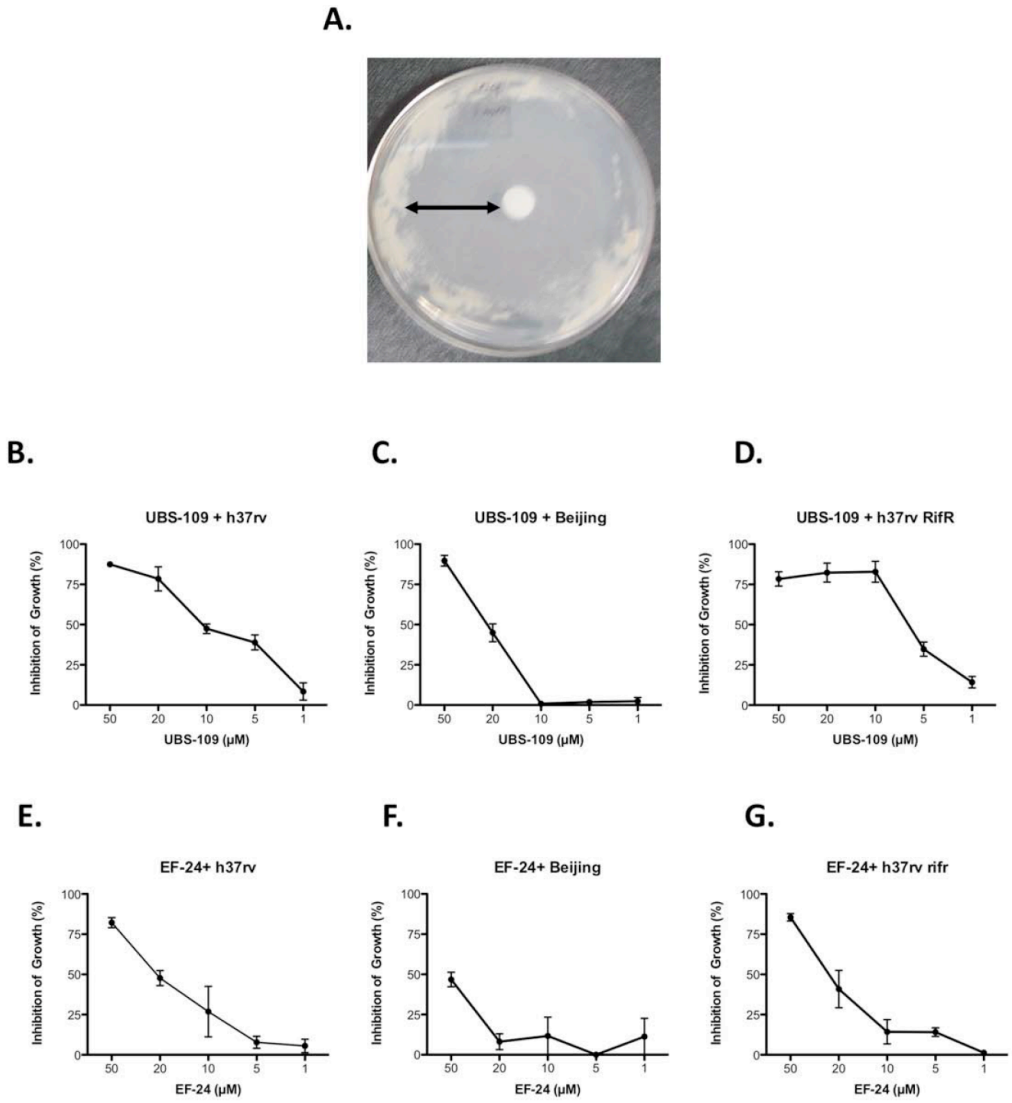


Figure 5. Monoketone curcumin analogues inhibit the growth of *Mycobacterium tuberculosis*. (A) Initial screening of inhibition properties was analyzed by disk diffusion assay (100mM analogues was added to *Mm containing* plate, incubated for 7 days at 32^{o}C, and zone of inhibition measured); (B) Liquid culture assay validating that UBS-109 inhibits H36Rv strain of Mtb (different concentration of analogues were added to *Mtb* culture, incubated for 8d, then the OD_{600} was measured); (C) Liquid culture assay validating that UBS-109 inhibits Beijing strain of *Mtb*; (D) Liquid culture assay validating that UBS-109 inhibits rifampicin-resistence strain of H36Rv *Mtb*; (E) Liquid culture assay validating that EF-24 inhibits H36Rv strain of *Mtb*; (F) Liquid culture assay validating that EF-24 inhibits Beijing strain of *Mtb*; (G) Liquid culture assay validating that EF-24 inhibits rifampicin-resistence strain of H36Rv *Mtb*.

Beijing strain with and MIC of 20 µM (Figure 5B and C). EF-24 also inhibited *Mtb*, though not as effectively as UBS-109 (MIC of ~20 µM for H37Rv and 50 µM for Beijing stains; Figure 5D and E). Finally, the effect of UBS-109 and EF-24 on the rifampcin-resistant *Mtb* (H37Rv RifR) was evaluated. UBS-109 inhibited H37Rv RifR *Mtb* with MIC of ~7 µM (Figure 5D). EF-24 also inhibited the rifampcin-resistant *Mtb* strain, though with lower efficacy compared to UBS-109 (MIC of 20 µM; Figure 5G). These data suggest that curcumin analogues are as effective against Mm as they are against *Mtb* H37Rv, but less effective against *Mtb* Beijing.

Discussion:

The use of curcumin as a potential therapeutic is limited due to poor bioavailability. One approach to overcoming this disadvantage is the synthesis of structural analogues. Analogues developed by us displayed a range of increased pharmacological effects [11-13]. Here we have identified monocarbonyl curcumin analogues that inhibit growth of pathogenic Mycobacterium species (*Mtb* and *Mm*). UBS-109 showed the best inhibitory effect, as determined by its MIC and its activity against multiple Mycobacteria species. UBS-109 and several other analogues had significantly lower MICs compared to curcumin, which reportedly inhibited growth of Mtb [9].

We identified several structural domains that confer anti-mycobacterial activity for these analogues. First, the presence of a mono-carbonyl group linking the aromatic rings in these analogues improved growth inhibition in comparison to curcumin, which has two carbonyl groups. Furthermore, our data suggests that a fluorine group in the

aromatic ring reduces anti-mycobacterial activity by ~2-fold compared to UBS-109. Fluorine groups generally stabilize compounds and increase their reactivity with proteins, though it is not clear how fluorine contributes to reduced antibacterial activity. In addition, the presence of large side chains on the central ring (e.g. U2-262 and U2-277) appeared to hinder anti-bacterial activity.

Perhaps the most important structural characteristic of these analogues is the two unsaturated bonds flanking the carbonyl, which constitutes a Michael Acceptor. In various studies involving curcumin analogues, this domain is required for biological activity. While it is recognized that these bonds are needed for most biological effects, they also represent a potential limitation in the usefulness of these compounds as therapeutics. Michael acceptors can render compounds unstable and facilitate their degradation. In addition, Michael acceptors can undergo numerous reactions with proteins, which can contribute to nonspecific effects including toxicity. This limitation may likewise complicate the use of these particular curcumin analogues in treating TB patients. Our data concur with previous studies by Changtam *et al.*, who showed that the elimination of unsaturated bonds decreased anti-mycobacterial activity of curcumin [9].

Importantly, our data suggest that UBS-109 inhibits growth of antibiotic-resistant *Mycobacterium* species. Currently, a major impediment to TB treatment is the rise of strains that are resistant to some or all first and second line antibiotics. Our data indicate that monocarbonyl analogues are effective against rifampicin-resistant strains of *Mtb* and *Mm*. However, these effects are complicated by the observation that the

presence of antibiotics inhibited the efficacy of UBS-109. This effect is most likely due to the antibiotic-induced up-regulation of efflux pumps that reduce the effective concentration of UBS-109. A similar effect of rifampicin on effux pumps results in loss of efficacy of other anti-tuberculosis drugs [15]. This property complicates the use of curcumin analogues on people with MDR TB because these individuals are not always identifiable and are often treated with an antibiotic cocktail containing rifampicin.

While this study focused directly on the effects of these analogues on the bacterial growth, it did not characterize potential effects on infected cells. Recently, Napier *et al.* reported the successful clearance of *Mtb* by targeting tyrosine kinases within infected host cells with imatinib [14]. It is possible that these monocarbonyl analogues may likewise affect clearance by targeting cellular pathways within infected host cells. Such dual targeting has been observed previously with curcumin treatment of the bacterial pathogen *H. pylori*, a gram-negative bacterium that is responsible for causing peptic ulcers and gastric cancer [16]. For *H. pylori*, curcumin prevents and eliminates infection by targeting critical bacterial proteins and by inhibiting inflammatory responses in infected cells. It remains to be determined whether such dual targeting occurs with curcumin against TB.

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Chapter 3:

A Monocarbonyl Analogue of Curcumin Induces Necroptosis and Autophagy Cell Death

A Monocarbonyl Analogue of Curcumin Induces Necroptosis and Autophagy Cell Death

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Abstract:

Many types of cancer cells contain mutations that facilitate their survival in response to stressors including chemotherapeutic agents. As a result, several therapeutic agents have been introduced that specifically target pathways that induce cell death, such as apoptosis. While targeting apoptotic pathways has proven effective for certain types of cancer, selection of resistant mutants has reduced the efficacy of this approach. A solution to this problem is to concurrently target additional cell death pathways. We report here that a monocarbonyl analogue of curcumin, called UBS-109, may represent a novel class of compounds with such capability. Using cells and mice deficient in RIP-3, which controls necroptosis, and caspase 8, which controls extrinsic apoptosis, we show that UBS-109 induces necroptotic cell death at low concentrations, whereas it induces apoptosis and autophagy at higher concentrations. Using a specific inhibitor of necroptosis, we determined that the effect of UBS-109 on necroptosis is independent of its effect on autophagy. Finally, we show that UBS-109 induces the autophagy pathway by targeting the phagolysosomal fusion. Together, these data indicate that UBS-109 stimulates both necroptosis and autophagy. These data provide important information on development of therapeutics that target multiple cell death pathways.

Introduction:

Curcumin is a naturally occurring metabolite isolated rhizome of perennial plant of the ginger family, *Curcumin longa* [1]. For thousands of years, curcumin has been used as traditional medicine to treat various illnesses such as arthritis and gastrointestinal diseases [2]. Recent work has indicated that curcumin may be useful as

a therapeutic for treating disorders associated with inflammation, microbial infections, and cancer. Furthermore, curcumin has been found to be relatively free of toxic side effects both *in vitro* and *in vivo*. While these properties have shown great promise, curcumin development as a therapeutic compound is being severely limited due to its poor bioavailability [2].

One approach to improving both bioavailability and therapeutic properties of curcumin is the synthesis of structural analogues. We have synthesized a library of analogues based on modification of the keto-enol moiety within the central sector of the molecule. One of these analogues, called UBS-109, contains a monocarbonyl group embedded in a heterocyclic six-membrane ring conjugated with flanking double bonds [3-4]. UBS-109 was originally screened *in vitro* for activity against a variety of transformed cell lines for anti-growth or antiangiogenic activity. Subsequently, UBS-109 was found to have specific cytotoxic effects on tumor growth *in vivo*, which was attributed to apoptosis [3]. Recently, these analogues have been identified as pleiotropic inhibitors of various kinases [4].

Cancer is responsible for over 6 million deaths annually [5]. While some success has been achieved in the controlling the disease with drugs such as Gleevec, most cancers remain intransigent to treatment. One focus in anti-cancer drug development has been to target cellular death pathways, particular apoptosis. Apoptosis is vital for normal development and function of cells [6]. However, dysregulation of this pathway in cancer cells can facilitate survival and thereby acquisition of additional mutations that contribute to tumor progression. Extensive effort has been devoted to inducing

apoptosis with drugs or to restoring sensitivity of cancer cells to therapeutics that promote apoptosis as a mean to eradicate cancer cells. One complication of targeting apoptosis alone is that cells will up-regulate compensatory pathways, for example anti-apoptotic pathways controlled by Bcl-2, which allows the cancer cell to survive [7]. In addition, targeting apoptosis may have promoting tumor growth by activation of cellular regeneration pathways [8]. For example, Li *et al* reported that the activation of caspase 3/7 induces prostaglandin E_2, which induces stem cell proliferation and tumor regrowth (the so called "rising phoenix" pathway).

To overcome these limitations, we reasoned that it may be possible to target several cell death pathways, and thereby facilitate tumor elimination and preclude development of resistance. Curcumin and its analogues have been extensively reported to affect the apoptotic pathway. However, the effect of these small molecules on non-apoptotic death pathways is poorly understood. Given the plieotropic effects of curcumin, we reasoned that the compound might affect more than just apostosis. Our data suggest that UBS-109 induces not only apoptosis, but also necroptosis, and autophagy. These data expand the understanding the mechanisms of cell death induced by these analogues and provide insight that will aid in the development of compounds that target multiple cell death pathways.

