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Identification of Small Molecule Inhibitors of I-κB Kinase

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Identification of Small Molecule Inhibitors of I-κB Kinase

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Abstract

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By Xuesong Yang

Nuclear factor κB (NF-κB) transcription factor and its pivotal role in many physiological and pathological processes have been intensely studied for decades. Aberrant regulation of NF-κB and its controlling signaling pathway can result in many autoimmune diseases as well as cancer, making it a desirable therapeutic target for drug development. Many natural products such as curcumin have demonstrated their inhibition to NF-κB signaling pathway, which contributes to their anticancer therapeutic potential. To circumvent the problems of poor bioavailability and low potency with curcumin, while retaining its safety profile, many synthetic analogs derived from curcumin have been developed. EF24, a monoketone analog of curcumin, has been reported to have ten-fold better bioactivity than curcumin. In this study, a novel class of compounds derived from curcumin was tested for their anticancer activity. One compound, 3, 5-bis [(6-chloropyridin-2-yl) methylidene]-1-methyl-4-piperidone, named AM5, showed greater cytotoxic effect than EF24. Furthermore, AM5 was indicated to directly target NF-κB signaling pathway, by effectively blocking the nuclear translocation of NF-κB, impairing tumor necrosis factor (TNF)-α-induced I-κB phosphorylation and degradation, and directly inhibiting the catalytic activity of I-κB kinase. AM5 represents a new structure of curcumin analogs that targets NF-κB signaling more effectively, and the activities of compounds from the same class have shed lights on further optimization and modification of AM5 to improve its bioactivity for therapeutic application.

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List of Abbreviations

ANK	ankyrin
ATP	adenosine triphosphate
BCL-3	B-cell CLL/lymphomas 3
CMI	cell mediated immune
COX	cyclooxygenase
DD	death domain
DNA	deoxyribonucleic acid
GST	glutathionine S-transferase
HCA	high-content assay
I-κB	inhibitor of kappa B
IFN	interferon
IKK	inhibitor of kappa B kinase
IL	interleukin
κB	kappa B
LZ	leucin zipper
NEMO	NF-κB essential modifier
NF-κB	nuclear factor kappa-B
NIK	NF-κB inducing kinase
PEST	proline-, glutamic acid-, threonine-rich domain
PGE_2	prostaglandin E2
RHD	Rel homology domain
SRB	sulforhodamine B
STAT5a	signal transducer and activator of transcription 5a
TAD	transactivation domain
TGF-β	transforming growth factor beta

Th-1	T-helper cells class 1
Th-2	T-helper cells class 2
TNF-α	tumor necrosis factor alpha

Chapter 1:

Introduction

1.1. NF-κB

1.1.1. Introduction of NF-κB

NF-κB is a transcription factor that was first discovered in B cells in 1986[1]. It was considered to play a pivotal role in the initiation of immune response[2]. After further exploration, NF-κB was determined to be ubiquitously expressed in virtually all cells, and to function in multiple ways in different physiological and pathological processes. The activation of NF-κB can result from different stimuli, such as chemokines, free radicals, stress, and ultraviolet radiation. Activated NF-κB, accompanied by different transcriptional coactivators, regulates a range of genes responsible for cell death and survival.

The mammalian NF-κB family consists of five members, RelA (also known as p65), RelB, CRel, p50/p105, and p52/p100 (Figure 1.1)[3]. Each member contains a 300 amino-acid Rel-homology domain (RHD), which regulates the dimerization of NF-κB at the interface near the N-terminus[4-6]. In fact, NF-κB functions as homo- or hetero-dimers formed by any of the above members. Different combinations of the family members act differently in the regulation of NF-κB transcription factor units, either activation or repression[7-9]. NF-κB dimers bind tightly to a highly conserved nucleotide sequence (GGGGACTTTCC) named κB site, and this is how NF-κB got its name as nuclear factor κB[1]. The presence of transactivation domain (TAD) in NF-κB dimers partly determines their ability to activate target genes, and vice versa[10]. Among the five family members, RelA, RelB and CRel all contain TAD, so they regulate target genes positively. However,

p100 and p105 need to heterodimerize with any of the TAD-containing family member to obtain the function to activate transcription of target genes, since there is no TAD within these two family members[11]. On the other hand, p100 and p105 form homodimers or heterodimers of each other to repress transcription of target genes[12-14].

1.1.2. The role of NF-κB in immune response

It is well known that NF-κB plays a central role in the immune response[15, 16]. One typical example is the growth and proliferation of T-cells regulated by the activation of NF-κB[17-19]. In rapidly proliferating T-cells, STAT5a (signal transducer and activator of transcription 5a) activation induced by NF-κB is required for their further proliferation[20]. Conversely, when NF-κB is inhibited in T-cells, STAT5a is not activated, leading to the induction of T-cell apoptosis and the failure of their growth. However, T-cell mediated immune response can be altered by sustained NF-κB activation through cytokine production[21, 22]. Th-1 and Th-2, the two classes of helper T-cells, produce their specific cytokines, which influence different aspects of normal and disease physiological processes[23-25]. The first class of Th cells, Th-1 cells, releases IL-2, IL-12 and interferon (IFN)-γ[23]. These cytokines are necessary for the activation of a cell mediated immune (CMI) response, which is found to be decreased in many chronic infectious diseases. While the second class of Th cells, Th-2 cells, produces IL-4, IL-5, IL-6 and IL-10, which suppress the CMI response and influence the humoral response[23].

Certain immune responses were discovered to be associated with development of cancer[26]. In cancers, the population of each class of Th cells and the level of the expressed cytokines are altered[27]. In fact, various cancers have been correlated to inhibition of CMI response and promotions in the humoral response[28]. For example, activation of cytokines, such as IL-10 and TGF-β, in cancers results in the suppression of CMI response[29, 30]. Besides, in curable tumors like lymphomas, the activation of Th-2-immune response causes lethal outcome. Moreover, IL-10 production is also directly induced by NF-κB activation, which is additional evidence for tumorigenesis induced by immune response associated with NF-κB[31, 32].

1.1.3. The role of NF-κB in tumorigenesis

Many genes implicated in cell survival and anti-apoptosis are found to be up-regulated by NF-κB in tumorigenesis[33]. Cyclooxygenase (Cox)-2 is one of the common targets of NF-κB to be promoted in both inflammatory diseases and cancer. In response to pro-inflammatory cytokines, Cox-2 expression is elevated. Cox-2 enzymes not only regulate immune response, but also control several important aspects in carcinogenesis, such as angiogenesis, cell proliferation, cell migration, and inhibition of apoptosis[34, 35]. Actually, Cox-2 was discovered to be constitutively expressed in all types of cancer[36, 37]. One example lies in the fact that Cox-2 expression is associated with the formation of tumor blood vessels in human prostate cancer[38]. Tumorigenesis is usually induced by downstream products of Cox-2. For instance, Cox enzyme catalyzes the conversion of

arachidonic acid into prostaglandins. One such prostaglandin, PGE_2, produced by Cox-2 catalysis, leads to angiogenesis for further tumor growth[39].

Since the aberrant regulation of NF-κB and signaling pathways it controls have been found in many types of cancer, this pro-survival pathway has become a potential therapeutic target for treating inflammatory diseases, as well as cancer[40].

1.1.4. NF-κB signaling pathway

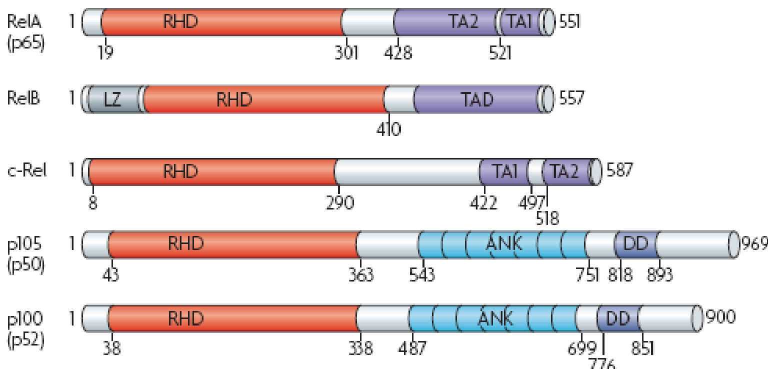
1.1.4.1. *Classical NF-κB signaling pathway*

The classical NF-κB pathway regulates immune recognition, cell survival and proliferation, so it draws much interest as a desirable drug target[41]. In unstimulated cells, NF-κB is sequestered in the cytoplasm in an inactive complex with I-κB, the inhibitor of kappa B. The family of I-κB mainly includes I-κBα, I-κBβ, I-κBε, and B-cell CLL/lymphoma 3 (BCL-3) (Figure 1.2)[3]. Each member contains a core domain with five to seven ankyrin repeats, which masks the nuclear localization sequence on NF-κB through binding to its RHD domain[14]. In response to stimuli such as cytokines and growth factors, I-κB is phosphorylated at Ser32 and Ser36 by IKK, the inhibitor of kappa B kinase (Figure 1.3)[42]. IKK is essential for the activation of NF-κB pathway. It is a complex containing three subunits, two catalytic subunits (IKKα and IKKβ), and one regulatory subunit (IKKγ, also named NF-κB essential modulator (NEMO))[43, 44]. Among all three subunits, IKKβ is believed to be responsible for I-κB phosphorylation[45]. Upon

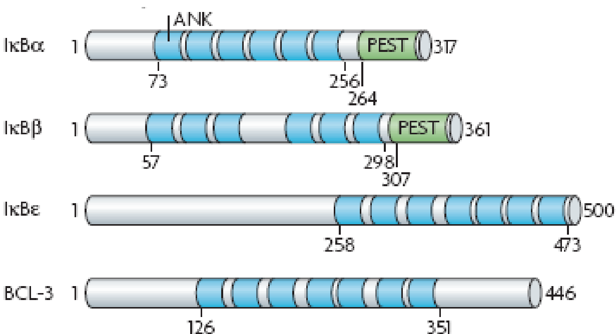
phosphorylation by IKK, I-κB undergoes a rapid polyubiquitination by E3 ubiquitin ligase, followed by 26s proteasome degradation (Figure 1.3)[46]. After the degradation of I-κB, the nuclear localization sequence on NF-κB is exposed, leading to its translocation to the nucleus[47]. Finally, NF-κB binds to its target genes at κB site in the nucleus, and regulates their expression (Figure 1.3)[46].

