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**Inhibition of IKK–NF- κ B signaling pathway by EF24, a novel monoketone analogue
of curcumin**

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c. Text: 31 pages

Tables: 1

Figures: 7

References: 33

Words:

Abstract (220)

Introduction (718)

Discussion (917)

d. A list of nonstandard abbreviations used in the paper

FBS, fetal bovine serum; Hepes, N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid; I- κ B, inhibitor of kappa B; IKK, inhibitor of kappa B kinase; NF- κ B, nuclear factor kappa-B; NP-40, Nonident P-40; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gels

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Abstract

The nuclear factor kappa-B (NF- κ B) signaling pathway has been targeted for therapeutic applications in a variety of human diseases such as cancer. A number of naturally occurring substances including curcumin have been investigated for their actions on the NF- κ B pathway because of their significant therapeutic potential and safety profile. A synthetic monoketone compound termed EF24 was developed from curcumin and exhibited a potent anticancer activity. Here we report a mechanism by which EF24 potently suppresses the NF- κ B signaling pathway through direct action on the I- κ B kinase (IKK). We demonstrate that (i) EF24 induces death of lung, breast, ovarian, and cervical cancer cells with a potency about 10 times higher than curcumin does, (ii) EF24 rapidly blocks the nuclear translocation of NF- κ B with an IC₅₀ of 1.3 μ M compared with curcumin with an IC₅₀ of 13 μ M, (iii) EF24 effectively inhibits TNF α -induced I- κ B phosphorylation and degradation, suggesting a role of this compound in targeting IKK, and (iv) EF24 indeed directly inhibits the catalytic activity of IKK in an *in vitro* reconstituted system. Our study identifies IKK as an effective target for EF24 and provides a molecular explanation for a superior activity of EF24 over curcumin. The effective inhibition of TNF α -induced NF- κ B signaling by EF24 extends the therapeutic application of EF24 to other NF- κ B-dependent diseases, including inflammatory diseases such as rheumatoid arthritis.

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Introduction

Curcumin, isolated from the rhizomes of the plant *Curcuma longa* L, is the major component of the spice curry. This compound, freely available in the human food supply, is associated with numerous therapeutic benefits including chemoprevention and chemotherapy in cancer, anti-inflammatory, antioxidant, and antiviral activities (Cheng et al., 2001; Commandeur and Vermeulen, 1996; Levi et al., 2001; Osawa T, 1995). In addition, the pharmacological safety of curcumin is evident by its consumption for centuries at levels up to 100 mg/day by people in certain countries (Satoskar et al., 1986). These beneficial properties have attracted numerous efforts for the development of curcumin as a safe therapeutic agent (Aggarwal et al., 2005; Anand et al., 2007). Recent therapeutic efficacy against pancreatic cancer in a phase II clinical trial further supports the use of curcumin as a lead for the development of a new class of anticancer agents (Dhillon N et al., 2006). Unfortunately, due to the low potency and poor absorption characteristics of curcumin, its clinical potential remains to be limited (Shoba et al., 1998). However, curcumin represents an ideal lead compound for further chemical modifications and optimization (Adams et al., 2005; Adams et al., 2004).

In an attempt to retain curcumin's favorable medicinal properties and safety profile, while increasing its potency, computer-assisted topological searches of the NCI database were carried out to identify lead analogs. Two such analogs incorporating a monoketone were identified and exhibited improved cytotoxic effect over curcumin (Adams et al., 2004). These results stimulated a more thorough search for easily prepared and readily functionalized analogs with improved potency. The strategy adopted is captured by the following manipulation of curcumin. Two carbons and an

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oxygen were removed from the center of the molecule to produce a monoketone, terminal ring substituents were varied, and an extensible heterocyclic six-membered ring including the ketone was installed (see Fig. 1 for an example). Approximately 100 analogs were tested. A subset of 10 was further evaluated in the 60 panel of NCI cancer cell lines and in several in vitro anti-angiogenesis screens (Adams et al., 2004). EF24 (Fig. 1) surfaced as one of the top candidate compounds. EF24 has shown to induce apoptosis in cancer cells and inhibit the growth of human breast tumors in a mouse xenograft model with relatively low toxicity and at a dose much less than that of curcumin (Adams et al., 2005; Adams et al., 2004). Studies with various cancer cells have suggested a model that EF24 impairs cell growth by inducing G2/M arrest followed by induction of apoptosis, which is accompanied by caspase-3 activation, phosphatidylserine externalization, and an increased number of cells with a sub-G1 DNA content (Adams et al., 2005). However, the cell signaling pathways that mediate the EF24 effect remain to be elucidated. This study examines the effect of EF24 on a key survival pathway in lung cancer cells mediated by the NF- κ B transcription factor.