Experimental Procedures:

Cell Culture. J774A.1, 3T3 ($ATG5^{+/+}$), $ATG5^{-/-}$ mouse embryonic fibroblasts were purchased from ATCC (Manassas, VA). $Caspase8^{+/+}Rip3^{+/+}$, $Caspase8^{+/+}Rip3^{-/-}$, $Casp8^{-/-}Rip3^{-/-}$, $ATG5^{+/+}$, and $ATG5^{-/-}$ primary macrophages were derived from bone marrow. $ATG5^{+/+}$ and $ATG5^{-/-}$ cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 1% MEM nonessential amino acid (NEAA) and 1% HEPES. Media for J774A.1 and 3T3 was supplemented with 10% fetal bovine serum (FBS) and 2mM L-glutamine.

Mice. The $Caspase8^{+/+}Rip3^{-/-}$ mice were generated as previous described by Newton *et al* [9]. The $Casp8^{-/-}Rip3^{-/-}$ mice were generated as described by Kaiser *et al* [10]. The $Caspase8^{+/+}Rip3^{+/+}$ and $ATG5^{+/+}$ mice were obtained from Jackson Laboratory (Bar Harbor, Me).

Chemicals. UBS-109 and ECMN-00742 were synthesized as previous described [3 and 4]. Necrostatin-1 was purchased from Calbiochem (EMD Millipore, Billerica, MA). Curcumin, dimethyl sulfoxide (DMSO), copper(II) sulfate, tris(2-carboxyethyl phosphine hydrochloride (TCEP), and tris[(1-benzyl-1H-1,2,3-triaol-4-yl)methyl] amine (TBTA), and bafilomycin A-1 was purchased from Sigma-Aldrich (St. Louis, MO). PEG4 carboxamide-6-azidohexanyl biotin and D-biotin were purchased from Invitrogen (Grand Island, NY). Immobilized monomeric avidin resin beads were purchased from Therma Scientific (Rockford, IL). The primary antibody anti-biotin-HRP was purchased from Cell Signaling Technology (Danvers, MA).

Generation of Primary Macrophagys from Mouse Bone Marrow. ATG5$^{-/-}$, Caspase8$^{+/+}$Rip3$^{+/+}$, Caspase8$^{+/+}$Rip3$^{-/-}$, Caspase8$^{-/-}$Rip3$^{-/-}$ primary macrophages were derived knockout mice. Briefly, mice were sacrificed, femur and tibia collected, and bone marrow cells flushed from the bones with cold RPMI-1640 with L-glutamine media supplemented with sodium bicarbonate (1.5g/L), sodium pyruvate (1mM), HEPES (10mM), 0.05mM 2-mecraptoethanol, and 10% fetal bovine serum (bone marrow media). The cell suspension was then passed up and down with a syringe to disperse cell clumps, centrifuged for 5min, and then the supernatant was removed. The cells were resuspened in fresh bone marrow media (10mL), transferred to a T-75 Falcon culture flask, and incubated at 37C for 4h. After 4h, culture supernatant was transferred to a new T-75 Falcon culture flask and M-CSF (10ng/mL) was added. The culture was incubated at 37C and monitored daily for the development of mature macrophages.

Drug Effect on Cell Viability. J774A.1, ATG5$^{+/+}$, ATG5$^{-/-}$, Caspase8$^{+/+}$Rip3$^{+/+}$, Caspase8$^{+/+}$Rip3$^{-/-}$, or Caspase8$^{-/-}$Rip3$^{-/-}$ cells were plated at 20,000 cells/well in 96-well plate (Costar white plate with clear bottom, Corning Incorporated, NY) and allowed to adhere overnight. UBS-109, ECMN-00742, or curcumin was dissolved in DMSO, diluted to desired concentrations in cell culture medium, applied to cell, and then the cells were incubated for 24h at 37˚C. Cell viability was determined by measuring the intracellular ATP levels using the Cell Titer-Glo luminescent Cell Viability Assay kit (Promega, Madison, WI) according to the manufacturer's instructions. Luminescence was

measured on a Synergy HT Multi-Detection Microplate Reader (BioTek, Winooski, VT). Results were normalized relative to vehicle control (DMSO) treated cells.

Fluorescence Microscopy of UBS-109-treated RIP3$^{-/-}$ bone marrow-derived macrophagys. Bone marrow-derived Caspase8$^{+/+}$Rip3$^{-/-}$ and Caspase8$^{+/+}$Rip3$^{+/+}$ cells were plated at 50,000 cells/coverslip in a 12-well (Costar cell culture cluster flat bottom plate, Corning Incorporated, New York) and allowed to adhere overnight at 37□C. Next, UBS-109 was diluted to desired concentrations, applied to cells, and then cell were incubated for 12h at 37□C. Following incubation, the media was removed, washed PBS (2x), fixation buffer (1x cytoskeleton buffer, 4% formaldehyde, 0.32M sucrose, and water) was added to each coverslip, and then incubated for 20min at room temperature. Next, the cells were with PBS (2x) and PBS+0.1% TritonX was added permeabilize cell membranes. The coverslips were blocked with 2% milk in PBS+0.1% TritonX for 1h at room temperature. Dilution of the primary antibodies Alex 488 and DAPI were prepared, applied to the coverslips, and then incubated for 1h at room temperature. Finally, the coverslips were mounted and observed using a scientific-grade cooled charge-coupled device (Cool-Snap HQ with ORCA-ER chip) on a multi-wavelength, wide-field, three-dimensional microscopy system (Intelligent Imaging Innovations, Denver, CO), based on a 200M inverted microscope using a 63x numerical aperture 1.4 lens (Carl Zeiss, Thornwood, NY). Samples were imaged at room temperature.

Transmission Electron Microscopy (TEM) of UBS-109-treated Mammalian Cells. J774A.1, bone marrow-derived Caspase8$^{+/+}$Rip3$^{+/+}$ and bone marrow-derived

Caspase8$^{+/+}$Rip3$^{-/-}$ cells were plated at 50,000 cells/well in a 12-well (Costar cell culture cluster flat bottom plate, Corning Incorporated, New York) and allowed to adhere overnight at 37□C. The next day, fresh stocks of UBS-109 were diluted to desired concentrations (10μM), applied to the cells, and then the cells were incubated for 24h at 37˚C. After 24h, the solutions were removed, washed with sterilized PBS and then 2.5% gluteraldehyde in 0.1 M cacodylate buffer was added to each well. The samples were then embedded in resin and analyzed by the Robert P. Apkarian Integrated Electron Microscopy Core of Emory University.

Effect of UBS-109-treated on RIP3$^{-/-}$ mice *in vivo*. Age-matched Caspase8$^{+/+}$Rip3$^{-/-}$ and Caspase8$^{+/+}$Rip3$^{+/+}$ mice received daily intraperitoneal injections of 30mg/kg UBS-109 (in DMSO). The animals were monitored daily for signs of weight loss, sickness, and behavior differences as indicators of drug effect.

Role of Necroptotic Inhibitor on UBS-109-induced Cell Death. 3T3 and ATG5$^{-/-}$ MEFs cells were plated at 20,000 cells/well in 96-well plate (Costar white plate with clear bottom, Corning Incorporated, NY) and allowed to adhere overnight. Next day, solutions of UBS-109 and necrostatin-1 (Nec-1) were prepared (0.1μM, 0.5μM, 1μM, 5μM, 10μM, 20μM, and 100μM for UBS-109; and 60μM Nec-1). Prior to treatment with UBS-109, the half of the cells were pretreated with 60μM Nec-1 and incubated at 37□C for 3h. After 3h, pre-treatment were removed and compound solutions (dmso-only, UBS-109-only, 60μM Nec-1-only and UBS-109+60μM Nec-1) were applied to each well. The cells were incubated overnight at 37□C. Cell viability was determined by measuring the intracellular ATP levels using the Cell Titer-Glo luminescent Cell Viability Assay kit

according to the manufacturer's instructions. Luminescence was measured on a Synergy HT Multi-Detection Microplate Reader. Results were normalized relative to vehicle control (DMSO) treated cells.

Target Identification using Drug Affinity Responsive Target Stability (DARTS). This method for target identification was conducted as previous described by Lomenick *et al* [11]. J774 cells were plated onto 100mm x 20mm culture dish and incubated until confluence. The monolayer of cells were washed three times with PBS and then scraped off the dish in M-PER lysis buffer into a 1.5ml microfuge tubes. Tubes were incubated for 30min and following by centrifugation at 16,000rcf at 4C for 5min. The supernatant was removed and the pellet was discarded. UBS-109 (100µM) was addition one of the 1.5ml microfuge tubes. For comparison, the vehicle control DMSO was addition to the second tube. Both tubes were incubated for 30min at 37C, and then the solutions (40µL) were transferred to a series of labeled tubes. Various concentration of the protease thermolysin were added to each tube and then the tubes were incubated at room temperature. Finally, samples were separated on a 10% SDS-PAGE and stain overnight with coomasie blue.

Target Identification using Photo-Crosslinking and Click Chemistry. ATG5$^{+/+}$ was plated onto 100mm x 20mm culture dish and incubated until confluence. The monolayer of cells were washed three times with PBS and then scraped off the dish in 0.1% Triton X-100 lysis buffer into a 1.5ml microfuge tubes. Tubes were incubated for 30min and following by centrifugation at 16,000rcf at 4C for 10min. The supernatant was removed and the pellet was discarded. The protein concentration of the supernatant was

quantified using Bio-Rad Dc Protein Assay. The supernatant was diluted with PBS to make 1mg/mL protein stocks. Photo-crosslinking and click chemistry method for target identification was conducted as previous described by Mackinnon *et al* [8]. Briefly, 0.8µL of DMSO was added to 19µL of protein stock. 0.2µL of EMCN-00742 (10mM) was added and the sample solution was incubated on ice for 30min. Following incubation, the sample solution was irradiated at 365nm for 10min. Following irradiation, 2.5µL of 10% SDS was added and vortex mixed. Next, 0.5µL of 5mM biotin-azide was added. A copper(I)-catalyzed click chemical reaction was then conducted. The click reaction catalyst was prepared by combining 1.5 volumes 1.7mM TBTA in 80% t-butanonl/20% DMSO, 0.5 volumes 50mM $CuSO_4$, and 0.5 volumes 50mM TCEP. 2.5µL of catalyst was added to sample and incubated at 32˚C for 30min. Finally, the sample was run on 10% SDS-PAGE gel and detection of biotin by western blot using an anti-biotin antibody. To validate specific-labeling of analogue targets, four controls were conducted (cell lysate-only, no ECMN-00742, no UV-crosslinking, no click reaction).

To identify protein by mass spectroscopy, this identification process was scaled-up. Following click reaction step, acetone purification was conducted to remove any uncoupled biotin-azide from the samples (repeated 3x). The resulting pellet was air-dry and then redissolved in affinity buffer (50mM HEPES pH7.4, 100nM NaCl, and 1% Triton X-100). 100µL of monomeric avidin agarose beads were added to the solution and then incubated overnight at 4˚C with slow rotation. Next, the beads were sedimented using centrifugation (2min at 10,000rcf and 4˚C). Beads were washed using 1mL of the affinity buffer, slow rotation at 4˚C, and then centrifugation. This process was repeated 3x and

then the affinity buffer was replaced with washing buffer (50mM HEPES pH7.4, 500nM NaCl, and 1% Triton X-100). This process was repeated for addition 4x. Finally, the biotin-labeled proteins were eluted from the beads using a 2mM D-biotin elution buffer. Samples were incubated at room temperature for 2h and then heated at 96˚C for 5min. The eluent was then separated on a 10% SDS-PAGE and stain overnight with coomasie blue. Following destaining, the visible protein bands were cut from the gel and submitted for identification by LC-MS/MS (Protea Biosciences Group, Morgantown, WV).

Role of Autophagic Flux on UBS-109-induced Cell Death. $ATG5^{+/+}$ and $ATG5^{-/-}$ MEFs cells were plated at 20,000 cells/well in 96-well plate (Costar white plate with clear bottom, Corning Incorporated, NY) and allowed to adhere overnight. Next, solutions of UBS-109 and bafilomycin A1 was prepared to the desired concentrations (0.1µM, 1µM, and 10µM for UBS-109; 50nM bafilomycin A1), applied to the cells, and then incubated for 24h at 37˚C. Cell viability was determined by measuring the intracellular ATP levels using the Cell Titer-Glo luminescent Cell Viability Assay kit according to the manufacturer's instructions. Luminescence was measured on a Synergy HT Multi-Detection Microplate Reader. Results were normalized relative to vehicle control (DMSO) treated cells.

Results:

UBS-109 Induces Necroptosis.