1.1.4.2. *Alternative NF-κB signaling pathway*

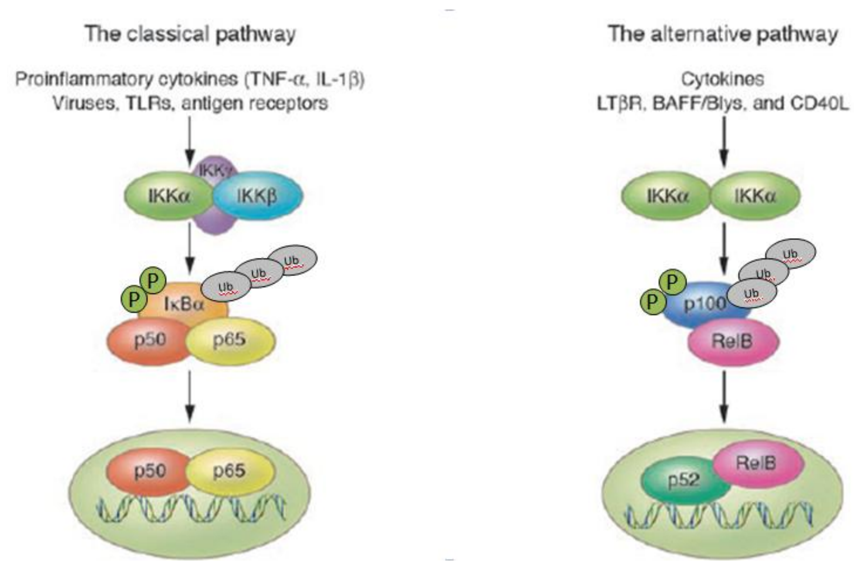
The alternative NF-κB pathway regulates immune system response in several ways, such as control of development of B and T lymphocytes, and lymphoid organogenesis[48]. Cytokines, such as CD40 ligand and lymphotoxin B, first activate NF-κB-inducing kinase (NIK), which is specific to the alternative NF-κB pathway[49]. Then the homodimer of IKKα is phosphorylated by NIK. The activation of IKKα homodimer results in the phosphorylation, ubiquitination and cleavage of p100, converting it into p52[49]. Consequently, the NF-κB heterodimer formed by p52 and RelB translocates to the nucleus and binds to its target genes (Figure 1.3)[49].



<u>Figure 1.1</u> Members of NF-κB family. N-terminal Rel-homology domain (RHD) is shared by all members; TAD, transactivation domain; LZ, leucine zipper; GRR, Glycine-rich region; DD, region with homology to a death domain.



<u>Figure 1.2</u> Main members of I-κB family. I-κBα is the canonical member of I-κB. ANK, ankyrin repeats; PEST, proline (P), glutamate (E), serine (S) and threonine (T).



<u>Figure 1.3</u> Two classes of NF-κB signaling pathway. In classical NF-κB pathway, I-κB is phosphorylated by IKK, followed by degradation of I-κB and release of NF-κB to the nucleus. In alternative NF-κB pathway, p100 is phosphorylated by IKKα, then processes to p52, and binds to RelB to form a heterodimer of NF-κB, leading to its nuclear translocation.

1.1.5. Development of IKK inhibitors for therapeutic application

Compounds that can block NF-κB signaling pathway have demonstrated inhibition to the growth of tumor cells[50, 51]. Therefore, developing small molecule inhibitors of NF-κB signaling pathway can be a promising means toward tumor therapy[52]. On the overview of NF-κB signaling events, different strategies to inhibit NF-κB pathway can be taken, such as inhibiting proteasome activity to prevent the degradation of I-κB, impairing the binding of NF-κB to its target genes, or directly blocking the activation of IKK[53, 54]. IKK has been revealed to phosphorylate only proteins involved in NF-κB pathway, making IKK inhibitors the most effective and specific class among all classes of inhibitors targeting the NF-κB pathway[55]. Since IKKβ plays a key role in the activation of classical NF-κB pathway, it is being pursued as an effective target for anti-inflammatory or anti-cancer drug development[55].

An increasing number of IKKβ inhibitors have been identified mainly by the screening of large compound libraries and the use of combinational chemistry tools[55]. However, the toxicity has prevented most of these compounds from the entry of clinical trial. As such, identification of novel IKK inhibitors with safety profile has become the new aim for the development of drugs treating both inflammation and cancer. One approach to meet this challenge is to develop inhibitors derived from natural sources[56, 57]. My research seeks to identify effective, small molecule inhibitors of NF-κB pathway from a group of novel synthesized analogs of the natural product, curcumin.

1.2. Curcumin

1.2.1. Introduction of curcumin

Curcumin is a polyphenolic compound that has been identified as the main bioactive component extracted from the plant *Curcuma longa* L. This yellow colored compound has a long history of human consumption as a natural pigment and the ingredient of the spice curry. For centuries, curcumin has been applied in diet for treatment of ailments like arthritis by people in many regions of the world. Not until recent years has curcumin been discovered to be effective in preventing and treating chronic inflammatory diseases as well as cancer[58, 59]. Although the detailed mechanism driven by curcumin for the suppression of inflammation and tumorigenesis remains to be understood, curcumin has demonstrated its ability to block the signaling pathway of NF-κB[60].

1.2.2. Therapeutic potential of curcumin

Curcumin has long been considered as a beneficial agent for the prevention of cancer. It effectively inhibits the initiation, promotion, and progression of tumor during carcinogenesis, attributing to its activities against inflammation, virus, and oxidation[58, 61-65]. This was first tested in a mouse model, in which animals were injected with Dalton's lymphoma cells before the treatment of curcumin. A significantly increased percentage of cancer-free survival was observed in animals receiving curcumin, compared with control animals[66]. Further pre-clinical studies on animals have supported the role of curcumin in preventing cancer in multiple organs, such as breast, stomach, prostate, and colon[67-70].

The clue of this chemopreventive effect on human by curcumin can be suggested by the statistics from epidemiologists[71]. The rate of colorectal cancer is relatively lower in regions with a high consumption of curcumin, compared with countries where curcumin consumption is low. Moreover, the second generations of migrants from Eastern countries to the Western world exhibit a higher risk for colorectal cancer than their first-generation predecessors, suggesting a correlation to the lower consumption of curcumin in their diet in the Western countries.

Although the prevention of cancer is imperative, the effective treatment for existing tumor is even more significant. The use of traditional chemotherapeutics usually causes severe side effects to healthy tissues. As such, the well established safety profile of curcumin has triggered researchers' interest to use it as a chemotherapeutic agent[72]. However, this attempt was unsuccessful because of certain drawbacks of curcumin in chemotherapy[73].

1.2.3. Therapeutic limitations of curcumin

Despite its efficacy and safety, the use of curcumin as a therapeutic agent has been hindered by its low potency and poor bioavailability[73-75]. The low concentration of curcumin in serum was observed in an early study, in which rats were given curcumin orally[76]. It was mainly attributed to the poor absorption of curcumin from the gut. Since not all compounds can be absorbed well through the mechanism of oral administration, another study was set up to examine whether the administration mode of curcumin (orally or intraperitoneally) had any effect on its bioavailability. However, it was observed that

both means of administration lead to rapid clearance of curcumin from the animal[77]. Further study has identified the increased metabolism of curcumin as an important factor causing its poor bioavailability, besides the reduced absorption of this compound[74]. In fact, curcumin quickly forms conjugates with glucurnoic acid in the liver during the process of glucuronidation, resulting in the increase of its water solubility, and subsequent excretion by the kidneys[74]. In summary, the low absorption and rapid metabolism of curcumin greatly limit its effect in therapeutic application.

1.2.4. Improving the pharmacological properties of curcumin

The safe toxicity to human and the low molecular weight of curcumin together make it an ideal lead compound for drug design. Endeavors were undertaken by Dr. Dennis Liotta's research group at Emory University, by which a series of curcumin analogs were devised, aiming at enhancing the potency of curcumin while retaining its safety profile[78]. Two monoketone analogs were identified by computational modeling, exhibiting the effect of increased cytotoxicity over curcumin. More than 100 compounds were then prepared according to the results from the topological search for analogs with improved potency. These compounds were screened by in vitro cell viability assays. A lead compound, EF24, was identified to inhibit the tumor cell growth by 50% with a 10.4-fold better potency than curcumin, and inhibit the tumor growth by 100% with a 9.8-fold better effect (Figure 1.4)[78, 79]. Further *in vivo* study of EF24 demonstrated its effective inhibition to the growth of solid breast tumors with no side effects on normal tissues[79]. A more comprehensive study on the molecular events of this novel curcumin analog has revealed that EF24

effectively inhibits NF-κB signaling pathway by blocking the nuclear translocation of NF-κB, by inhibiting TNF-α induced I-κB phosphorylation and degradation, and by directly inhibiting the catalytic activity of IKK[80].

As a relatively successful analog of curcumin, EF24 was further modified and optimized for improved potency and efficacy. As such, another series of curcumin analogs were designed and prepared by Drs. Alessandra Mancini and Dennis Liotta (Table 1.1). My research seeks to screen these compounds, in order to discover analogs more potent than EF24, as well as to elucidate their molecular mechanisms of action.

MeO
HO
O
O
OMe
OH
Curcumin

F
O
F
N
H

EF24

Figure 1.4 Structures of curcumin and EF24

<u>Table 1.1</u> Structures of AM compounds.

Name	Structure	Name	Structure
AM1		AM2	
AM3		AM4	
AM5		AM6	
AM7		AM8	
AM9		AM10	
AM12		AM13	

AM14		AM15	
AM16		AM17	
AM21			

Chapter 2:

Identification of a novel unsaturated monoketone analog of curcumin as a direct inhibitor of IKK-NF-κB signaling pathway

2.1. Introduction

In this report, I screen the anticancer activities of 17 synthetic curcumin analogs named AMs, and EF24. I demonstrate that AM5 exhibited a remarkable decrease in cell viability (i.e., significantly better potency than EF24). Further exploration of the molecular mechanism of AM5 identifies it as a direct inhibitor of IKK-NF-κB signaling pathway. AM5 provides a new structural feature that can be introduced in future design of novel curcumin analogs for improved efficacy and potency.

2.2. Materials and Methods

Materials – EF24 (3, 5-bis (2-fluorobenzylidene) piperidin-4-one) was prepared as reported[78]. All of the AM compounds were prepared by Dr. Alessandra Mancini in Dennis Liotta's group at Emory University. All compounds were dissolved in DMSO with a stock concentration of 5 mM. Tumor necrosis factor (TNF)-α (Sigma-Aldrich Chemicals, St Louis, MO) was dissolved in water to a final concentration of 10 μg/ml. Glutathione S-transferase-I-κBα (1-54) was expressed and purified as previously described[81]. Antibodies against pS32-I-κBα and I-κBα were purchased from Cell Signaling (Beverly, MA). Antibody against IKKβ was purchased from Imegenex (San Diego, CA). Secondary antibody conjugates, HRP-goat anti-rabbit and HRP-goat anti-mouse antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA).

Cell Cultures – Cells were cultured in medium RPMI (A549, H157, H226, H1299), or DMEM/F-12(SKBR3) with 10% fetal bovine serum (FBS) and 1% of penicillin/streptomycin in a 37°C incubator with 5% of CO_2. For western blotting, cells were lysed with 1% NP-40 lysis buffer (1% Nonident P-40, 150 mM NaCl, 10 mM Hepes [N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.4, 5 mM NaF, 2 mM Na_3VO_4, 5 mM $Na_4P_2O_7$, 10 μg/ml aprotonin, 10 μg/ml leupeptin, 1 mM PMSF [phenylmethylsulfonyl fluoride]).

Cell Viability Assay – Cells were plated at 1,000 cells/well in 384-well plates. They were incubated over night before triplicated treatments with test compounds on the following day. Cells were incubated for another 48 hr before the viability assay. The sulforhodamine B assay was carried out to measure the cell viability and to obtain the IC_{50} values[82, 83]. Cellular protein content is evaluated in this assay to determine cell density. The mean and standard error for each treatment was obtained and the cell viability index compared to control (no treatment) was calculated. The IC_{50} value is defined as the concentration of compounds when the cell viability is decreased by 50% in a total cell population relative to control group at the end of the incubation.

NF-κB Translocation Assay – A549 cells were plated in 384-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at 6,000 to 7,000 cells/45 μl/well and were incubated for 20 hr. Compounds to be tested were added to the cells in triplicate, and

cells were incubated at 37°C for 30 min. Then cells were stimulated by TNF-α as previously indicated. After 30 min stimulation, medium was removed and cells were fixed with paraformaldehyde (2% solution in phosphate-buffered saline [PBS], 50 μl) for 15 min at room temperature. Plates were washed for three times with PBS. Cells were then permeabilized with Triton X-100 (0.1% in PBS, 50 ml) for 10 min. Plates were again washed for three times with PBS. Cells were incubated with rabbit anti-p65 NF-κB antibody (25 μl; Santa Cruz biotechnology, Inc., Santa Cruz, CA) for 1 hr at room temperature. Plates were washed for three times with PBS. Cells were incubated with goat anti-rabbit IgG with conjugated Alexa Fluor 488 (25 μl; Molecular Probes, Inc., Eugene, OR) with Hoechst 33342 (1 μg/ml; Promega, Madison, WI). Plates were washed for three times with PBS. Cells were scanned with the ImageXpress 5000 with the setting of filter for FITC (Ex: 490 nm, Em: 525 nm, and dichroic mirror: 505 nm), and DAPI (Ex: 350 nm, Em: 479 nm, and dichroic mirror: 400 nm) (Molecular Devices, Sunnyvale, CA). Scanned images were analyzed and quantified through MetaXpress software (Molecular Devices, Sunnyvale, CA). NF-κB subcellular translocation was analysis according to the "Translocation Enhanced" module. Nucleus was stained by Hoechst 33342. The translocation of NF-κB was calculated from the difference between the average fluorescence intensity in nucleus and in cytoplasm (Nuc - Cyt). The inhibitory activity of test agents was expressed as percentage of the difference of fluorescence intensity (Nuc - Cyt) of the control wells (TNF-α only, no compounds) after deducting background (No TNF-α, no compounds).

Western Blotting – Cells were lysed in 1% NP-40 lysis buffer. Cell lysates were equally loaded in SDS-PAGE (12.5%) gel for electrophoresis. Proteins were then electrotransferred from the SDS-PAGE gel to a nitrocellulose membrane (GE water and Process Technologies, Trevose, PA). Membranes transferred were then incubated with a solution of 5% non-fat dry milk in TBS-T buffer (500 mM NaCl, 20 mM Tris pH 7.6, 0.5% Tween-20) for 30 min at room temperature. Membranes were then incubated with primary antibody over night at 4°C. Membranes were washed with TBS-T buffer for three times, and were incubated with the corresponding horseradish peroxidase-conjugated anti-mouse immunoglobulin (Ig) or anti-rabbit Ig. Detection of the immunoblotting signals was performed with West Dura (Pierce), followed by imaging scanning on Kodak's Image Stattion 2000R (New Haven, CT).

In Vitro Kinase Assay – Recombinant IKKβ was resuspended in MOPS buffer (8 mM MOPS-NaOH, pH 7.0, 200 μM EDTA, 15 mM $MgCl_2$). (Upstate Cell Signaling Solutions, Lake Placid, NY). Test compounds were incubated with 40 ng IKKβ for 15 min at room temperature. Then Mg-ATP cocktail (15 mM $MgCl_2$, 100 μM ATP, 5 mM β-glycero-phosphate, 8 mM MOPS-NaOH, pH 7.0, 1 mM EGTA, 200 nM DTT, 200 nM sodium orthovanadate), purified GST-I-κBα (5 μg), and [γ-^{32}P]ATP (0.5 mCi) were added to the reaction to reach the total volume of 25 μl, reaction for each test compound was carried out at room temperature for 30 min. Reaction was terminated by adding 6× SDS sample buffer to the reaction solution, and boiled for 3 min. Samples were resolved by electrophoresis in SDS-PAGE (12.5%) gel. The SDS-PAGE gel was cut in half, and

the top part was transferred to a nitrocellulose membrane and was immunoblotted with IKKβ antibody. The bottom part was stained with Coomassie Blue dye (0.05%). The levels of radiolabeled phosphate incorporated into GST-I-κBα were detected by the PhosphoImager and were quantified by the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). Additionally, the same assay was terminated by spotting the reaction mixture (10 μl) on to P81 phosphocellulose paper (Whatman). The paper with spotted reaction mixures was washed for three times with phosphoric acid (0.75%), and once with acetone in the end. Then the levels of radiolabeled phosphate incorporated into GST-I-κBα were quantified by liquid scintillation counting using a scintillation counter (Beckman, LS 6500, Beckman Coulter, Fullerton, CA).

2.3. Results

2.3.1. AM5 represents a favorable structure with improved cytotoxicity over EF24

Cell viability assay was carried out for the evaluation of the cytotoxic potency of AM compounds in comparison to EF24. The cytotoxic effect of all compounds was tested in a panel of lung cancer and breast cancer cells. Treatment of the cells with all AM compounds and EF24 exhibited decreased cell viability in a dose dependent manner. The IC_{50} value for each compound was calculated as summarized in Table 2.1, with varied range in different cell lines. The cytotoxic effect of all compounds on these cell lines were ranked according to their IC_{50} values (Table 2.2). Interestingly, five compounds with the same core structure rarely dropped below EF24 in the ranking, including AM5, AM 12, AM15, AM16, and AM17 (Figure 2.1). In addition, AM10, AM13, and AM21, with a similar core structure but more extensive modification, also demonstrated improved cytotoxicity over EF24. Among all these analogs, AM5 and AM12 consistently exhibited the most improved potency. As described in Figure 2.2, AM5 showed a more potent cytotoxic effect than EF24 in all cell lines tested, suggesting its role as a competitive novel analog of curcumin with a more favorable structure than EF24.

2.3.2. AM5 effectively impairs the nuclear translocation of NF-κB

Since NF-κB signaling pathway is suggested to be a direct target of EF24, the molecular mechanism that accounts for the improved anticancer activity of AM5 was examined in this pathway[80]. An NF-κB translocation assay was carried out to test the effect of AM5

on the subcellular translocation of the NF-κB transcription factor to the nucleus. As previously established, a method based on high content analysis (HCA) was utilized to visualize the status of NF-κB in the cytoplasm and nucleus[84]. The p65 subunit of NF-κB with an immunofluorescent probe was detected under a fluorescence microscope. Compared with control, the stimulation of A549 cells by TNF-α (10ng/ml) resulted in thorough translocation of NF-κB from cytoplasm to the nucleus (Figure 2.3). However, the pretreatment of A549 cells with AM5 before TNF-α stimulation effectively blocked NF-κB nuclear translocation. This effect was examined with all other AM compounds as well as EF24. Dose-response curves were obtained for each compound through the quantification of the confocal images, and all the IC_{50} values were summarized and ranked in Table 2.3. Consistently, compounds showed improved activities over EF24 in cell viability assay also stayed at the top half of this ranking. AM5 demonstrated better potency with an IC_{50} of 0.6 μM, compared with 1.3μM for EF24, which was previously reported to be 10-folds more potent than curcumin in blocking NF-κB nuclear translocation (Figure 2.3)[80]. This result indicated that AM5 impaired the survival of cancer cells probably through the interference to NF-κB signaling.