To determine whether monocarbonyl curcumin analogues affect additional cell death signaling pathways other than apoptosis, UBS-109 was screened using various cell death assays. To do this, we isolated primary macrophages from wild type mice or mice lacking the receptor-interacting protein 3 ($RIP3^{-/-}$), which encodes a serine/threonine kinase required for the activation of the necroptotic death pathway [12]. Using a fluorescence microscopy assay, we observed that UBS-109 caused wild type but not $Rip3^{-/-}$ macrophages to detach from the coverslip, which is a crude indicator of cell death (Figure 1B). To quantitative this effect, we treated wild type and $RIP3^{-/-}$ macrophages with various concentration of UBS-109 for 24h and then measured cell viability directly using a luminescent cell viability assay, in which the number of photons produced by the addition of luminol to a cellular lysate depends on the concentration of cellular ATP in that lysate. Thus, dying cells produce less photons than living cells. As shown in Figure 1C, this assay showed that $RP3^{-/-}$ macrophages were significantly more viable than their wild type counterparts. To distinguish the effects on necroptosis from apoptosis, we next assessed the effects of UBS-109 on macrophages derived from mice lacking both Caspase 8 and Rip3 ($Caspase8^{-/-}$ $RIP3^{-/-}$). As shown in Figure 1C, $Caspase8^{-/-}$ $RIP3^{-/}$ macrophages exhibited significantly more survival than the wild type macrophages in presence of UBS-109. For contrast, curcumin was found to have no effect on the survival of either wild type or $RIP3^{-/-}$ macrophages at any concentration (data not shown).

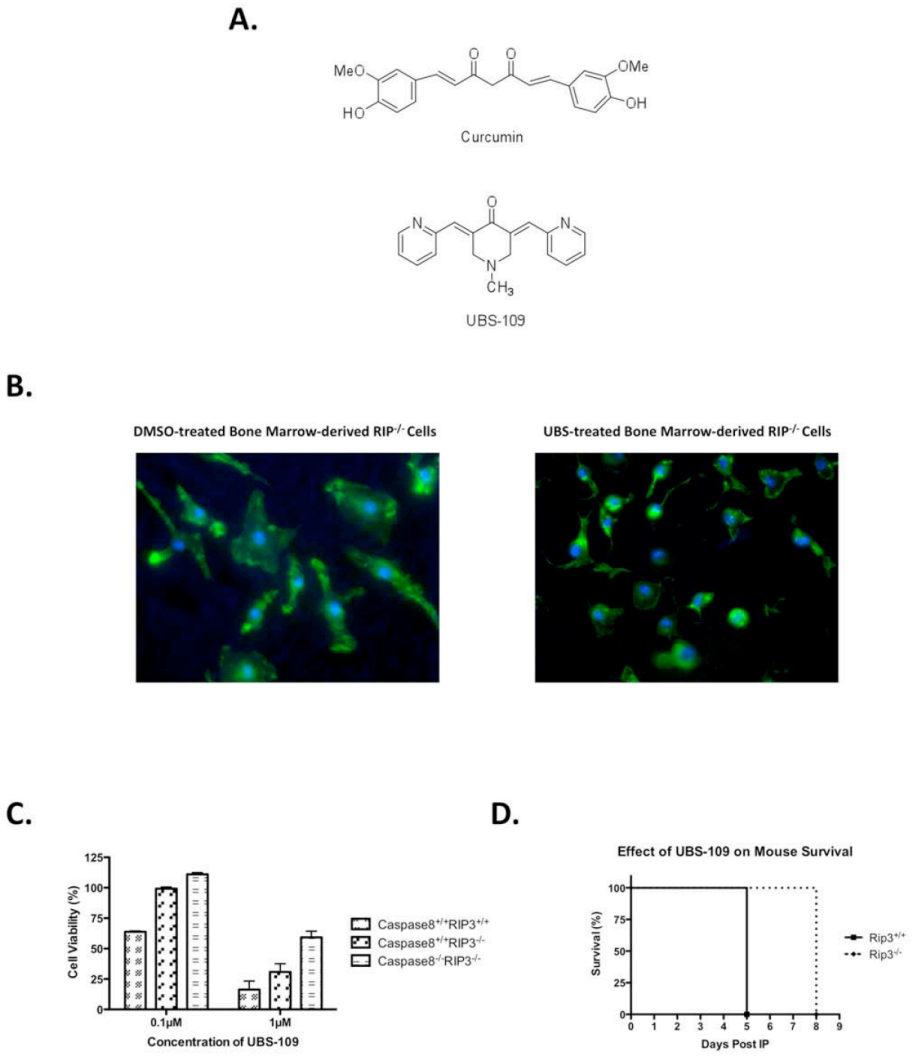

Figure 1. The curcumin analogue, UBS-109, induces necroptosis. (A) structure of curcumin and UBS-109; (B) Fluorescence microscopic examination of the mouse bone marrow-derived RIP3$^{-/-}$ macrophagys treated with DMSO for 12h and 10 μM UBS-109; (C) viability of wild type, rip3-knockout , and caspase8-knockout macrophages treated with various concentrations of UBS-109 (n=3). Cell viability was determine 24h post-treatment with UBS-109 by measuring ATP levels (Cell Titer-Glo Luminescent Cell Viability Assay Kit, Promega). Data normalized to dmso vehicle control.; (D) The effect of UBS-109 on mouse survival *in vivo*. Wild type and RIP3$^{-/-}$ mice received daily intraperitoneal injections of 30mg/kg UBS-109 (in DMSO) and montoried daily for drug effect.

These effects of UBS-109 were at least in part dependent on concentration. At low concentrations of UBS-109, RIP3$^{-/-}$ macrophages were found to have improved survival compared to wild type macrophages (100% for RIP3$^{-/-}$ versus 65% for wild type). By contrast, at higher concentrations (10μM), UBS-109 reduced survival of both wild type and knockout macrophages, though Caspase8$^{-/-}$ RIP3$^{-/-}$ macrophages survived better than the single knockout cells (~55% for Caspase8$^{-/-}$ RIP3$^{-/-}$, ~30% for RIP3$^{-/-}$, and ~18% for wild type). These data suggests that at low concentrations, UBS-109 induces RIP3-dependent necroptosis, whereas at higher concentrations, intrinsic apoptosis pathways are induced

Next, we assessed whether UBS-109 induced necroptosis *in vivo.* To do this, wild type and RIP3$^{-/-}$ mice were administered UBS-109 at 30mg/kg by IP injection daily and behavior changes, weight loss, appearance, and survival were monitored (Figure 1D). At this concentration all wild type mice succumbed by d5, whereas RIP3$^{-/-}$ animals survived until day 8. These data are in accordance with *in vitro* experiments and indicate that Rip3 mediates UBS-109 mediated cell toxicity both *in vitro* and *in vivo*.

UBS-109 Induces Autophagy.

Next, we determined whether UBS-109 induced autophagy a cellular pathway that controls both cell survival and cell death. First, using tramsmission elecron microscopy (TEM) on primary macrophages and J774 cells, we evaluated vesicle structures formed following treatment with UBS-109. We found that whereas no autophagosomes were detected in control cells, such structures were readily apparent in all cells treated with UBS-109 (Figure 2A). These data suggested that UBS-109 induced

autophagy. To confirm this possibility, we isolated mouse embryonic fibroblasts (MEFs) and macrophages from wt and ATG5$^{-/-}$ mice. ATG5 controls phagosomal trafficking

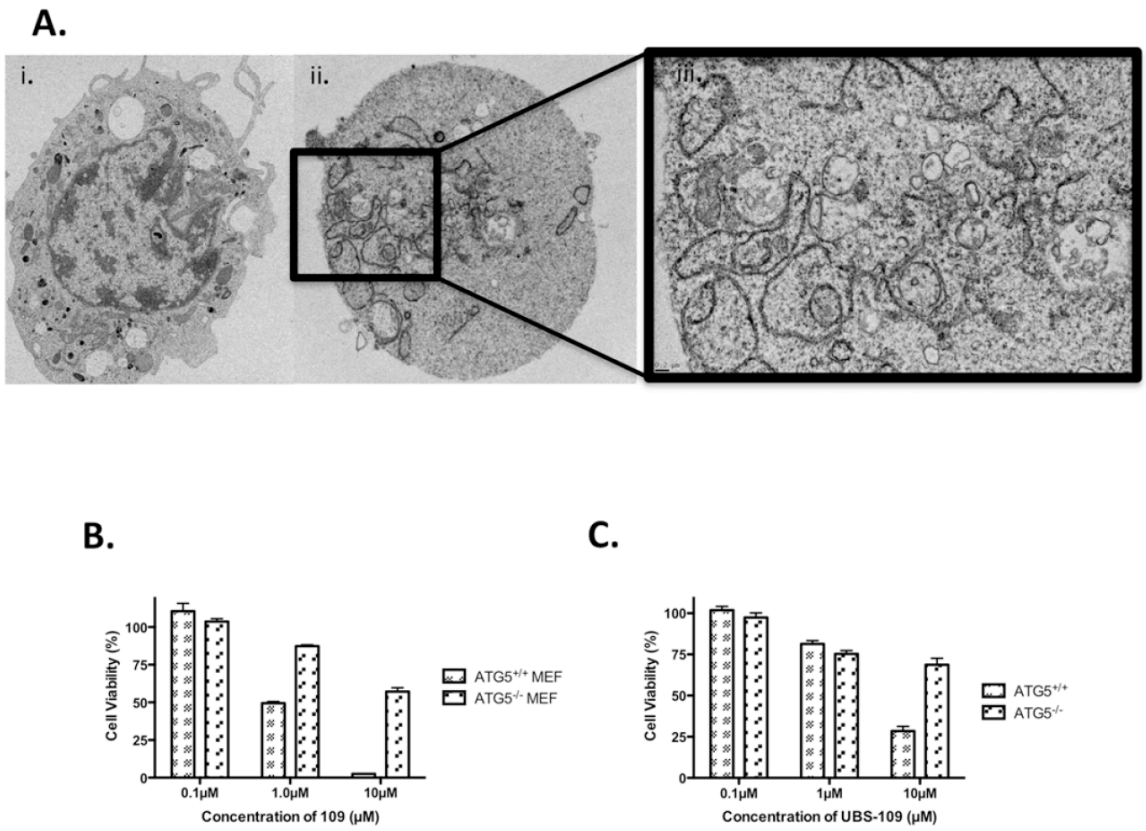

Figure 2. UBS-109 induces the autophagy pathway. (A) transmission electron microscopy images of mouse macrophagys (J774) treated with UBS-109 for 24h, (i) dmso vehicle control, (ii) 10μM UBS-109-treated, and (iii) close up of 10μM UBS-109-treated cells showing autophagosome; (B) viability of autophagy wild type and knockout mouse embryonic fibroblast cells (ATG5$^{-/-}$ MEFs). Cell viability was determine 24h post-treatment with UBS-109 by measuring ATP levels (Cell Titer-Glo Luminescent Cell Viability Assay Kit, Promega). Data normalized to dmso vehicle control.; n=3.; (C) viability of autophagy wild type and knockout mouse bone marrow-dervied primary fibroblast cells (ATG5$^{-/-}$ MEFs), n=3.

within the autophagy pathway, and cells lacking this protein do not form autophagosomes. We treated cells from wt or ATG5$^{-/-}$ mice with various concentrations

of UBS-109 for 24h and then measured cell viability using the luminescent assay, (Figure 2B). We found that ATG5$^{-/-}$ MEFs survived better than wild type MEFs at all concentrations of UBS-109. Similarly, primary macrophages derived from ATG5$^{-/-}$ mice survived better than primary wild type macrophages when treated with UBS-109 (Figure 2C). Together, these data suggest that UBS-109 induces autophagosome formation that depends on ATG5, and activates the autophagy pathway.

UBS-109 Induces Necroptosis and Autophagy By Independent Pathways.

We next determine whether induction of necroptotic cell death by UBS-109 depends on autophagy or whether both pathways are induced independently. To do this, we treated wild type and ATG5$^{-/-}$ cells with a necroptosis inhibitor, necrostatin-1 (Nec-1), together with various concentration of UBS-109 and then measured cell viability using the luminescence assay. We predicted that if necroptosis depended upon autophagy to cause cell death, then Nec-1 would improve the survival of wild type cells treated with UBS-109 similarly to the effect observed in cells lacking ATG5. However, if UBS-109 induced necroptosis and autophagy independently, then we would expect improved survival both in wild type and ATG5$^{-/-}$ cells due to blockage of necroptosis. In support of the independent model, we found that Nec-1 increased survival of ATG5$^{-/-}$ cells compared to cells not treated with Nec-1 (Figure 3A). These data suggest that UBS-109 induces both necroptosis and autophagy, but that necroptotic effects do not depend on autophagy.