2.3.3. AM5 inhibits I-κB phosphorylation and degradation induced by TNF-α

When unstimulated, NF-κB is sequestered in the cytoplasm as an inactivated complex with I-κB. Induced by stimuli like TNF-α, I-κB is phosphorylated at S32 and S36, followed by rapid ubiquitination and degradation, and the degradation of I-κB frees NF-

κB to translocate to the nucleus[42]. It is likely that AM5 blocks the nuclear translocation of NF-κB through inhibiting the phosphorylation and/or degradation of I-κB. EF24 was previously reported to inhibit TNF-α induced I-κB phosphorylation and degradation with 10-fold better potency than curcumin[80]. Based on this established method, the effect of AM5 on the same event was tested. A549 cells were pretreated with test compounds, followed by TNF-α stimulation. Phosphorylation and degradation of I-κB was detected after six minutes and 20 minutes of stimulation, respectively. The result revealed that AM5 effectively inhibited TNF-α induced I-κB phosphorylation and subsequent degradation at lower concentrations than EF24 (Figure 2.4 and Figure 2.6). The dose-response curves for the inhibition of I-κB phosphorylation demonstrated an IC_{50} of 1.3 μM for AM5, in comparison with 7.1 μM for EF24. These results suggested a role of AM5 in the inhibition of I-κB phosphorylation and degradation, leading to the blockade of NF-κB nuclear translocation. Additionally, this inhibitory activity was tested with several other compounds in comparison with AM5 (Figure 2.4 and Figure 2.5). Consistent with results in cell viability and NF-κB translocation assays, AM5 represented a favorable structure as a novel analog of curcumin for its improved anticancer activity.

2.3.4. AM5 directly inhibits the catalytic activity of IKKβ

In the classical NF-κB signaling pathway, the phosphorylation of I-κB is believed to be catalyzed by IKKβ of the kinase complex. It is possible that AM5 inhibits I-κB phosphorylation and degradation through direct inhibition of IKKβ kinase activity, as the

mechanism proposed and reported for EF24[80]. An *in vitro* kinase assay was performed to examine the effect of AM5 on the catalytic activity of IKKβ, which is defined by its ability to phospohrylate GST-I-κB in a reconstituted *in vitro* system. Active recombinant IKKβ was incubated with AM5 for 15 minutes before the addition of its substrate GST-I-κB, and [γ-^{32}P]ATP. The level of radiolabeld ^{32}P incorporated to GST-I-κB was detected by radiography, and a dose-dependent curve reflecting compound activity was obtained from quantification of the GST-I-κB bands by scintillation counting (Figure 2.7). Previous research reported the IC_{50} of curcumin in *in vitro* kinase assay as above 20 μM[80]. In this study, the IC_{50} of 2.3 μM for AM5 and 5.4 μM for EF24 indicated AM5 as a direct inhibitor of IKKβ, with improved potency over EF24.

Figure legends

Figure 2.1. The core structure of compounds with most improved cytotoxicity.

Figure 2.2. AM5 exhibits better potency of cytotoxic effect than EF24 in cancer cells. Cells were plated in 384-well plates, and were treated with AM5 or EF24 for 48 hr. Cell viability results were expressed in control with DMSO. Results are shown with four types of lung cancer cells, including A549 (lung adenocarcinoma), H157, H226 (squamous carcinoma), H1299 (non-small cell carcinoma), and one breast cancer cell line SKBR3 (breast carcinoma).

Figure 2.3. AM5 inhibits NF-κB nuclear translocation induced by TNF-α. A549 cells were plated in 384-well plates, and were pretreated with AM5 or EF24 for 30 min before stimulation by TNF-α for another 30 min. The effect of AM5 and EF24 on TNF-α induced NF-κB translocation was shown and quantified.

Figure 2.4. AM5 impairs TNF-α induced I-κB phosphorylation. A549 cells were pretreated with increasing concentration of test comounds (0 μM, 0.5 μM, 1 μM, 2.5 μM, 5 μM, 7.5 μM, and 10 μM) for 1 hr before stimulation by 10ng/ml TNF-α. Cell lysates were prepared after 6 min TNF-α stimulatin, and detected with pS32-I-κB antibody

through western blotting. Intensity of the bands were quantified with a Kodak imaging system, and expressed by percentage relative to DMSO-TNF-α control. Western blots for the effect of AM1, AM3, and AM4 were also demonstrated in comparison to AM5.

Figure 2.5. AM5 inhibits TNF-α induced I-κB phosphorylation with a better potency than AM16. Methods the same as described in Figure 2.4, except for the compound concentrations for AM5, EF24, and AM16 are 5 μM, 7.5 μM, and 10 μM. Total amount of I-κB was detected by I-κB antibody to show the stability of I-κB protein during phosphorylation.

Figure 2.6. AM5 impairs TNF-α induced I-κB degradation. A549 cells were treated with increasing doses of test compounds, and then were stimulated with 10 ng/ml TNF-α. After 20 min stimulation, cells were lysed and I-κB was detected through western blot. Effects of compounds AM1, AM3, and AM4 on blocking I-κB degradation were also shown here.

Figure 2.7. AM5 directly inhibits the catalytic activity of IKKβ. Recombinant IKKβ was incubated with increasing concentrations of AM5 (0.5 μM, 1 μM, 5 μM, 10 μM, and 25 μM) at room temperature for 15 min. Then GST-I-κB and [γ-^{32}P]ATP were added into the reaction system. Reactions were terminated after 15 min, and the mixtures were

resolved in a SDS-PAGE gel and stained with Coomassie Blue for total GST-I-κB. Total IKKβ was detected with an IKKβ antibody through western blot. Radiolabeled GST-I-κB was detected with a PhosphoImager after the gel was dried. Quantification was carried out by scintillation counting for the effect of both AM5 and EF24.

Compound	R_1, R_2
AM5	Cl
AM16	F
AM15	Br
AM12	H, Br
AM17	H, F

Figure 2.1 The core structure of compounds with most improved cytotoxicity.

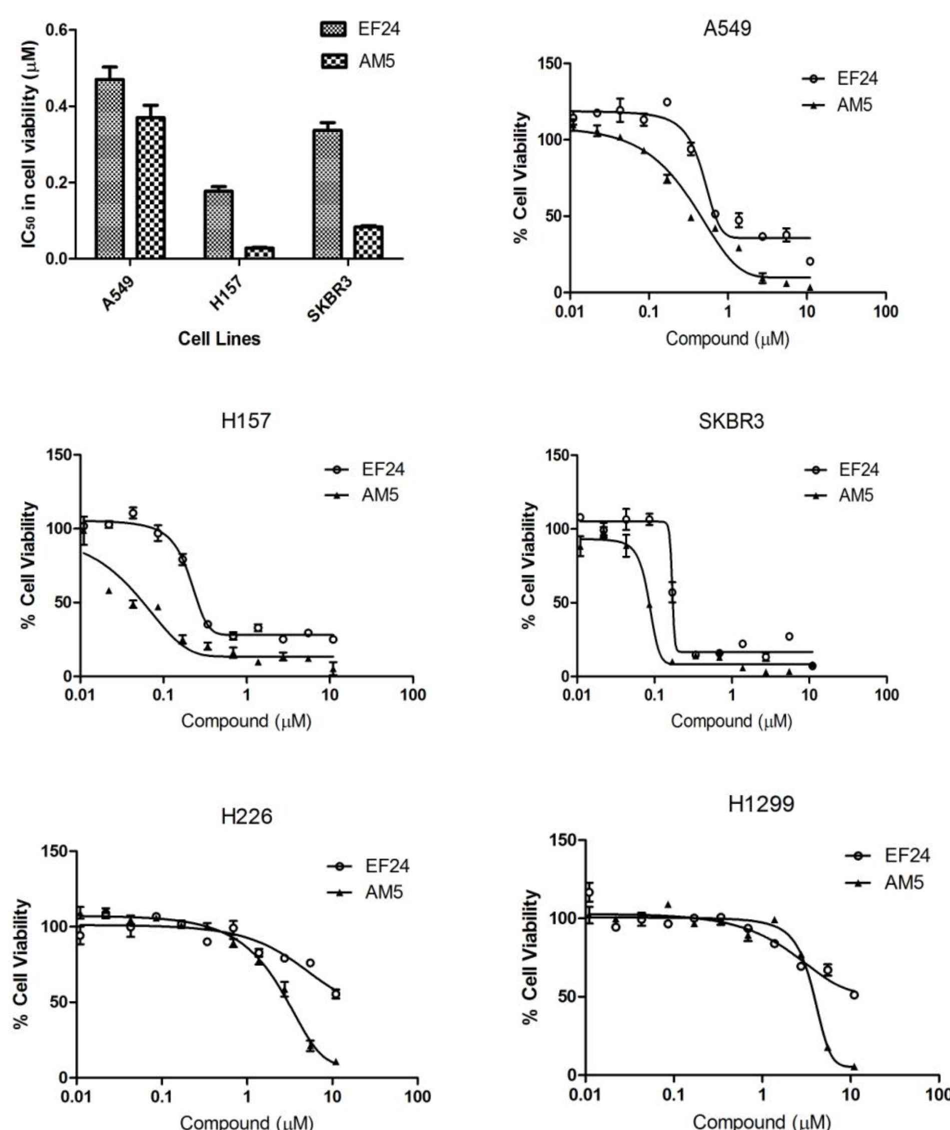
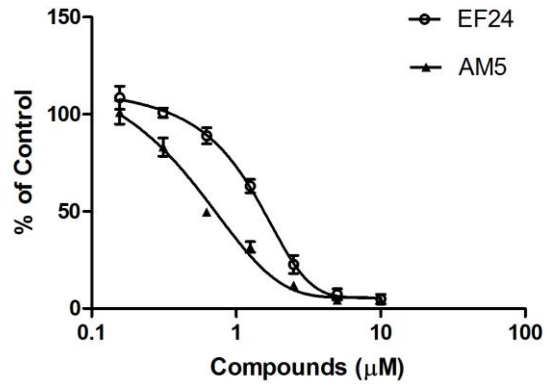


Figure 2.2 **AM5 exhibits better potency of cytotoxic effect than EF24 in cancer cells.** Cells were plated in 384-well plates and were treated with AM5 or EF24 for 48 hr. Cell viability results were expressed in control with DMSO. Results are shown with four types of lung cancer cells, including A549 (lung adenocarcinoma), H157, H226 (squamous carcinoma), H1299 (non-small cell carcinoma), and one breast cancer cell line SKBR3 (breast carcinoma).