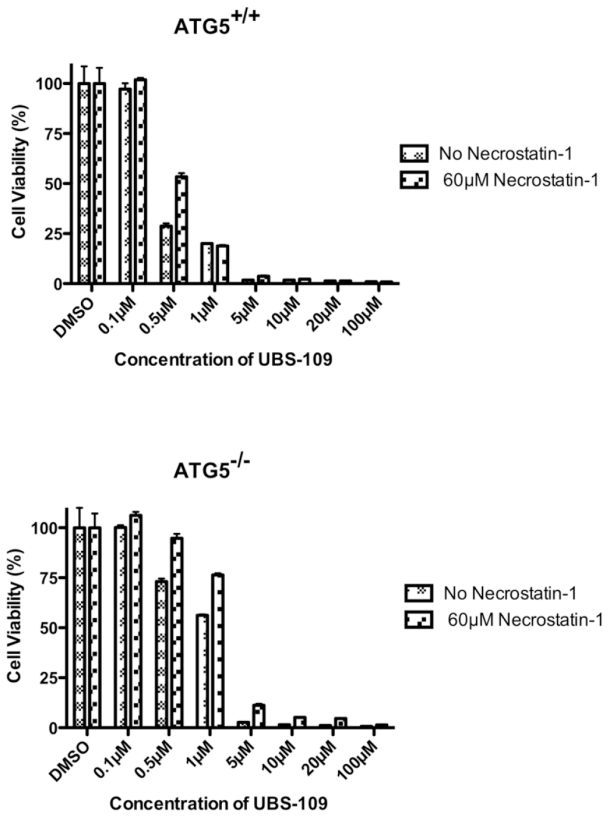

Figure 3. The effect of a necroptosis inhibitor on UBS-109-treated cells. (A) viability of autophagy wild type and knockout mouse MEFs cells (n=3) treated with various concentration of UBS-109 in the presence and absence of the RIP1 necroptosis inhibitor, necrostatin-1. Cell viability was determine 24h post-treatment with UBS-109 by measuring ATP levels (Cell Titer-Glo Luminescent Cell Viability Assay Kit, Promega). Data normalized to dmso vehicle control.

Identification of the Cellular targets of UBS-109.

We next set out to identify the possible direct binding targets of UBS-109 that induces the cell death pathways. To identify these targets, we used two technologies: drug affinity responsive target stabilization (DARTS) and photo-crosslinking/click chemistry. The basic concept of the DARTS method is that binding of a drug stabilizes

the target proteins in a specific conformation, which masks protease recognition sites and reduces the protease sensitivity of the target proteins [7]. As a result, protein targets of the drug will be relatively resistant to degradation, thereby allowing them to be identified subsequently by MS. However, after several attempts using different concentration of various proteases, this method failed to identify any targets of UBS-109.

As an alternative, we conducted a photo-crosslinking/click chemistry approach [13]. To do this, we developed a clickable UBS-109 (ECMN-00742), which contained an alkyne group for click chemistry (Figure 4A). ECMN-00742 was synthesized and shown to have similar biological activity as UBS-109 in cell viability assays (Figure 4B). To define targets, we incubated the analogue with cell lysates, washed, and then crosslinked the compound to putative targets using UV light. Next, click chemistry was used to a attach a biotin moiety to the compound (Figure 4C). The proteins were separated by SDS-PAGE, and visualized by western analysis with an anti-biotin-HRP antibody. As controls, we incubated lysates with DMSO, the carrier for ECMN-00742, or with ECMN-00742 without crosslinking, or with UV-crosslinking, but without the click reaction. As shown in Figure 3D, multiple biotin-labeled proteins were evident, while no protein was detected in controls. To determine the sequence of identified proteins, the reaction was scaled up, and the proteins purified by affinity chromatography using mono-avidin beads. Isolated proteins were then separated by SDS-PAGE and detected by coomassie blue staining. Multiple protein bands ranging from 30kDa to 150kDa were identified, isolated from the gel system, and submitted for protein identification by LC-ESI-MS/MS.

Mass spectroscopy identified multiple proteins targets for UBS-109. They included transcription factors, elongation factors, metabolic enzymes, ribosomal proteins, proliferation/survival proteins, and heat shock proteins. Several of these proteins have been shown previously to be targets of curcumin, including tubulin, actin, and thioredoxin domain-containing protein (Table 1). The most common proteins identified were heat shock proteins (Hsp). Hsp 90 and all of its isoforms, including GRP94, TRAP1, Hsp90α, and Hsp90β, were the most commonly detected Hsps. Interestingly, most of the identified proteins contained ATP-binding domains.

Several identified proteins (e.g. hsp90, survivin, etc) have been shown to affect cell death. In regards to necroptosis, none of the identified proteins are known to affect this pathway. However, one of the identified proteins, called transitional endoplasm reticulum ATPase, has been shown to affect the autophagy pathway. This protein plays a role in mediating phagolysosomal fusion stage of autophagy. An examination of this protein identified a cysteine residue near the ATP binding domain, which could serve as a Michael addition reaction site.

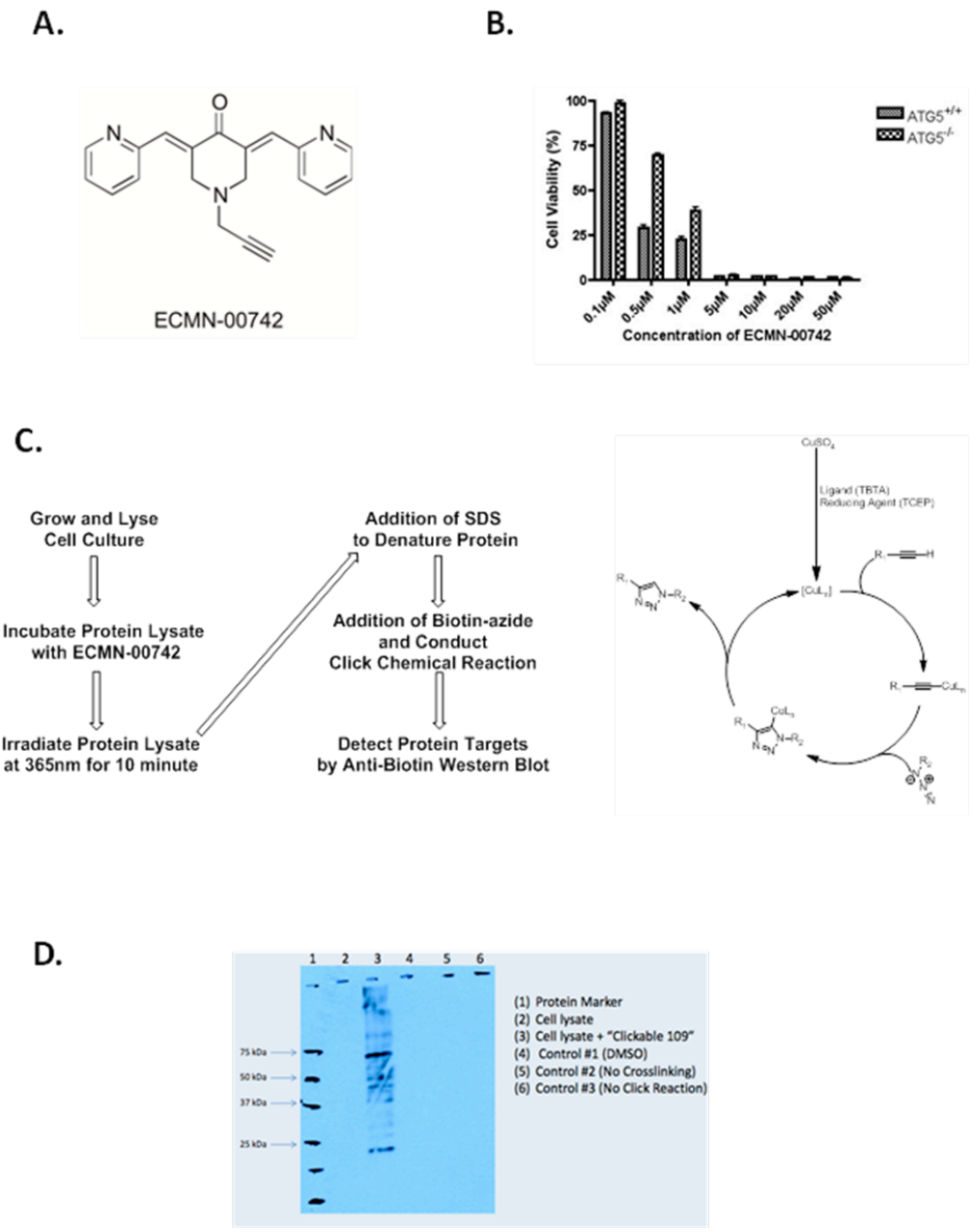

Figure 4. Identification of curcumin analogue targets in mammalian cells by photo-crosslinking/click chemistry. (A) structure of click chemistry curcumin analogue, ECMN-00742; (B) viability of autophagy wild type and knockout mouse MEFs cells (n=3) treated with various concentration of curcumin. Cell viability was determined 24h post-treatment with UBS-109 by measuring ATP levels (Cell Titer-Glo Luminescent Cell Viability Assay Kit, Promega). Data normalized to dmso vehicle control.; (C) method of photo-crosslinking and click chemistry reaction of wild type MEFs; (D) western blot

analysis of sample and controls from photo-crosslinking and click chemistry reaction. Proteins detected using anti-biotin antibody (1:2500 ratio).

Table 1- List of Most Common Protein Target Identified By Photo-crosslinking/Click Chemistry for ECMN-00742
Heat Shock Protein (Hsp)*
Hsp90α
Hsp90β
TRAP1
Hsp70
Hsp40
Heat Shock Cognate 71
Stress-70 Protein (mitochondrial Hsp)
Ribosomal Protein*
40S Ribosomal Protein S3a
60S Ribosomal Protein L7a
40S Ribosomal Protein S6
Metabolitic Enzymes
Glyceraldehyde-3-phosphate dehydrogenase*
Pyruvate Kinase Isozyme
Pyruvate Kinase
L-Lactate dehydrogenase
Phosphoglycerate Kinase
Citrate Synthase
Succinate Dehydrogenase Flavoprotein
ATP Synthase
α-Enolase
Elongation Factors*
1α-Elongation Factor
2-Elongation Factor
Elongation Factor Tu
Other
Tubulin*
Actin*
Importin
Thioredoxin Domain-containing Protein*

*Known Curcumin Target

UBS-109 Affects the Phagolysosomal Fusion Stage of Autophagy.

Taken together, these data suggest that UBS-109 both induces the autophagy pathway leading to an increases in autophagosomes, but may also prevent phagolysosomal fusion by inhibiting transitional endoplasm reticulum ATPase. As a result of preventing this fusion, the recycling of cellular material will be blocked and cause the induction of cell death. To test this idea, wild type or $ATG5^{-/-}$ cells were treated with bafilomycin A1, which directly inhibits vacuolar-type H^{+}-ATPases and prevents phagoloysomal fusion, together with various concentration of UBS-109 and then cell viability measured using the luminescent assay. If protein turnover in the phagolysosme is required for cell death, then we expected that bafilomycin A1 would reduce autophagic cell death induced by UBS-109, similar to observations in $ATG5^{-/-}$ cells. By contrast, if phagolysosomal fusion is not required for cell death, then bafilomycin A1 would be expected to have no effect on autophagic cell death. As seen in Figure 5, we found no difference in UBS-109-induced cell death upon addition of bafilomycin A1 to wild type cells, although some bafilomycin A1-specific toxicity was evident. Moreover, although less than in wild type cells, the extent of cell death evident in ATG5-/- cells treated with UBS-109 was likewise unaffected by treatment with bafilomycin A1. These data suggests that UBS-109 may induce autophagic cell death by preventing phagolysosomal fusion, and thereby turnover of cellular material.

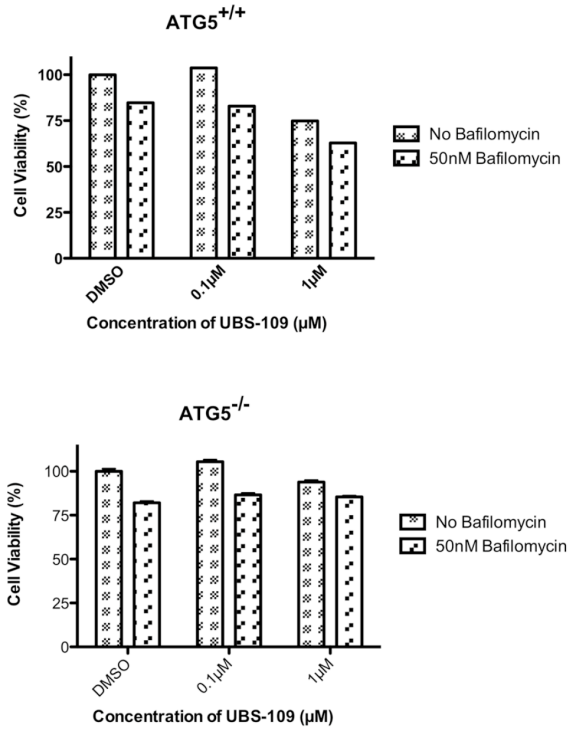

Figure 5. The effect of the phagolysosomal fusion inhibitor on UBS-109-treated cells. The viability of autophagy wild type and knockout mouse MEFs cells (n=3) treated with various concentration of UBS-109 in the presence and absence of the autophagic flux inhibitor, bafilomycin A1. Cell viability was determine 24h post-treatment with UBS-109 by measuring ATP levels (Cell Titer-Glo Luminescent Cell Viability Assay Kit, Promega). Data normalized to DMSO vehicle control.

Discussion:

Most anti-cancer therapeutics function by promoting the induction of apoptosis as a means to eradicate cancer cells. However, this approach is generally not effective because cancer cells up-regulate anti-apoptotic pathways. One means to eliminate such countermeasures in cancer cells is by developing compounds that target multiple cell death pathways. Curcumin and its analogues have been reported to affect multiple cellular pathways, including apoptosis [14-15]. However, the effect of these analogues on non-apoptotic cell death pathways has not been determined. Here, we demonstrate

using knockout cell lines and specific inhibitors that the curcumin analogue UBS-109 induces two non-apoptotic cell pathways, necroptosis and autophagy.

Recently, it has been proposed that the necroptosis and autophagy pathways may regulate each other. Some studies have reported that autophagy regulates and induces necroptosis [16-18]. For instance, palmitic acid-induced autophagy requires necroptosis to cause cell death. Alternatively, necroptois may induce autophagy [19]. In addition, autophagosomes have been observed in TEM images of cells undergoing necroptosis [20]. Our data using the necroptosis inhibitor Nec-1 suggest that UBS-109 induces necroptosis and autophagy by independent means. Thus, inhibition of the necroptosis improved survival in wild type and ATG5$^{-/-}$ cells (Figure 3). However, this inhibition of the necroptosis pathway in the wild type cells did not copy the effect of knocking out ATG5 cells. From these results, we conclude that these death pathways are independently activated by UBS-109.

Using our photo-crosslinking/click chemistry-based identification method, we identified multiple proteins (e.g. transcription factors, metabolic enzymes, heat shock proteins, etc) that may be potential targets for UBS-109, including several known targets of curcumin such as actin, tubulin, and thioredoxin-domain-containing protein. Several identified proteins (e.g. heat shock protein 90, survivin, etc) have also been shown to affect cell death. For example, inhibition of heat shock protein 90 (Hsp90) [21] or survivin induce apoptosis [22]. None of the identified proteins are known to affect the necroptosis pathway.

However, one of the identified proteins, called transitional endoplasm reticulum ATPase, has been shown to contribute to autophagic cell death [23]. Inhibition of this protein has been found to prevent the phagolysomal fusion stage of autophagy, which causes a buildup of autophagosomes and eventual death of the cell. Our TEM data also showed a buildup of autophagosomes with UBS-109 treatment. Using the autophagy inhibitor bafilomycin A1, which prevents the phagolysomal fusion stage of autophagy, we show that UBS-109 has no additional effect suggesting that it also inhibits phagolysomal fusion stage to induce cell death. Finally, an examination of the crystal structure of transitional endoplasm reticulum ATPase showed a cysteine residue near the ATP-binding domain, which is the preferred target of Michael acceptor in these curcumin analogues. Together, these data suggest that UBS-109 may induce autophagic cell death by preventing phagolysomal fusion stage from occurring.

In conclusion, our results revealed that the curcumin analogue UBS-109 induces necroptosis and autophagy. These finding demonstrated that UBS-109 or similar compounds could potentially be developed into a novel cancer therapeutic that targets multiple cell death pathways. Future work will focus on developing analogues that specifically target either the autophagy or necroptosis pathway.

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Chapter 4:

UBS-109 Binds To Heat Shock Protein 90

UBS-109 Binds To Heat Shock Protein 90

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Abstract:

Heat shock protein 90 (Hsp90) has been proposed as a target for therapeutic intervention in cancer due to its critical role in maintaining cell proliferation. While Hsp90 inhibitors have efficacy in killing cancers *in vitro*, no compound has proven successful in a clinical environment, mostly due to toxicity. Because of the critical role of Hsp90 in cancer cell survival, particularly for cancers resistant to other therapeutic agents, there is an urgent need for the development of new Hsp90 inhibitors that are less toxic and more selective. We report here that a monocarbonyl analogue of curcumin, called UBS-109, may represent a novel class of Hsp90 inhibitors. Using *in vitro* fluorescence polarization assays and mass spectroscopy, we validated that UBS-109 reversibly binds to Hsp90. Using computational modeling approaches, we show that the Michael acceptor in UBS-109 may not be critical for binding of UBS-109 to Hsp90. Finally, we have identified possible binding sites for UBS-109 using a peptide mapping strategy. Together, these data indicate that UBS-109 reversibly binds Hsp90 by a mechanism that does not depend upon its Michael acceptor. These date provide important information that will aid in the development of curcumin-based Hsp90 inhibitors.

Introduction:

Cancer is one of the leading causes of death worldwide with over 6 million deaths annually in US [1]. Because most cancers remain inflexible to treatment, there has been a rush to identify new cellular targets for therapeutic intervention. Recently, a

class of molecular chaperones, known as heat shock proteins (Hsp), has been proposed as a promising new target for eliminating cancer cells [2].

Hsps are a family of molecular chaperones (e.g. Hsp90 and Hsp70) that are expressed in all living organisms, and function to regulate cellular homeostasis by maintaining proper folding of proteins in the presence of cellular stressors [3]. Hsp90 is the most abundant member of this family and consists of multiple isoforms including Hsp90α and Hsp90β in the cytosol, TRAP1 in the mitochondria, and GRP94 in the endoplasmic reticulum. The molecular structure of Hsp90 consists of three major regions: an amino (N)-terminal domain with an ATP-binding and hydrolyzing pocket (ATPase activity), which regulates client protein folding; a middle domain that regulates protein interactions; and a carboxy (C)-terminal domain that directs Hsp90 dimerization. Hsp90 has been found to interact with over 200 different proteins, which may account for its critical role in regulating numerous signaling pathways controlling proliferation, differentiation, and cell death [3]. Hsp90 is frequently over-expressed in many cancer cells and may play a critical role in stabilizing specific oncogenic proteins, such as HER2 [2].

Recently, we have identified Hsp90 as a potential cellular target for the monocarbonyl curcumin analogue, UBS-109 (see chapter 3). Based on this data, the objective of this present work is to investigate the effect of UBS-109 on Hsp90. Initially, we validated that UBS-109 forms a reversible chemical interaction with Hsp90. By applying computational modeling methods, we proposed a possible mechanism for UBS-109 binding that does not require the Michael acceptor, which may contribute to

toxicity *in vivo*. Finally, we have conducted a peptide mapping strategy to identify potential UBS-109 binding sites. These data expand the understanding of the mechanisms of UBS-109 inhibition of Hsp90 and will aid in the development of curcumin-based inhibitors.

Experimental Procedures

Chemicals. UBS-109, ECMN-00742, ECMN-909, and ECMN-951 were synthesized as previous described [5 and 6]. Curcumin and demethyl sulfoxide (DMSO), was purchased from Sigma-Aldrich (St. Louis, MO).

Drug Effect on Binding to Nucleotide-binding pocket in the N-terminal of Hsp90. The binding of UBS-109 to the ATP-binding in Hsp90 by UBS-109 was determined by measuring the binding of fluorescein isothiocynate-labeled geldanamycin (FITC-GA) to UBS-109-treated Hsp90 using the fluorescence polarization Hsp90 assay kit (BPS Bioscience, San Diego, CA). Briefly, purified Hsp90 (α and β) was incubated with various concentration of UBS-109 for 30min at 4˚C. Following incubation, FITC-GA was added to samples and incubated for 2h at room temperature. Fluorescence was measured using Synergy HT Multi-Detection Microplate Reader (λ_{ex}= 485nm and λ_{em}= 525nm). Both reagent blank, (+) and (-) controls were conducted. Data was normalized to the positive control.

Binding Interaction of UBS-109 to Hsp90α. To determine whether the reversible or irreversible covalent binding interaction between UBS-109 and Hsp90α occurs, several solutions consisting of Hsp90α (5µg; BPS Bioscience, San Diego, CA) in cold PBS were prepared on ice. Various concentrations (0.1µM, 1.0µM, 10µM, and 100µM) of UBS-109

were added and then incubated for 30min on ice. Following incubation, solutions were flash frozen in liquid nitrogen and submitted for intact protein analysis by LC-MS/MS (Protea Biosciences Group, Morgantown, WV).

Determine Binding Location of UBS-109 to Hsp90. The binding location was determined using purified recombinant human Hsp90 (BPS Bioscience, San Diego, CA). Several solutions consisting of Hsp90α (15µg) in cold PBS were prepared on ice. Next, various concentrations (0.1µM, 1.0µM, 10µM, and 100µM) of UBS-109 were added and then incubated for 30min. Following incubation, the solution were transferred to a 96-well plate and irradiated at 365nm at 4˚C for 10min. Following photo-crosslinking, the solutions were flash freeze in liquid nitrogen and submitted for digestion and LC-MS/MS analysis (Protea Biosciences Group, Morgantown, WV). Briefly, the proteins were concentrated by Amicon centrifugation and then were digested by trypsin. The solutions were lyophilized and stored for analysis at -80˚C. The solutions were analyzed by LC-MS/MS. To determine the binding region, the mass of peptide fragments of the untreated Hsp90 was compared to the mass of peptide fragments from UBS-109 treated Hsp90.

Modeling of the Interaction of UBS-109 to Hsp90α. The structures of curcumin, ECMN-00746, UBS-109, EF-31, and SEF-31 were draw in 2D with ChemDraw and then submitted for ligand preparation in Maestro 9.1 to obtain the 3D structures. 1YET for Hsp90α was downloaded and processed by protein preparation wizard in Maestro 9.1. The receptor grid was prepared for the ATP-binding pocket. Ligand-docking was conducted with SP precision for flexible docking of the ligands.

Results:

Binding of Heat Shock 90 by UBS-109-

Hsp90 was isolated as a binding partner of UBS-109 in a click-chemistry based target identification scheme (see Chapter 3). To validate that Hsp90 is a direct binding target of UBS-109, we evaluated the ability of UBS-109 to inhibit the binding of a FITC-labeled heat shock 90 inhibitor, called geldanamycin (FITC-GM), to the ATP-binding pocket in the N-terminal domain of Hsp90α using a competitive fluorescence polarization assay. As shown in Figure 1A, UBS-109 inhibited binding of FITC-GM with an IC_{50} of 1.4μM and K_i of 1.3 μM. Similarly, UBS-109 inhibited the binding of FITC-GM to the Hsp90β isoform (data not shown). To further validate that Hsp90 is a direct binding target of UBS-109, we conducted a pull-down assay using purified Hsp90α with a clickable UBS-109 modified for click chemistry (ECMN-00742). To do this, we incubated the analogue with Hsp90α, UV-crosslinked the compound to the protein, and then attached a biotin moiety to the compound by click chemistry. The labeled proteins were separated by SDS-PAGE and visualized by western analysis with an anti-biotin-HRP antibody. In addition, we incubated purified Hsp90α with DMSO as a control. As shown in Figure 1B, a single biotin-labeled protein band was evident, while no protein was detected in the control. This biotin-label protein had a molecular weight of 86kDa, which corresponds to the weight of Hsp90. Together, these data suggest that Hsp90α is a direct binding target of UBS-109.

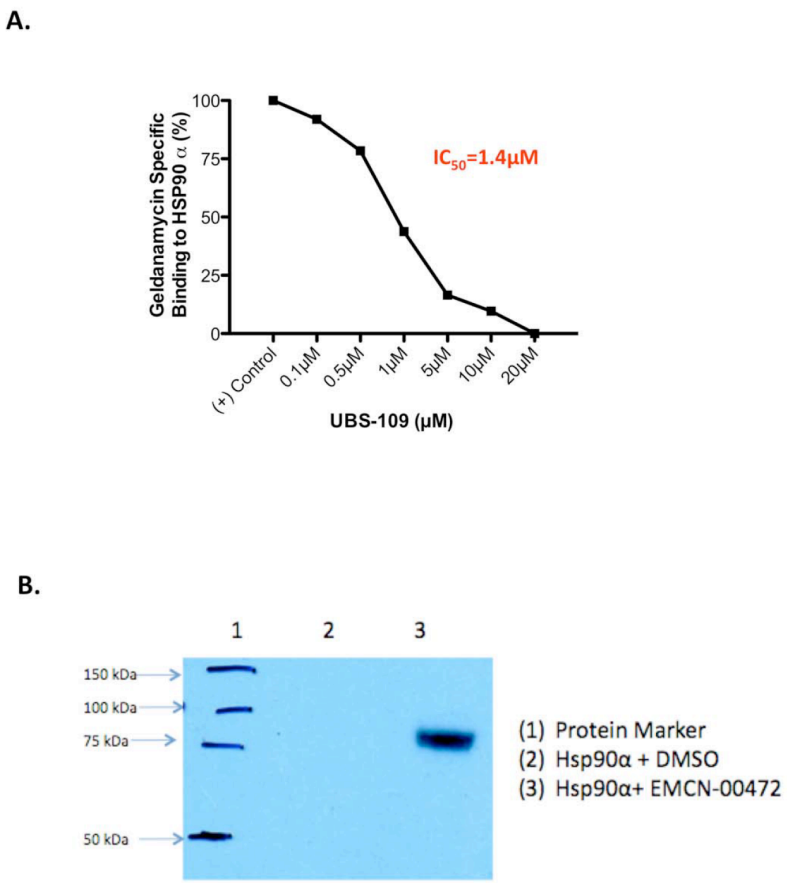


Figure 1. Binding of UBS-109 to Hsp90. (A) fluorescence polarization compete binding assay (Hsp90α Assay Kit, BPS Bioscience) for validating UBS-109 binding to Hsp90. Purified Hsp90α is incubated with various concentration of UBS-109 for 30min (n=2), following by addition of 100nM FITC-Geldanamycin and room incubation for 2h. Fluorescence was measured with excitation of 485nm and emission of 525nm. Binding of UBS-109 was determined by decrease fluorescent signal resulting from FITC-Gelanamycin binding to Hsp90. (B) Western blot analysis of photo-crosslinking and click chemistry of ECMN-00472 to purified Hsp90α. Proteins detected using anti-biotin antibody (1:2500 ratio).