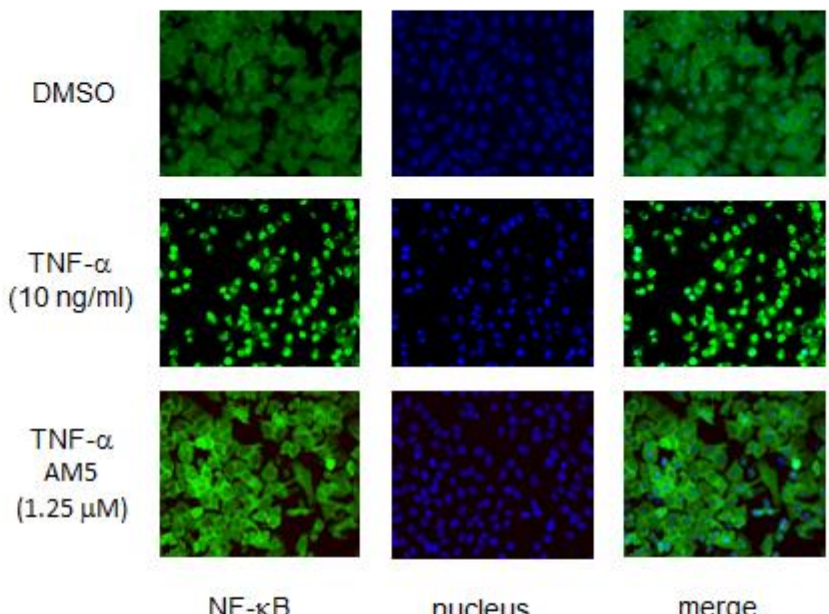
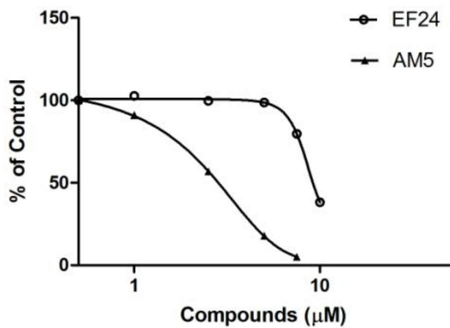
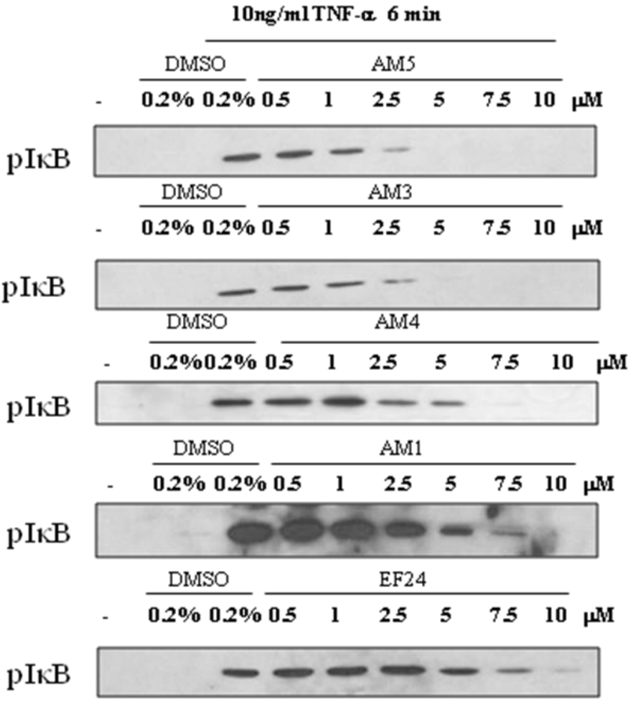
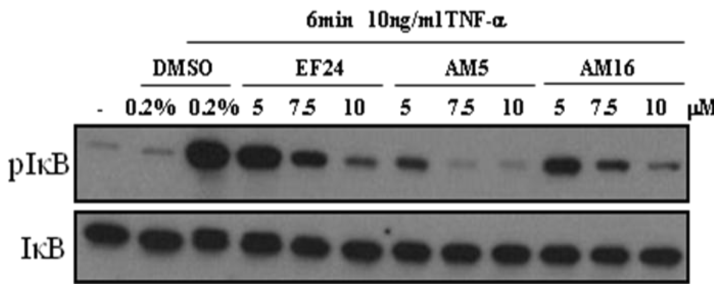


Figure 2.3 AM5 inhibits NF-κB nuclear translocation induced by TNF-α. A549 cells were plated in 384-well plates, and were pretreated with AM5 or EF24 for 30 min before stimulation by TNF-α for another 30 min. The effect of AM5 and EF24 on TNF-α induced NF-κB translocation was shown and quantified.

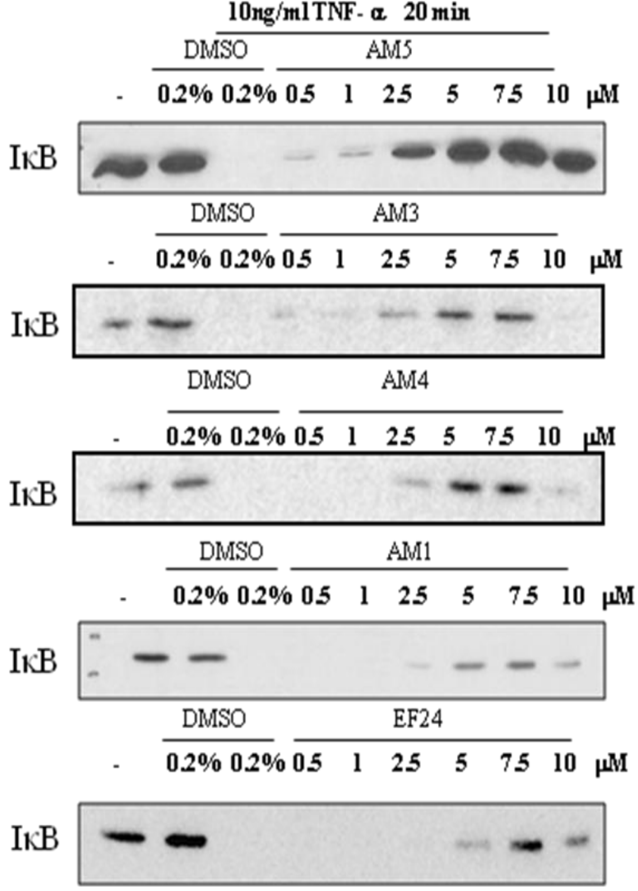




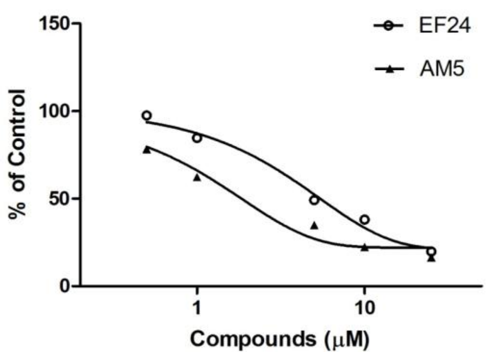
<u>Figure 2.4</u> AM5 impairs TNF-α induced I-κB phosphorylation. A549 cells were pretreated with increasing concentration of test comounds (0 μM, 0.5 μM, 1 μM, 2.5 μM, 5 μM, 7.5 μM, and 10 μM) for 1 hr before stimulation by 10ng/ml TNF-α. Cell lysates were prepared after 6 min TNF-α stimulatin, and detected with pS32-I-κB through western blotting. Intensity of the bands were quantified with a Kodak imaging system, and expressed by percentage relative to DMSO-TNF-α control. Western blots for the effect of AM1, AM3, and AM4 were also demonstrated in comparison to AM5.

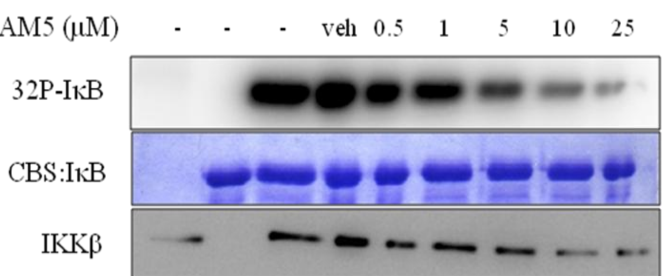


<u>Figure 2.5</u> AM5 inhibits TNF-α induced I-κB phosphorylation with a better potency than AM16. Methods the same as previously described in Figure 2.4, except for the compound concentrations for AM5, EF24, and AM16 are 5 μM, 7.5 μM, and 10 μM. Total amount of I-κB was detected by I-κB antibody to show the stability of I-κB protein during phosphorylation.