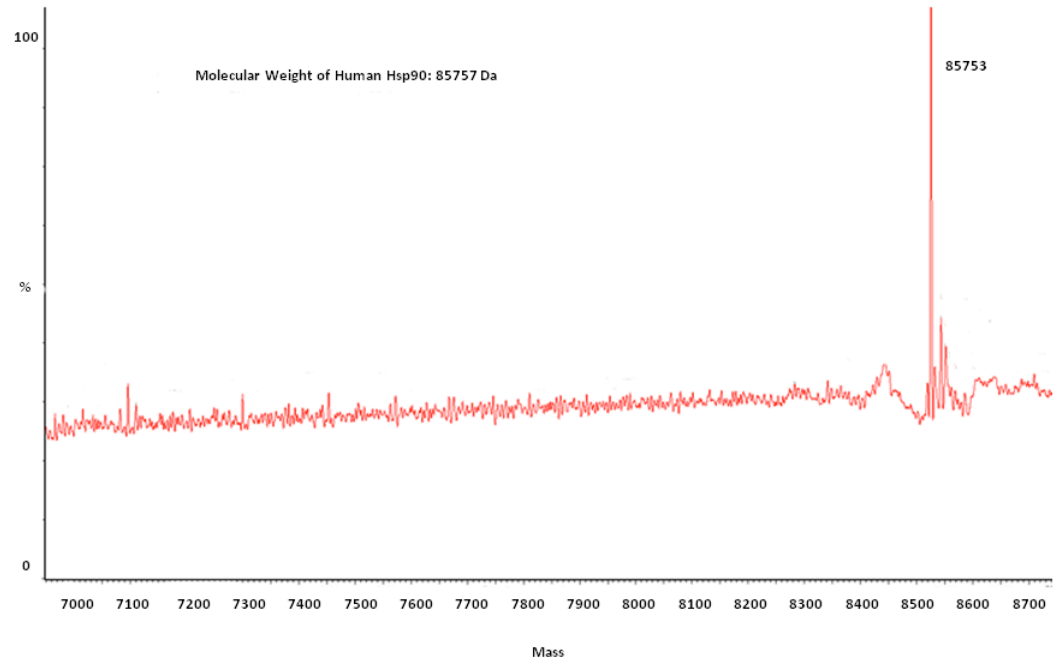
UBS-109 Does Not Form Irreversible Chemical Interactions with Hsp90-

To determine whether the binding of UBS-109 to Hsp90 chemically modifies the target, we treated purified HSP90α with various concentrations of UBS-109 and then assessed differences in the protein by LC-MS/MS. As a control, we treated HSP90α with

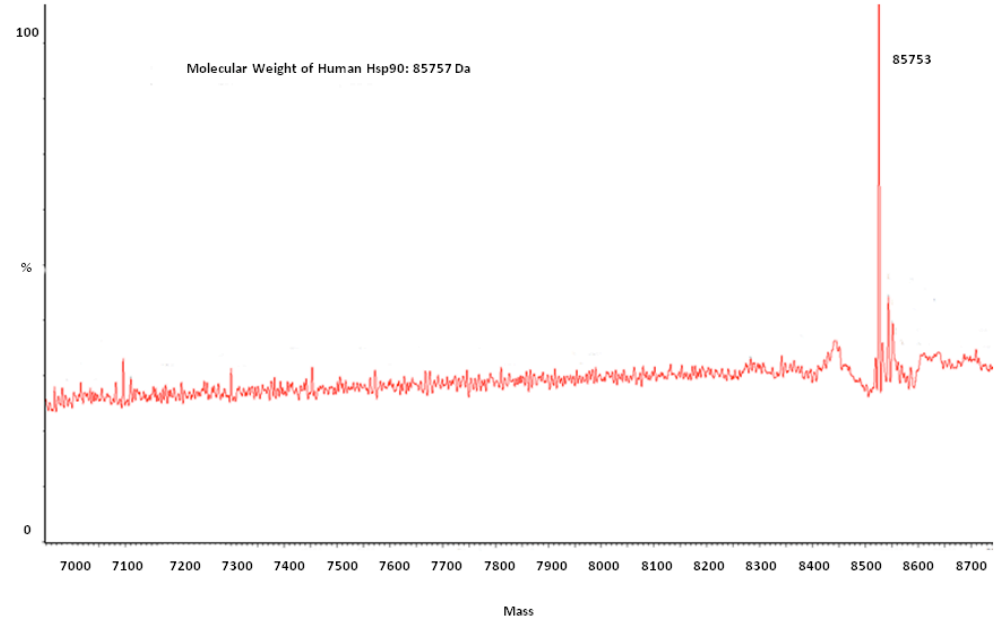
DMSO. To distinguish reversible from irreversible modifications, an increase mass for UBS-109-treated HSP90 in comparison with the control was interpreted as an irreversible modification. As seen in Figure 2, we observed a peak of 86kDa on the mass spectrum for the control, which corresponds to the mass of HSP90α. However, we did not observed any increase in mass upon the addition of UBS-109. These data suggest that UBS-109 does not irreversibly interact with HSP90α.

Evaluation UBS-109 Docking into the ATP-Binding Site on the N-Terminal of HSP90α- Previously, it has been reported that UBS-109 is a competitive inhibitor for the ATP-binding pocket of various kinases [6]. Using Schrodinger's Glide docking software and the crystal structure of geldanamycin-binding to the ATP pocket of N-terminus domain of HSP90α (1YET), we next assessed how UBS-109 inhibits the binding of geldanamycin to the ATP-binding pocket in Hsp90α. To do this, a receptor grid based on the binding interactions of geldanamycin to HSP90α was generated for the docking of UBS-109. As a control to validate that the receptor grid was correctly formed, we successfully re-docked geldanamycin into the ATP-binding pocket with a glide score of -9.400 and a docking score of -9.346 (Figure 3A). In addition, we found that overlaying the geldanamycin from both the crystal structure and the control docking had a very similar pose (Figure 3B). Together, our data suggest the receptor grid for was correctly

A.


100
%
0
Molecular Weight of Human Hsp90: 85757 Da
85753
7000 7100 7200 7300 7400 7500 7600 7700 7800 7900 8000 8100 8200 8300 8400 8500 8600 8700
Mass

B.


100
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0
Molecular Weight of Human Hsp90: 85757 Da
85753
7000 7100 7200 7300 7400 7500 7600 7700 7800 7900 8000 8100 8200 8300 8400 8500 8600 8700
Mass

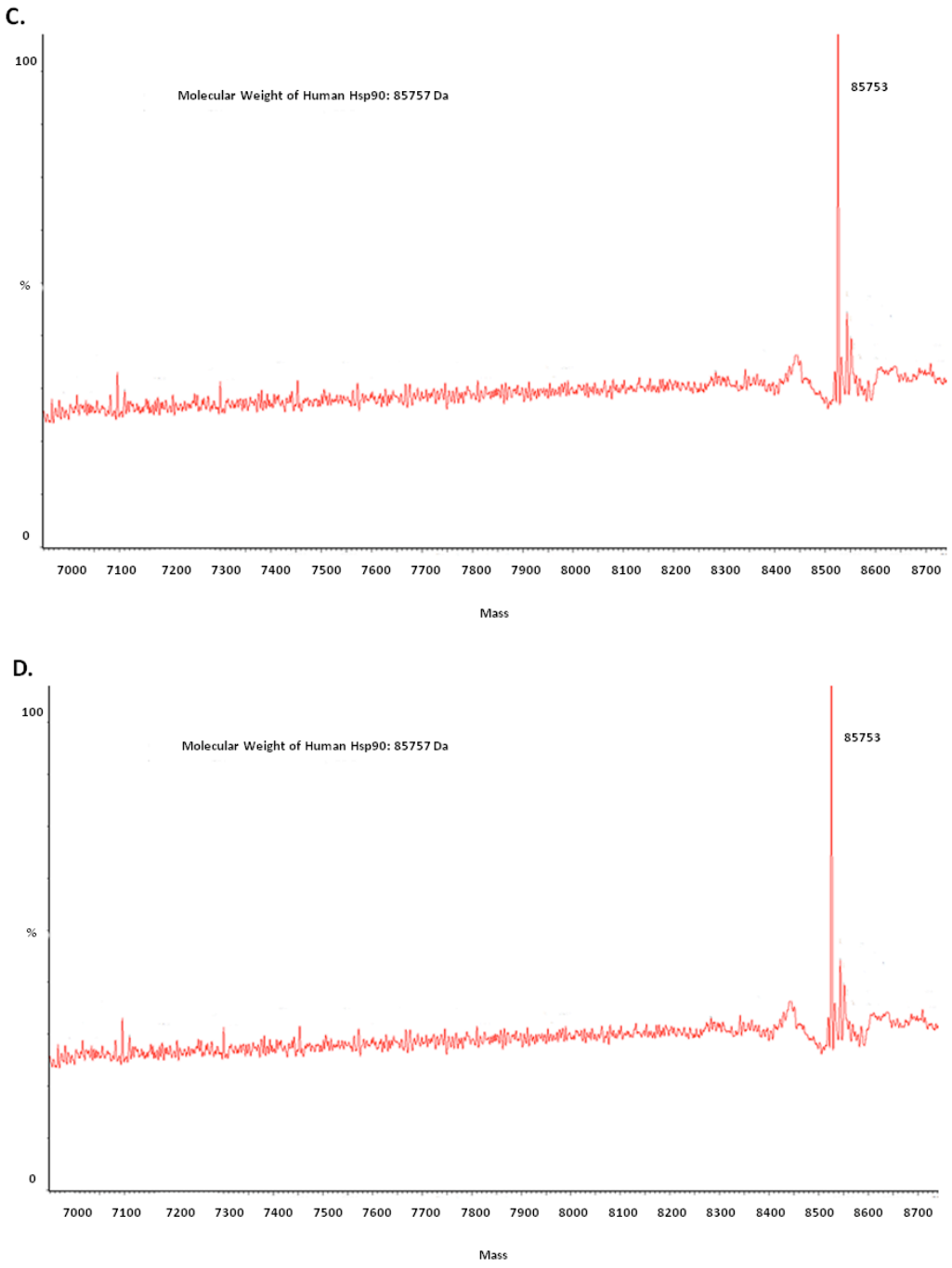


Figure 2. Determination of Reversible Covalent Modification of Hsp90α by UBS-109. Purified Hsp90α was incubated with various concentration of UBS-109 for 30min, flash frozen with liquid nitrogen, and submitted for analysis by LC-MS/MS (Protea Biosciences Group, Morgantown, WV). An increase in mass for UBS-109-treated HSP90 in comparison with the control was interpreted as an irreversible modification. (A) MS data showing interaction of purified Hsp90α with 0μM UBS-109 (control). (B) MS data showing interaction of purified Hsp90α with 0.1μM UBS-109. (C) MS data showing

interaction of purified Hsp90α with 1μM UBS-109. (D) MS data showing interaction of purified Hsp90α with 10μM UBS-109.

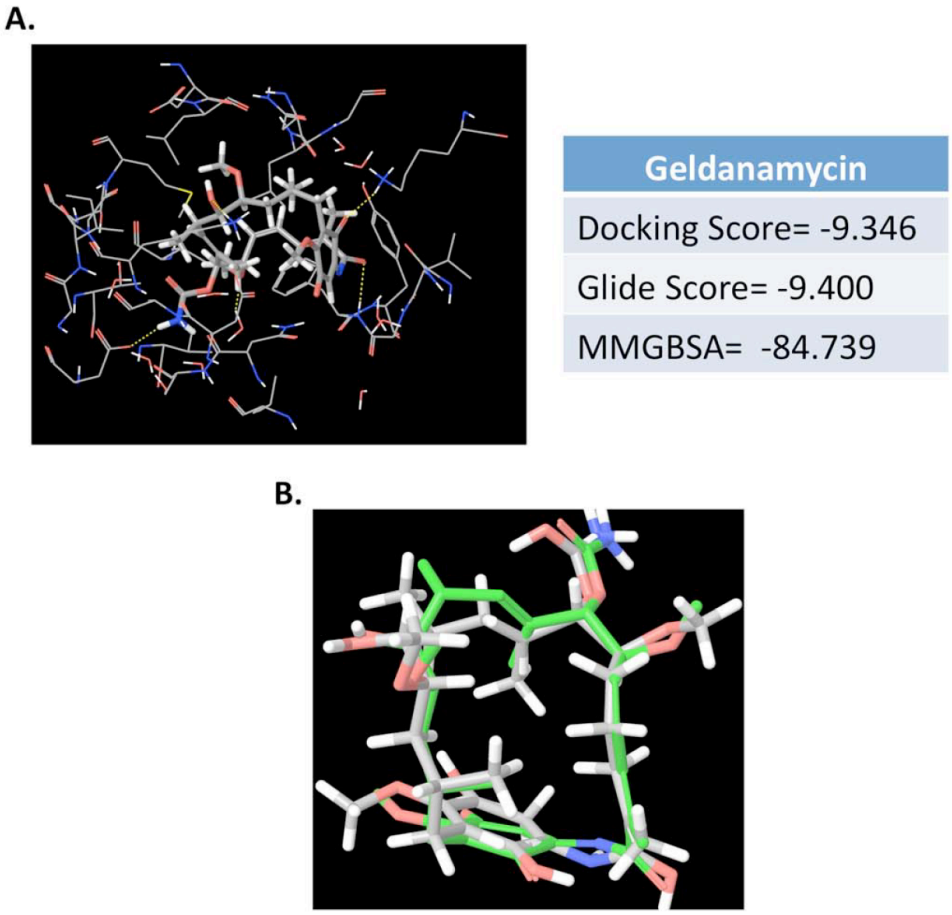


Figure 3. Glide ligand docking of geldanamycin to Hsp90α. (A) The glide ligand docking of geldanamycin to Hsp90α and its resulting docking scores . (B) Overlapping of glide-docked geldanamycin and geldanamycin from the Hsp90α crystal structure.

oriented for studying the binding of UBS-109 into HSP90α.

Using this receptor grid, we successful docked UBS-109 into the ATP-binding pocket of HSP90α with a docking score of -5.606 and a glide score of -5.606 (Figure 4A). This docking of UBS-109 into HSP90α was similar to that evident with binding of geldanamycin. This docking proposed that UBS-109 forms a hydrogen bond between

the nitrogen in one of its pyridine rings and lysine 58 (K58) in Hsp90, while the other pyridine ring in UBS-109 suggested that a pi-cation interaction with lysine 112 (K112). Both of the interactions involved amino acids used by geldanamycin in binding to Hsp90. In addition, this docking proposed that the monocarbonyl of UBS-109, which with its flanking double bonds constitutes a Michael acceptor, forms no chemical interaction with any amino acid in the binding pocket of Hsp90. Thus, we hypothesized that the Michael acceptor, a possible source of toxicity *in vivo*, may be dispensable for effects on Hsp90.

Besides UBS-109, we also docked curcumin and other curcumin analogues (SEF-31, and EF-31) into the ATP-binding pocket of HSP90α . EF-31 differs from UBS-109 in that the central ring lack a methyl side chain, while SEF-31 contains a sulfur group in central ring. As seen in Figure 4B and Table 1, we observed curcumin to have best docking scores of all compounds tested, because it formed three hydrogen bonds. Notably, both EF-31 and SEF-31 successfully docked into ATP-binding pocket with docking scores similar to the UBS-109 (-5.676 for SEF-31, -5.167 for EF-31, and -5.606 for UBS-109), and SEF-31 and EF-31 were judged to form similar chemical interactions as UBS-109. Together, these data suggest that UBS-109 may block specific residues used by geldanamycin in binding to Hsp90α.

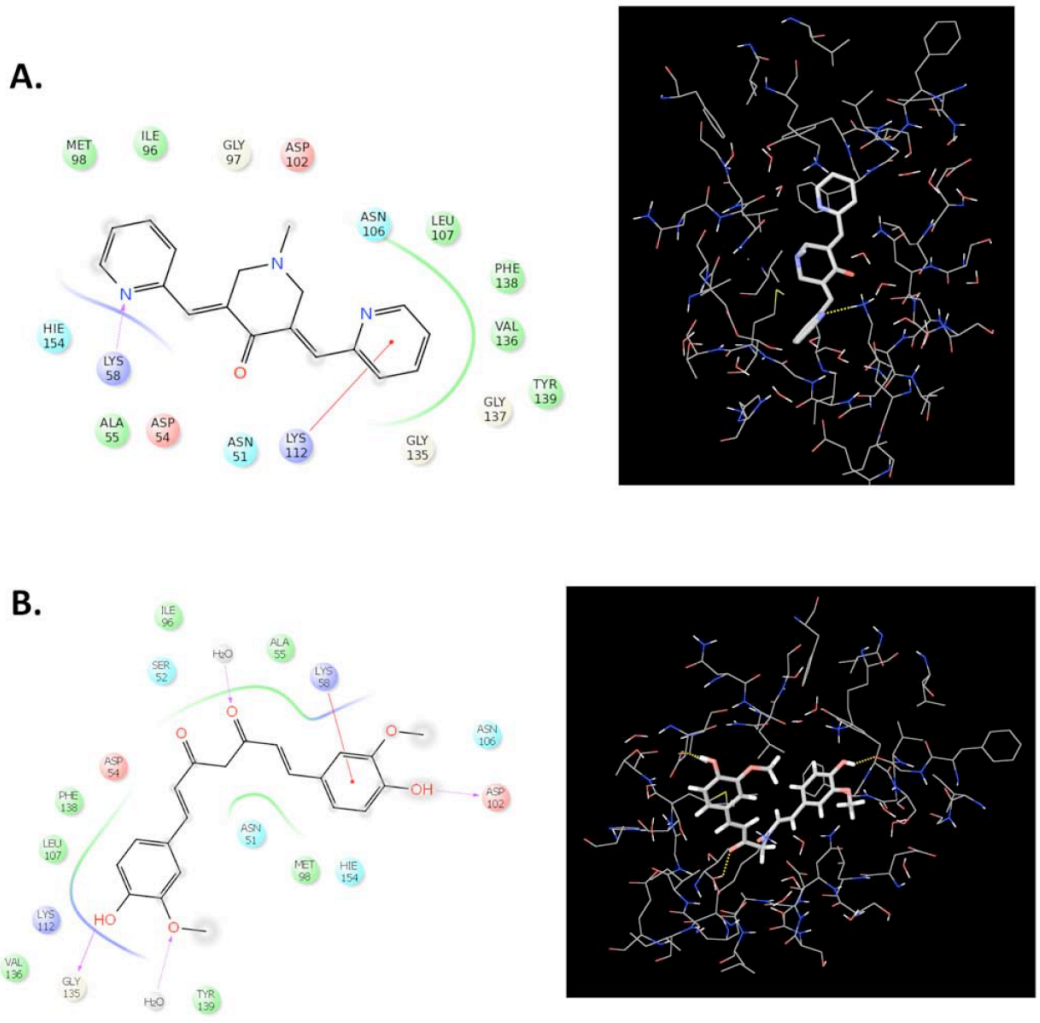


Figure 4. Glide Docking of UBS-109 and Curcumin to Hsp90α. (A) The ligand-protein interactions resulting from the glide-docking of UBS-109 to the ATP-binding pocket of Hsp90α. (B) The ligand-protein interactions resulting from the glide-docking of curcumin to the ATP-binding pocket of Hsp90α.

Table 1- Glide Docking Results of Curcumin Analogs to Hsp90α			
Compounds	**Docking Score**	**Glide Score**	**MMGBSA**
Curcumin	-5.771	-5.777	-56.107
UBS-109*	-5.606	-5.606	-43.896
Ubs-109**	-5.619	-5.619	-50.594
EF-31	-5.167	-5.167	-40.729
SEF-31	-5.676	-5.676	-48.096

Identification of Possible HSP90 Binding Sites Using Peptide Mapping-

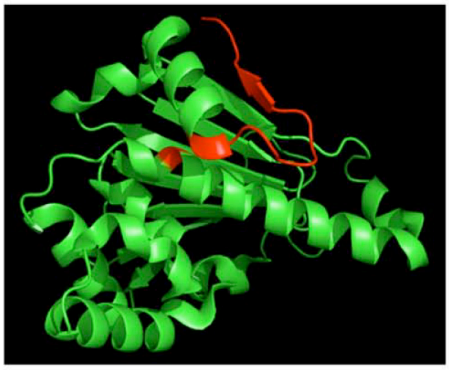
To validate the binding location for UBS-109 to HSP90α, we incubated HSP90α with various concentrations of UBS-109, UV-crosslinked the compound to HSP90α, digested the protein-compound complex with a protease, and then assessed changes in the mass of peptide fragments by LC-MS/MS. As seen in Figure 5 (red amino acids), several specific residues of HSP90α were found to have an increase in mass corresponding to the crosslinked UBS-109, whereas no mass increase was observed for the DMSO control. These residues were as follows: leucine 45 (L45), lysine 327 (K327), aspartic acid 706 (D706), and two or more possible residues located between histidine 210 (H210) and lysine 245 (K245). These crosslinked fragments do not correspond to binding residues predicted by modeling. However, these data do not rule out the possibility that UV treatment may have crosslinked UBS-109 to residues outside of the actual binding pocket.

A.

10 20 30 40 50 60
MPEETQTQDQ PMEEEEVETF AFQAEIAQLM SLIINTFYSN KEIFLRELIS NSSDALDKIR
70 80 90 100 110 120
YESLTDPSKL DSGKELHINL IPNKQDRTLT IVDTGIGMTK ADLINNLGTI AKSGTKAFME
130 140 150 160 170 180
ALQAGADISM IGQFGVGFYS AYLVAEKVTV ITKHNDDEQY AWESSAGGSF TVRTDTGEPM
190 200 210 220 230 240
GRGTKVILHL KEDQTEYLEE RRIKEIVKKH SQFIGYPITL FVEKERDKEV SDDEAEEKED
250 260 270 280 290 300
KEEEKEKEEK ESEDKPEIED VGSDEEEEKK DGDKKKKKKI KEKYIDQEEL NKTKPIWTRN
310 320 330 340 350 360
PDDITNEEYG EFYKSLTNDW EDHLAVKHFS VEGQLEFRAL LFVPRRAPFD LFENRKKKNN
370 380 390 400 410 420
IKLYVRRVFI MDNCEELIPE YLNFIRGVVD SEDLPLNISR EMLQQSKILK VIRKNLVKKC
430 440 450 460 470 480
LELFTELAED KENYKKFYEQ FSKNIKLGIH EDSQNRKKLS ELLRYYTSAS GDEMVSLKDY
490 500 510 520 530 540
CTRMKENQKH IYYITGETKD QVANSAFVER LRKHGLEVIY MIEPIDEYCV QQLKEFEGKT
550 560 570 580 590 600
LVSVTKEGLE LPEDEEEKKK QEEKKTKFEN LCKIMKDILE KKVEKVVVSN RLVTSPCCIV
610 620 630 640 650 660
TSTYGWTANM ERIMKAQALR DNSTMGYMAA KKHLEINPDH SIIETLRQKA EADKNDKSVK
670 680 690 700 710 720
DLVILLYETA LLSSGFSLED PQTHANRIYR MIKLGLGIDE DDPTADDTSA AVTEEMPPLE
730
GDDDTSRMEE VD

Red Amino Acids= Possible UBS-109 Candidate Binding Sites for Photo-Crosslinking

B.



C.

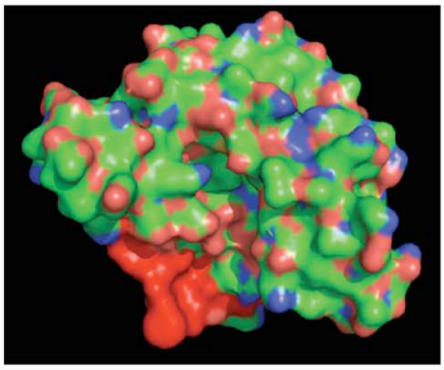


Figure 5. Peptide Mapping of Possible UBS-109 Binding Site to Hsp90α. Purified Hsp90α is incubated with various concentration of UBS-109 for 30min, irratitated at 365nm for 10 minutes and 4□C, then flash freeze with liquid nitrogen, and submitted for analysis by LC-MS/MS (Protea Biosciences Group, Morgantown, WV). (A) Peptide mapping showing interaction of purified Hsp90α with 10μM UBS-109 (red amino acids are proposed sites of crosslinking. (B) Crystal structure of the N-terminal of Hsp90α with the red regions being possible sites of UBS-109 binding.

Discussion:

Heat shock protein 90 (Hsp90) is a highly conserved molecular chaperone that interacts with over 200 different protein and is critical for regulating numerous biological processes such as proliferation and cell death [3]. In cancer cells, Hsp90 is frequently over-expressed and involved in stabilizing specific oncogenic proteins (e.g. HER2, c-KIT, AKT, etc) [4]. As a result, inhibitors of Hsp90 represent a promising target for the development of new anti-cancer therapeutics.

While several Hsp90 inhibitors have been developed, none of these compounds has proven successfully in a clinical environment. With its biological activity and reduced toxicity, curcumin and its analogues may represent a new source of Hsp90 inhibitors. Here, we report the binding of Hsp90 by our lead compound UBS-109, a monocarbonyl curcumin analogue. Hsp90 was detected as a binding target for UBS-109 using a click chemistry target identification scheme (see chapter 3). Using an Hsp90 competitive fluorescence polarization assay and other methods, we provide evidence that UBS-109 binds to Hsp90. Further, we showed that UBS-109 does not form irreversible chemical modifications to HSP90.