<u>Figure 2.6</u> AM5 impairs TNF-α induced I-κB degradation. A549 cells were treated with increasing doses of test agents, and then were stimulated with 10 ng/ml TNF-α. After 20 min stimulation, cells were lysed and I-κB levels were detected through western blot. Effects of compounds AM1, AM3, and AM4 on blocking I-κB degradation were also shown here.





<u>Figure 2.7</u> **AM5 directly inhibits the catalytic activity of IKKβ.** Recombinant IKKβ was incubated with increasing concentrations of AM5 (0.5 μM, 1 μM, 5 μM, 10 μM, and 25 μM) at room temperature for 15 min. Then GST-I-κB and [γ-^{32}P]ATP were added into the reaction system. Reactions were terminated after 15 min, and the mixtures were resolved in a SDS-PAGE gel and stained with Coomassie Blue for total GST-I-κB. Total IKKβ was detected with an IKKβ antibody through western blot. Radiolabeled GST-I-κB was detected with a PhosphoImager after the gel was dried. Quantification was carried out by scintillation counting for the effect of both AM5 and EF24.

Table 2.1 **IC_{50} (μM) values of cell toxicity for all 17 AM compounds and EF24**

Compounds	A549	H157	SKBR3	H226	H1299
EF24	0.47	0.17	0.34	>11	>11
AM1	0.27	0.18	0.26	>11	>11
AM2	>11	0.24	0.34	>11	>11
AM3	0.55	0.11	0.21	>11	6.1
AM4	0.82	0.55	0.19	5	2.1
AM5	0.37	0.028	0.083	5.8	3.2
AM6	>11	3.8	1.3	>11	>11
AM7	>11	0.27	0.42	>11	>11
AM8	5.3	2.2	0.37	>11	7.5
AM9	1.9	0.23	0.42	10	2.8
AM10	0.11	0.11	0.16	>11	5.9
AM12	0.26	0.03	0.078	5.2	3.9
AM13	0.31	0.096	0.18	>11	1.9
AM14	0.57	0.35	0.15	6.4	2.6
AM15	0.28	0.39	0.15	4.5	4.8
AM16	0.16	2.8	0.12	8.1	2.5
AM17	0.41	0.064	0.18	>11	4.8
AM21	0.65	0.24	0.072	4.8	2.9

Table 2.2 Ranking of IC_{50} values of cell toxicity for all 17 AM compounds and EF24

Ranking	A549	H157	SKBR3	H226	H1299
1	AM10	AM12	AM21	AM15	AM13
2	AM16	AM5	AM5	AM21	AM4
3	AM1	AM17	AM12	AM4	AM16
4	AM12	AM3	AM16	AM12	AM14
5	AM13	AM10	AM3	AM5	AM9
6	AM15	AM13	AM4	AM14	AM21
7	AM17	EF24	AM10	AM16	AM5
8	AM5	AM1	AM13	AM9	AM12
9	EF24	AM2	AM14	AM17*	AM15
10	AM14	AM9	AM15	EF24*	AM17
11	AM3	AM21	AM17	AM10*	AM10
12	AM21	AM7	EF24	AM13*	AM3
13	AM4	AM14	AM1	AM1*	AM8
14	AM9	AM15	AM2	AM2*	EF24*
15	AM8	AM4	AM7	AM3*	AM1*
16	AM2*	AM8	AM8	AM6*	AM2*
17	AM6*	AM16	AM9	AM7*	AM6*
18	AM7*	AM6	AM6	AM8*	AM7*

* IC_{50}>11 µM, no ranking available

<u>Table 2.3</u> Ranking of IC_{50} values from NF-κB nuclear translocation assay for all 17 AM compounds and EF24.

Ranking	Compound	IC_{50} (μM)	Ranking	Compound	IC_{50} (μM)
1	AM5	0.6	**10**	AM17	1.6
1	AM12	0.6	**11**	AM9	1.9
3	AM21	0.9	**12**	AM1	2.0
4	AM10	1.0	**13**	AM7	2.4
4	AM13	1.0	**14**	AM4	2.9
6	AM16	1.1	**15**	AM3	3.1
6	AM14	1.1	**16**	AM6	>5
8	EF24	1.3	**16**	AM8	>5
9	AM15	1.4	**18**	AM2	>10

Chapter 3:

Discussion

Discussion

A group of 17 synthetic analogs of curcumin was screened for their cytotoxic effects on a panel of lung cancer and breast cancer cells with different genetic background. This screening yielded 5 compounds with a particular core structure, which might account for their improved cytotoxicity over EF24. The most potent compound from this group, AM5, was identified as a direct inhibitor of IKKβ. Similar to the molecular mechanism of EF24 as previously reported, AM5 acted to block the nuclear translocation of NF-κB, to impair TNF-α induced phosphorylation and degradation of I-κB, and to directly inhibit the kinase activity of IKKβ (Figure 3.1)[80]. In all these assays, AM5 exhibited two to four-fold better potency than EF24, supporting its role as a novel analog of curcumin with improved inhibitory activity over EF24 in targeting NF-κB signaling pathway. In the same set of assays reported in a former study, curcumin was examined to be at least ten folds less potent than EF24[80]. These results together demonstrated notable progress in the structural modification of curcumin for the further improvement of its therapeutic potency.

Although the detailed interaction mode of AM5 with IKKβ requires further investigation to explain why the structure of AM5 is more favorable than EF24 and curcumin as an inhibitor of IKK, several instructive insights can be obtained from the relationship between the structure and activity of different AM compounds, as references for future modification of AM5.

1. The activity of AM5 and AM21 were similar, indicating that the elimination of methyl group from the central ketone ring did not affect much of AM5 activity.

2. AM10 apparently showed better activity than EF24, which suggested that the replacement of the fluoro-substituent by nitrogen in the terminal phenyl ring to form a terminal pyridine might improve the activity of EF24.

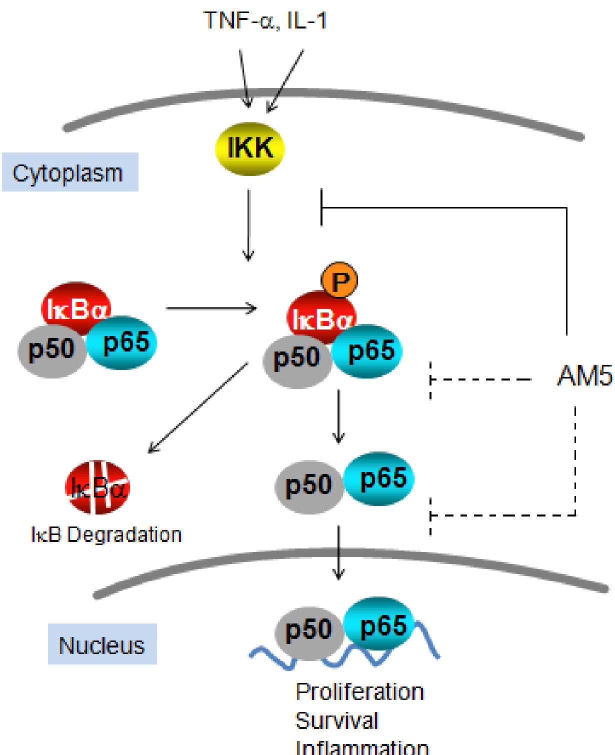
3. AM5 demonstrated better potency than AM3, suggesting that the second nitrogen in the terminal ring might not have a positive role in the improvement of activity. This was also supported by the relatively lower activity of all the other AM compounds with two nitrogen atoms in their terminal ring, with a slight exception of AM14.

4. AM5, AM15 and AM16 are compounds with the same symmetrically substituted core structure, but different halogen substituents. The double bromo-substituted AM15 showed the least activity. AM5 and AM16 exhibited similar cytotoxic effects in cell viability assay, while the western blot results indicated a clearly better activity of AM5 than AM16 in inhibiting the phosphorylation of I-κB. Thus, the double chloro-substituted AM5 was the most favorable of these three compounds in targeting NF-κB signaling.

5. Interestingly, the asymmetrically bromo-substituted AM12 consistently showed as good activity as AM5. While AM15, which is symmetrically bromo-substituted, demonstrated apparent decreased activity compared with AM12. In contrast, AM17 with the single fluoro-substituent was examined to be less potent than AM16, which was symmetrically fluoro-substituted.

6. AM13 also showed good activity close to AM5. However, whether this activity was related to the elimination of the chloro-substituents of AM5, or the substitution at the central ketone ring remained to be understood in further investigation.

In summary, this work has revealed a new class of curcumin analogs with improved bioactivity over EF24. AM5 represents the most favorable structure of this class, which has been identified as a direct IKK inhibitor. The activities of AM compounds with different modifications have provided instructive information for further optimization of AM5. Hopefully, more effective analogs of curcumin with therapeutic value and improved potency based on this study will be discovered in future.



<u>Figure 3.1</u> A molecular mechanism for the action of AM5. Nuclear translocation of NF- κB can be effectively impaired by AM5, which is probably resulted from the inhibition of I-κB phosphorylation and degradation through direct inhibition of IKK activity by AM5.

References

1. Sen, R.; Baltimore, D., Multiple nuclear factors interact with the immunoglobulin enhancer sequences. *Cell* **1986,** *46* (5), 705-16.
2. Sen, R.; Baltimore, D., Inducibility of kappa immunoglobulin enhancer-binding protein Nf-kappa B by a posttranslational mechanism. *Cell* **1986,** *47* (6), 921-8.
3. Perkins, N. D., Integrating cell-signalling pathways with NF-kappaB and IKK function. *Nat Rev Mol Cell Biol* **2007,** *8* (1), 49-62.
4. Ghosh, S.; Gifford, A. M.; Riviere, L. R.; Tempst, P.; Nolan, G. P.; Baltimore, D., Cloning of the p50 DNA binding subunit of NF-kappa B: homology to rel and dorsal. *Cell* **1990,** *62* (5), 1019-29.
5. Gilmore, T. D., NF-kappa B, KBF1, dorsal, and related matters. *Cell* **1990,** *62* (5), 841-3.
6. Kieran, M.; Blank, V.; Logeat, F.; Vandekerckhove, J.; Lottspeich, F.; Le Bail, O.; Urban, M. B.; Kourilsky, P.; Baeuerle, P. A.; Israel, A., The DNA binding subunit of NF-kappa B is identical to factor KBF1 and homologous to the rel oncogene product. *Cell* **1990,** *62* (5), 1007-18.
7. Ruben, S. M.; Klement, J. F.; Coleman, T. A.; Maher, M.; Chen, C. H.; Rosen, C. A., I-Rel: a novel rel-related protein that inhibits NF-kappa B transcriptional activity. *Genes Dev* **1992,** *6* (5), 745-60.
8. Liou, H. C.; Baltimore, D., Regulation of the NF-kappa B/rel transcription factor and I kappa B inhibitor system. *Curr Opin Cell Biol* **1993,** *5* (3), 477-87.
9. May, M. J.; Ghosh, S., Rel/NF-kappa B and I kappa B proteins: an overview. *Semin Cancer Biol* **1997,** *8* (2), 63-73.
10. Nolan, G. P.; Baltimore, D., The inhibitory ankyrin and activator Rel proteins. *Curr Opin Genet Dev* **1992,** *2* (2), 211-20.
11. Ruben, S. M.; Narayanan, R.; Klement, J. F.; Chen, C. H.; Rosen, C. A., Functional characterization of the NF-kappa B p65 transcriptional activator and an alternatively spliced derivative. *Mol Cell Biol* **1992,** *12* (2), 444-54.
12. Hatada, E. N.; Nieters, A.; Wulczyn, F. G.; Naumann, M.; Meyer, R.; Nucifora, G.; McKeithan, T. W.; Scheidereit, C., The ankyrin repeat domains of the NF-kappa B precursor p105 and the protooncogene bcl-3 act as specific inhibitors of NF-kappa B DNA binding. *Proc Natl Acad Sci U S A* **1992,** *89* (6), 2489-93.
13. Bours, V.; Burd, P. R.; Brown, K.; Villalobos, J.; Park, S.; Ryseck, R. P.; Bravo, R.; Kelly, K.; Siebenlist, U., A novel mitogen-inducible gene product related to p50/p105-NF-kappa B participates in transactivation through a kappa B site. *Mol Cell Biol* **1992,** *12* (2), 685-95.
14. Naumann, M.; Wulczyn, F. G.; Scheidereit, C., The NF-kappa B precursor p105 and the proto-oncogene product Bcl-3 are I kappa B molecules and control nuclear translocation of NF-kappa B. *EMBO J* **1993,** *12* (1), 213-22.
15. Atchison, M. L.; Perry, R. P., The role of the kappa enhancer and its binding factor NF-kappa B in the developmental regulation of kappa gene transcription. *Cell* **1987,** *48* (1), 121-8.

16. Queen, C.; Foster, J.; Stauber, C.; Stafford, J., Cell-type specific regulation of a kappa immunoglobulin gene by promoter and enhancer elements. *Immunol Rev* **1986,** *89*, 49-68.
17. Gerondakis, S.; Strasser, A.; Metcalf, D.; Grigoriadis, G.; Scheerlinck, J. Y.; Grumont, R. J., Rel-deficient T cells exhibit defects in production of interleukin 3 and granulocyte-macrophage colony-stimulating factor. *Proc Natl Acad Sci U S A* **1996,** *93* (8), 3405-9.
18. Liou, H. C.; Jin, Z.; Tumang, J.; Andjelic, S.; Smith, K. A.; Liou, M. L., c-Rel is crucial for lymphocyte proliferation but dispensable for T cell effector function. *Int Immunol* **1999,** *11* (3), 361-71.
19. Gerondakis, S.; Grumont, R.; Rourke, I.; Grossmann, M., The regulation and roles of Rel/NF-kappa B transcription factors during lymphocyte activation. *Curr Opin Immunol* **1998,** *10* (3), 353-9.
20. Welte, T.; Leitenberg, D.; Dittel, B. N.; al-Ramadi, B. K.; Xie, B.; Chin, Y. E.; Janeway, C. A., Jr.; Bothwell, A. L.; Bottomly, K.; Fu, X. Y., STAT5 interaction with the T cell receptor complex and stimulation of T cell proliferation. *Science* **1999,** *283* (5399), 222-5.
21. Dunn, G. P.; Bruce, A. T.; Ikeda, H.; Old, L. J.; Schreiber, R. D., Cancer immunoediting: from immunosurveillance to tumor escape. *Nat Immunol* **2002,** *3* (11), 991-8.
22. Dunn, G. P.; Old, L. J.; Schreiber, R. D., The immunobiology of cancer immunosurveillance and immunoediting. *Immunity* **2004,** *21* (2), 137-48.
23. Mosmann, T. R.; Coffman, R. L., TH1 and TH2 cells: different patterns of lymphokine secretion lead to different functional properties. *Annu Rev Immunol* **1989,** *7*, 145-73.
24. Swain, S. L.; McKenzie, D. T.; Weinberg, A. D.; Hancock, W., Characterization of T helper 1 and 2 cell subsets in normal mice. Helper T cells responsible for IL-4 and IL-5 production are present as precursors that require priming before they develop into lymphokine-secreting cells. *J Immunol* **1988,** *141* (10), 3445-55.
25. Ciavarra, R. P., T helper cells in cytotoxic T lymphocyte development: analysis of the cellular basis for deficient T helper cell function in the L3T4-independent T helper cell pathway. *Cell Immunol* **1991,** *134* (2), 427-41.
26. Duyao, M. P.; Buckler, A. J.; Sonenshein, G. E., Interaction of an NF-kappa B-like factor with a site upstream of the c-myc promoter. *Proc Natl Acad Sci U S A* **1990,** *87* (12), 4727-31.
27. Romagnani, S., Lymphokine production by human T cells in disease states. *Annu Rev Immunol* **1994,** *12*, 227-57.
28. Ferrara, J. L., Cytokines and the regulation of tolerance. *J Clin Invest* **2000,** *105* (8), 1043-4.
29. Ganss, R.; Hanahan, D., Tumor microenvironment can restrict the effectiveness of activated antitumor lymphocytes. *Cancer Res* **1998,** *58* (20), 4673-81.
30. Ganss, R.; Limmer, A.; Sacher, T.; Arnold, B.; Hammerling, G. J., Autoaggression and tumor rejection: it takes more than self-specific T-cell activation. *Immunol Rev* **1999,** *169*, 263-72.

31. Cao, S.; Zhang, X.; Edwards, J. P.; Mosser, D. M., NF-kappaB1 (p50) homodimers differentially regulate pro- and anti-inflammatory cytokines in macrophages. *J Biol Chem* **2006,** *281* (36), 26041-50.
32. Mosser, D. M.; Zhang, X., Interleukin-10: new perspectives on an old cytokine. *Immunol Rev* **2008,** *226*, 205-18.
33. Wang, S.; Liu, Z.; Wang, L.; Zhang, X., NF-kappaB signaling pathway, inflammation and colorectal cancer. *Cell Mol Immunol* **2009,** *6* (5), 327-34.
34. Kanaoka, S.; Takai, T.; Yoshida, K., Cyclooxygenase-2 and tumor biology. *Adv Clin Chem* **2007,** *43*, 59-78.
35. Stoeltzing, O.; Liu, W.; Fan, F.; Wagner, C.; Stengel, K.; Somcio, R. J.; Reinmuth, N.; Parikh, A. A.; Hicklin, D. J.; Ellis, L. M., Regulation of cyclooxygenase-2 (COX-2) expression in human pancreatic carcinoma cells by the insulin-like growth factor-I receptor (IGF-IR) system. *Cancer Lett* **2007,** *258* (2), 291-300.
36. Saukkonen, K.; Rintahaka, J.; Sivula, A.; Buskens, C. J.; Van Rees, B. P.; Rio, M. C.; Haglund, C.; Van Lanschot, J. J.; Offerhaus, G. J.; Ristimaki, A., Cyclooxygenase-2 and gastric carcinogenesis. *APMIS* **2003,** *111* (10), 915-25.
37. Denkert, C.; Winzer, K. J.; Hauptmann, S., Prognostic impact of cyclooxygenase-2 in breast cancer. *Clin Breast Cancer* **2004,** *4* (6), 428-33.
38. Hussain, T.; Gupta, S.; Mukhtar, H., Cyclooxygenase-2 and prostate carcinogenesis. *Cancer Lett* **2003,** *191* (2), 125-35.
39. Liu, X.; Lazenby, A. J.; Siegal, G. P., Signal transduction cross-talk during colorectal tumorigenesis. *Adv Anat Pathol* **2006,** *13* (5), 270-4.
40. Luque, I.; Gelinas, C., Rel/NF-kappa B and I kappa B factors in oncogenesis. *Semin Cancer Biol* **1997,** *8* (2), 103-11.
41. Ghosh, S.; Hayden, M. S., New regulators of NF-kappaB in inflammation. *Nat Rev Immunol* **2008,** *8* (11), 837-48.
42. Karin, M.; Ben-Neriah, Y., Phosphorylation meets ubiquitination: the control of NF-[kappa]B activity. *Annu Rev Immunol* **2000,** *18*, 621-63.
43. Yamaoka, S.; Courtois, G.; Bessia, C.; Whiteside, S. T.; Weil, R.; Agou, F.; Kirk, H. E.; Kay, R. J.; Israel, A., Complementation cloning of NEMO, a component of the IkappaB kinase complex essential for NF-kappaB activation. *Cell* **1998,** *93* (7), 1231-40.
44. Hacker, H.; Karin, M., Regulation and function of IKK and IKK-related kinases. *Sci STKE* **2006,** *2006* (357), re13.
45. Solt, L. A.; Madge, L. A.; May, M. J., NEMO-binding domains of both IKKalpha and IKKbeta regulate IkappaB kinase complex assembly and classical NF-kappaB activation. *J Biol Chem* **2009,** *284* (40), 27596-608.
46. Luo, J. L.; Kamata, H.; Karin, M., IKK/NF-kappaB signaling: balancing life and death--a new approach to cancer therapy. *J Clin Invest* **2005,** *115* (10), 2625-32.
47. Ghosh, S.; Karin, M., Missing pieces in the NF-kappaB puzzle. *Cell* **2002,** *109 Suppl*, S81-96.
48. Vallabhapurapu, S.; Karin, M., Regulation and function of NF-kappaB transcription factors in the immune system. *Annu Rev Immunol* **2009,** *27*, 693-733.
49. Li, Q.; Verma, I. M., NF-kappaB regulation in the immune system. *Nat Rev Immunol* **2002,** *2* (10), 725-34.