Our modeling data provide evidence that UBS-109 binds to the ATP binding in the N-terminal domain of Hsp90. Previous studies have shown that Hsp90 activity depends on binding of ATP to drives the structural rearrangements required for binding and stabilizing client proteins [7-8]. Previously, our target identification screening for UBS-109 detected that the largest majority of identified proteins contained ATP binding domains (see chapter 3). Our data concur with Brown *et al*, who previously showed

that monocarbonyl curcumin analogues competitively block the binding of ATP in various kinases [6]. Thus, these data suggest that UBS-109 may mechanistically inhibit proteins by competitively inhibiting ATP binding.

Modeling of UBS-109 binding into the ATP-binding pocket of the N-terminal domain in Hsp90 using Schrodinger's Glide docking software suggested that UBS-109 will form bonding interactions with two of the amino acids (K58 and K112) used by geldanamycin, a Hsp90 inhibitor that binds to the ATP binding. These data may explain why µM concentrations of UBS-109 are needed to inhibit the binding of geldanamycin to Hsp90.

Interestingly, our docking data suggest that the monocarbonyl of UBS-109 may not be involved in the binding to Hsp90. The monocarbonyl together with flanking double bonds, which constitutes a Michael acceptor, has been shown in various studies to be required for biological activity [5 and 8]. While it is recognized that this acceptor is required for most biological effects, it also represents a potential limitation in the usefulness of this compound due to non-specific effects including toxicity. This limitation may likewise complicate the use of UBS-109 and other monocarbonyl analogues as an Hsp90 inhibitor in cancer patients due to toxic side effects. However, if the monocarbonyl is not required for binding to Hsp90, it may be possible to develop more efficacious inhibitors that lacking this monocarbonyl group.

Using a peptide mapping strategy, we identified several potential binding sites for UBS-109 (e.g. leucine 45). However, the likelihood of these sites is complicated due to the requirement of UV-crosslinking of UBS-109 to Hsp90. Crosslinking was necessary

because UBS-109 likely forms reversible interactions with protein targets, which may be lost during the required denaturation step for this mapping strategy. However, this crosslinking may have resulted in non-specific labeling of residues. It remains to be determined whether any of potential sites is the binding location of UBS-109 to Hsp90. Future studies will focus on determining the binding location of UBS-109 and developing new analogues for selective binding of Hsp90.

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Chapter 5:
Overall Conclusion of Research

While the advancement of chemistry and biological sciences has resulted in the development of therapeutics for previously incurable diseases, a limitation on their utility is the development of drug resistance or, alternatively, toxicity [1]. As a result, there is a mandate to develop new therapeutics with improved activity, do not easily engender drug-resistance, and have limited toxic side effects. Currently, natural products are being exploited as a potential new source for therapeutic agents due their biological activity and relative safety [2-3). One potential source for new therapeutic compounds is the natural product curcumin, a polyphenol compound isolated from perennial herb *Curcuma longa.* Curcumin and has long been used as a traditional medicine to treat various ailments [4]. In the last forty years, curcumin has been identified as promising therapeutic agent to treat a wide-range of diseases including cancer, inflammatory, and infectious diseases. However, the utility of curcumin as a therapeutic agent is currently being limited due to extremely poor bioavailability. One approach in alleviating this limitation is the development of synthetic analogues of curcumin, which could potentially improve both bioavailability and biological activity.

Previous work in our research group has developed a library of curcumin analogues in which the keto-enol moiety of central sector was substituted with a monocarbonyl group embedded in a heterocyclic six-membrane ring conjugated with flanking double bonds [5]. Extensive screening of this library has identified specific analogues (e.g. EF-24) with significant anti-cancer effects [6]. However, specific cellular targets or pathways responsible for these effects are not fully understood. In addition,

the effect of these analogues on other diseases, such as infectious disease, has not been extensively studied.

In chapter two, we investigated the effect of these monocarbonyl analogues on inhibiting the growth of pathogenic *Mycobacteria* species (*Mycobacteria tuberculosis* [*Mtb*] and closely related *Mtb* model organism *M. marinum* [*Mm*]). *Mtb* is the etiologic agent for tuberculosis (TB), a disease of major public health concern that is responsible for infecting 2 billion worldwide and causing >1.8 million deaths annually [7]. While a drug regimen has been developed to treat TB, the utility of this regimen has been limited by compliance issues, which has resulted in the rise of drug-resistant strains of TB [8]. As a result, there is a critical need for the development of new TB drugs with improved biological activity and less toxicity.

Utilizing disc diffusion and liquid assays, we observed that specific monocarbonyl analogues will inhibit the growth of *Mm* with UBS-109 having the greatest inhibitory effect (MIC= ~10 μM). An examination of the structural differences between these analogues identified specific modifications that are critical for inhibiting the growth of *Mm*. For instance, the presence of the Michael acceptor is essential for inhibitory activity, while the presence of a fluorine group on aromatic rings reduces activity. Based on these positive data, we tested whether UBS-109 would also inhibit *Mtb*. Similarly to our *Mm* data, UBS-109 was found to inhibit both H37Rv (MIC= ~10 μM) and Beijing strain (MIC=~20 μM). Together, these data suggest that UBS-109 and structurally similar compounds could be used to hinder the growth of drug-sensitive pathogenic *Mycobacteria* species.

Currently, the major issue in treating TB is the rise of multi-antibiotics resistant TB stains (MDR-TB, XDR-TB, and TDR-TB). Because curcumin and its analogues are believed to target multiple cellular proteins, we proposed that these monocarbonyl analogues would be effective against antibiotics-resistant strains of *Mtb* and *Mm*. In this regard, we used a rifampicin-resistant *Mm* and *Mtb* to demonstrate that UBS-109 will inhibit the growth of antibiotics-resistant bacteria. Because monocarbonyl analogues would most likely be administered with other TB therapeutics, we investigated the effects of simultaneous analogues and antibiotics treatments on the growth of *Mm*. We observed that the presence of rifampicin reduces the efficacy of UBS-109 in inhibiting the growth of *Mm*, which we hypothesized was due to rifampicin promoting the up-regulation of efflux pumps that reduces the cellular concentration of UBS-109. While UBS-109 has potential in treating MDR-TB, its conjunction with front and second line antibiotics will need to be evaluated in order to avoid a loss of efficacy in UBS-109.

In chapter three, we investigated possible cell death pathways that are responsible for the cytotoxic effect of monocarbonyl analogues in cancer cells. Cancer is responsible for 6 million deaths annually with over 22 million living with some form of this disease [9]. Currently, extensive pharmaceutical research has focused on developing therapeutics to induce apoptosis in cancer cells. While certain cancer therapeutics have been successful in eliminating cancer cell by apoptosis, many of these therapies have failed to completely eliminate the disease due to the development of resistance [9-10]. An approach to improving the elimination of cancer cells is the use of

compounds such as curcumin and its analogues, which affect multiple signaling pathways. While curcumin and its analogues are known to affect apoptosis, their effect on non-apoptotic cell death pathways is poorly understood.

Utilizing cell lines derived from knockout mice and cell death inhibitors, we provided evidence that UBS-109 induces RIP3-dependent necroptosis ("program necross") and autophagy pathways. Using cells derived from $RIP3^{-/-}$ mice, we showed that blocking of RIP3 increase the survival of the cells to UBS-109 treatment. However, higher concentrations of UBS-109 were found to negate this improved survival in these cells by the induction other cell death pathways (e.g. apoptosis). Using wild type and $RIP^{-/-}$ mice, we observed that UBS-109 induced necroptosis *in vivo*. Because the only identified function of RIP3 is the induction of necroptosis [11], these data suggest that UBS-109 induces necroptosis.

Next, we assesed whether UBS-109 would also induce autophagy pathway. Autophagy is both a cell survival and death pathway. Generally this pathway is induced by cellular stressors and responses by promoting a limited degradation of cell components into basic building blocks used to maintain survival. However, if this process is prolonged for an extended time, then cell death will occur. Using an autophagy cell line lacking a protein critical for autophagy ($ATG5^{-/-}$), we observed that the $ATG5^{-/-}$ cells survived better than wild type cell. In addition, we observed using TEM an increase in the occurrence of autophagosomes, an autophagy biomarker, by TEM cells treated with UBS-109. Together, these data suggests that UBS-109 induces autophagic cell death.

Our observations of the effects of UBS-109 on both necroptosis and autophagy lead us to assess whether these cell death mechanisms are related. Recently, it has been proposed that the necroptosis and the autophagy pathways are linked, with some studies reporting that autophagy induces necroptosis and others reporting that necroptosis regulates autophagy [12-14]. To determine whether UBS-109 induces these pathways independently of each other, the necroptotic inhibitor Nec-1 was applied toward UBS-109-treated wild type and ATG5$^{-/-}$ cells. While we did observed that Nec-1 improved survival in UBS-109 treated wild type and ATG5$^{-/-}$ cells, inhibiting necroptosis did not mirror effect of blocking the expression of ATG5 in the wild type cells. These data suggested that UBS-109 independently affects necroptosis and autophagy.

We next sought to identify the cellular protein targets of UBS-109 to explain our cell death effects. Using a photo-crosslinking/click chemistry-based identification strategy, we identified multiple proteins that are involved in all aspects of cellular function, including several known targets of curcumin (e.g. actin). While none of these identified proteins were found to have a role in necroptosis, one identified protein, called transitional endoplasm reticulum ATPase, has been shown to affect the autophagy pathway. Previous studies have shown that inhibiting this ATPase prevents the phagolysomal fusion stage of autophagy, which results in a buildup of autophagosomes and eventual death of the cell [15]. Interestingly, our TEM data showed a buildup of autophagosomes in UBS-109 treated cells. Also our data with the phagolysosome fusion inhibitor bafilomycin A1 suggested that autophagic cell death may be due to the effect of UBS-109 on bafilomycin A1-sensitive ATPases. Together,

these data suggest that UBS-109 may prevent the final stage of autophagy, phagolysomal fusion, from occurring. As a result, we hypothesize that loss of resources due to the buildup of autophagosome and the failure to turnover cellular components results in cell death.

In chapter four, we investigated the effect of UBS-109 on binding to heat shock protein 90 (Hsp), a molecular chaperone that is critical for regulating numerous biological processes such as proliferation and cell death [16]. In cancer cells, Hsp90 is frequently over-expressed and is involved in stabilizing specific oncogenic proteins (e.g. HER2, c-KIT, AKT, etc), which make this protein a promising target for new anti-cancer therapeutics. Previously, we identified Hsp90 as a potential direct target of UBS-109 (see chapter 3). Using a Hsp90 competitive fluorescence polarization assay and other methods, we provide evidence that Hsp90 is a direct target of UBS-109.

Our data suggested that UBS-109 inhibits Hsp90 activity by blocking ATP binding in the N-terminal domain of this protein, which concur with previous studies that showed monocarbonyl analogues inhibiting kinases by blocking ATP-binding [17]. Generally, these monocarbonyl curcumin analogues have been shown to form Michael additions with sulfhydryl group of cysteine residues in their protein targets. However, an examination of the peptide sequence failed to identify any cysteine residues in the N-terminal domain of Hsp90. To explain how UBS-109 may be binding to Hsp90, we conducted modeling experiments. Using Schrodinger's Glide docking software, we successful docked UBS-109 into the ATP-binding pocket of the N-terminal domain in

Hsp90; however docking score indicated weak binding. Interestingly, our docking data suggest that the monocarbonyl of UBS-109 may not be involved in the binding to Hsp90.

In summary, curcumin, like other natural products, are becoming an important source for the development of new therapeutic agents for numerous diseases. While curcumin is limited in its therapeutic applications due to poor bioavailability, structural analogues have been developed with improved biological activation and bioavailability. The work presented in thesis reports new potential applications for these monocarbonyl curcumin analogues. We have provided evidence that these monocarbonyl analogues inhibit the growth both sensitive and resistant strains of *Mycobacteria* sepecies (*Mtb* and *Mm*), which may represent a potential source for developing new TB drugs. In addition, we provided evidence that UBS-109 binds to Hsp90, which may represent a potential source for developing new Hsp90 inhibitors for treating cancer.

UBS-109 may also serve as a potential basis for the development of new therapeutic inhibitors for treating cancer via inhibition of multiple cell death pathways. Thus, our work has identified new pathways affected by these analogues. We provided evidence that UBS-109 may induce the necroptosis or "programmed necrosis" pathway. To our knowledge, this evidence is the first identified effect of a "curcumin-like" molecule on the induction of RIP3-dependent necroptosis pathway. Currently, the clinical benefit for inducing the necroptosis pathway as an anti-cancer therapy is unknown [18]. Besides the necroptosis, we also provided evidence that UBS-109 may affect the autophagy pathway, possible by blocking the phagolysosome fusion stage. The induction of autophagy as anti-cancer therapy is complicated due to the paradoxical

relationship of autophagy in cancers [19]. During early stages of tumorogenesis, the induction of autophagy may suppress cancer, whereas at later stages, autophagy may protect cancer cells. However, UBS-109 may avoid this complication due to potentially targeting the final stage of autophagy (phagolysosome fusion stage). By blocking this stage of autophagy, the cellular protein and organelles cannot be broken down into basic cellular component (e.g. amino acids, etc) needed to maintain survival of cancer cell, which results in the eventual death of the cell. Thus, these analogues may represent a potentially useful cancer drug, in which several major cell death pathways are targeted.

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