50. Yamamoto, Y.; Gaynor, R. B., Therapeutic potential of inhibition of the NF-kappaB pathway in the treatment of inflammation and cancer. *J Clin Invest* **2001,** *107* (2), 135-42.
51. Calzado, M. A.; Bacher, S.; Schmitz, M. L., NF-kappaB inhibitors for the treatment of inflammatory diseases and cancer. *Curr Med Chem* **2007,** *14* (3), 367-76.
52. Olivier, S.; Robe, P.; Bours, V., Can NF-kappaB be a target for novel and efficient anti-cancer agents? *Biochem Pharmacol* **2006,** *72* (9), 1054-68.
53. Ludwig, H.; Khayat, D.; Giaccone, G.; Facon, T., Proteasome inhibition and its clinical prospects in the treatment of hematologic and solid malignancies. *Cancer* **2005,** *104* (9), 1794-807.
54. Nakanishi, C.; Toi, M., Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* **2005,** *5* (4), 297-309.
55. Lee, D. F.; Hung, M. C., Advances in targeting IKK and IKK-related kinases for cancer therapy. *Clin Cancer Res* **2008,** *14* (18), 5656-62.
56. Newman, D. J.; Cragg, G. M., Natural products as sources of new drugs over the last 25 years. *J Nat Prod* **2007,** *70* (3), 461-77.
57. Harvey, A. L., Natural products in drug discovery. *Drug Discov Today* **2008,** *13* (19-20), 894-901.
58. Aggarwal, B. B.; Sung, B., Pharmacological basis for the role of curcumin in chronic diseases: an age-old spice with modern targets. *Trends Pharmacol Sci* **2009,** *30* (2), 85-94.
59. Aggarwal, B. B.; Harikumar, K. B., Potential therapeutic effects of curcumin, the anti-inflammatory agent, against neurodegenerative, cardiovascular, pulmonary, metabolic, autoimmune and neoplastic diseases. *Int J Biochem Cell Biol* **2009,** *41* (1), 40-59.
60. Marin, Y. E.; Wall, B. A.; Wang, S.; Namkoong, J.; Martino, J. J.; Suh, J.; Lee, H. J.; Rabson, A. B.; Yang, C. S.; Chen, S.; Ryu, J. H., Curcumin downregulates the constitutive activity of NF-kappaB and induces apoptosis in novel mouse melanoma cells. *Melanoma Res* **2007,** *17* (5), 274-83.
61. Arora, R. B.; Kapoor, V.; Basu, N.; Jain, A. P., Anti-inflammatory studies on Curcuma longa (turmeric). *Indian J Med Res* **1971,** *59* (8), 1289-95.
62. Jurenka, J. S., Anti-inflammatory properties of curcumin, a major constituent of Curcuma longa: a review of preclinical and clinical research. *Altern Med Rev* **2009,** *14* (2), 141-53.
63. Jagetia, G. C.; Aggarwal, B. B., "Spicing up" of the immune system by curcumin. *J Clin Immunol* **2007,** *27* (1), 19-35.
64. Menon, V. P.; Sudheer, A. R., Antioxidant and anti-inflammatory properties of curcumin. *Adv Exp Med Biol* **2007,** *595*, 105-25.
65. Johnson, J. J.; Mukhtar, H., Curcumin for chemoprevention of colon cancer. *Cancer Lett* **2007,** *255* (2), 170-81.
66. Kuttan, R.; Bhanumathy, P.; Nirmala, K.; George, M. C., Potential anticancer activity of turmeric (Curcuma longa). *Cancer Lett* **1985,** *29* (2), 197-202.
67. Chen, A.; Xu, J.; Johnson, A. C., Curcumin inhibits human colon cancer cell growth by suppressing gene expression of epidermal growth factor receptor

through reducing the activity of the transcription factor Egr-1. *Oncogene* **2006,** *25* (2), 278-87.

68. Kawamori, T.; Lubet, R.; Steele, V. E.; Kelloff, G. J.; Kaskey, R. B.; Rao, C. V.; Reddy, B. S., Chemopreventive effect of curcumin, a naturally occurring anti-inflammatory agent, during the promotion/progression stages of colon cancer. *Cancer Res* **1999,** *59* (3), 597-601.
69. Huang, M. T.; Lou, Y. R.; Ma, W.; Newmark, H. L.; Reuhl, K. R.; Conney, A. H., Inhibitory effects of dietary curcumin on forestomach, duodenal, and colon carcinogenesis in mice. *Cancer Res* **1994,** *54* (22), 5841-7.
70. Conney, A. H.; Lysz, T.; Ferraro, T.; Abidi, T. F.; Manchand, P. S.; Laskin, J. D.; Huang, M. T., Inhibitory effect of curcumin and some related dietary compounds on tumor promotion and arachidonic acid metabolism in mouse skin. *Adv Enzyme Regul* **1991,** *31*, 385-96.
71. Chauhan, D. P., Chemotherapeutic potential of curcumin for colorectal cancer. *Curr Pharm Des* **2002,** *8* (19), 1695-706.
72. Suresh, D.; Srinivasan, K., Influence of curcumin, capsaicin, and piperine on the rat liver drug-metabolizing enzyme system in vivo and in vitro. *Can J Physiol Pharmacol* **2006,** *84* (12), 1259-65.
73. Anand, P.; Kunnumakkara, A. B.; Newman, R. A.; Aggarwal, B. B., Bioavailability of curcumin: problems and promises. *Mol Pharm* **2007,** *4* (6), 807-18.
74. Shoba, G.; Joy, D.; Joseph, T.; Majeed, M.; Rajendran, R.; Srinivas, P. S., Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med* **1998,** *64* (4), 353-6.
75. Suresh, D.; Srinivasan, K., Studies on the in vitro absorption of spice principles--curcumin, capsaicin and piperine in rat intestines. *Food Chem Toxicol* **2007,** *45* (8), 1437-42.
76. Washstrom, B. B., G.A, A study on the fate of curcumin in the rat. *Acta Pharmacol Toxicol (Copenh)* **1978,** *43*, 6.
77. Pan, M. H.; Huang, T. M.; Lin, J. K., Biotransformation of curcumin through reduction and glucuronidation in mice. *Drug Metab Dispos* **1999,** *27* (4), 486-94.
78. Adams, B. K.; Ferstl, E. M.; Davis, M. C.; Herold, M.; Kurtkaya, S.; Camalier, R. F.; Hollingshead, M. G.; Kaur, G.; Sausville, E. A.; Rickles, F. R.; Snyder, J. P.; Liotta, D. C.; Shoji, M., Synthesis and biological evaluation of novel curcumin analogs as anti-cancer and anti-angiogenesis agents. *Bioorg Med Chem* **2004,** *12* (14), 3871-83.
79. Adams, B. K.; Cai, J.; Armstrong, J.; Herold, M.; Lu, Y. J.; Sun, A.; Snyder, J. P.; Liotta, D. C.; Jones, D. P.; Shoji, M., EF24, a novel synthetic curcumin analog, induces apoptosis in cancer cells via a redox-dependent mechanism. *Anticancer Drugs* **2005,** *16* (3), 263-75.
80. Kasinski, A. L.; Du, Y.; Thomas, S. L.; Zhao, J.; Sun, S. Y.; Khuri, F. R.; Wang, C. Y.; Shoji, M.; Sun, A.; Snyder, J. P.; Liotta, D.; Fu, H., Inhibition of IkappaB kinase-nuclear factor-kappaB signaling pathway by 3,5-bis(2-flurobenzylidene)piperidin-4-one (EF24), a novel monoketone analog of curcumin. *Mol Pharmacol* **2008,** *74* (3), 654-61.

81. Mercurio, F.; Zhu, H.; Murray, B. W.; Shevchenko, A.; Bennett, B. L.; Li, J.; Young, D. B.; Barbosa, M.; Mann, M.; Manning, A.; Rao, A., IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **1997,** *278* (5339), 860-6.
82. Rubinstein, L. V.; Shoemaker, R. H.; Paull, K. D.; Simon, R. M.; Tosini, S.; Skehan, P.; Scudiero, D. A.; Monks, A.; Boyd, M. R., Comparison of in vitro anticancer-drug-screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J Natl Cancer Inst* **1990,** *82* (13), 1113-8.
83. Skehan, P.; Storeng, R.; Scudiero, D.; Monks, A.; McMahon, J.; Vistica, D.; Warren, J. T.; Bokesch, H.; Kenney, S.; Boyd, M. R., New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **1990,** *82* (13), 1107-12.
84. Rothe, M.; Sarma, V.; Dixit, V. M.; Goeddel, D. V., TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* 1995, 269 (5229), 1424-7.