NF- κ B is maintained in an inactive state in the cytoplasm by the inhibitor of kappa B (I κ -B), which masks the nuclear localization signal of NF- κ B. Phosphorylation of I κ -B by the inhibitor of kappa B kinase (IKK), via the canonical NF- κ B pathway, results in subsequent ubiquitination of I κ -B and proteasomal degradation. It is this degradation of I κ -B that liberates NF- κ B, allowing its localization to the nucleus and the transcriptional activation of its target genes. On the other hand, aberrant activation of NF- κ B contributes to deregulated growth, resistance to apoptosis, and propensity to metastasize observed in many cancers (Rayet and Gelinas, 1999; Richmond, 2002). The

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NF- κ B effect is in part due to the upregulation of NF- κ B -controlled genes that promote survival function of tumor cells, including c-myc, Bcl-xL, and members of IAP (Inhibitor of Apoptosis) family of genes. In addition, many anticancer agents can induce the activation of NF- κ B, resulting in reduced therapeutic efficacy (Nakanishi and Toi, 2005). Thus, agents that effectively impair the NF- κ B pathway are expected to have significant therapeutic potential.

In this report, we identify the molecular mechanism of action of EF24. We show that treatment with EF24 results in a marked decrease in cellular viability. The dose necessary to reduce viability correlates well with that needed for EF24 to impair the nuclear translocation of NF- κ B and to inhibit IKK. EF24 is at least 10 times more potent than curcumin and represents a lead compound for further therapeutic development of a new generation of natural product-derived anticancer agents.

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Materials and Methods

Materials –Curcumin was purchased from Alfa Aesar and its structural analog, namely EF24 (3,5-bis(2-fluorobenzylidene)piperidin-4-one), was prepared as previously reported (Adams et al., 2004). Both compounds were dissolved in DMSO with a stock concentration of 10 mM. TNF α (Sigma-Aldrich Chemicals, St Louis, MO) was resuspended in water to a final concentration of 10 μ g/ml. Glutathione S-transferase-I- κ B α (1-54) was purified from expression plasmid as previously described (Mercurio et al., 1997). Antibodies against pS32-I- κ B α and I- κ B α were purchased from Cell Signaling (Beverly, MA). Antibodies against IKK α and IKK β were purchased from Imgenex (San Diego, CA). Antibodies to Raf-1, and secondary antibody conjugates, HRP-goat anti-mouse and HRP-goat anti-rabbit antibodies, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA)

Cell Cultures – Cells were maintained in either in RPMI (A549, H157, H460, Calu-1, H358, PC3, 1A9, MDA-MB231), or DMEM (HeLa) with 10% fetal bovine serum (FBS) and penicillin/streptomycin in a 37°C incubator with 5% CO₂. For Western blot analysis, unless otherwise noted, cells were lysed in 1% NP-40 lysis buffer (1% Nonident P-40, 10 mM Hepes [N-2hydroxyethylpiperazine-N'-2-ethanesulfonic acid], pH 7.4, 150 mM NaCl, 5 mM NaF, 2 mM Na₃VO₄, 5 mM Na₄P₂O₇, 10 μ g/ml aprotinin, 10 μ g /ml leupeptin, 1 mM phenylmethylsulfonyl fluoride (PMSF).

Cytotoxicity Assay - Cells were plated at 5,000 cells/well in 96-well plates. They were treated with test agents in triplicate in the following day and further incubated for 48 hr

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before viability assay was carried out. The sulforhodamine B assay was performed to evaluate cell viability and to obtain the IC₅₀ values (Rubinstein et al., 1990; Skehan et al., 1990). It measures cellular protein content in order to determine cell density. The mean value and standard error for each treatment were determined and the cell viability index relative to control (untreated) was calculated. The IC₅₀ is defined as the concentration of agents that decrease viability by 50% in a total cell population (cell viability index = 0.5) as compared to control cells (cell viability index = 1) at the end of the incubation period.

High content analysis of NF-κB subcellular translocation - A549 cells were plated in 96-well plates (Becton Dickinson Labware, Franklin Lakes, NJ) at 10,000 cells/90 μl/well and grown for 20 hr. Test compounds were added to each well and incubated at 37°C. All samples were performed in triplicates. The cells were stimulated with TNFα as indicated. Reactions were terminated by washing the plates with ice-cold phosphate-buffered saline (PBS) followed by fixation with paraformaldehyde (2%, 100 μl) for 30 min at room temperature. Cells were permeabilized with Triton X-100 (0.1%, 100 μl) for 20 min, washed three times with PBS, and blocked with BSA (1%, 100 μl) for 1 hr. Rabbit anti-p65 NF-κB antibody (Santa Cruz biotechnology, Inc., Santa Cruz, CA) was added and incubated overnight at 4°C. Cells were washed three times in PBS and incubated with goat anti-rabbit IgG with conjugated Alexa Fluor 488 (50 μl; Molecular Probes, Inc., Eugene, OR) along with Hoechst 33342 (1 μg/ml; Promega, Madison, WI). After washing with PBS, cells were imaged with the ImageXpress 5000 with the filter set for FITC (Ex: 490 nm, Em: 525 nm and dichroic mirror at 505 nm) and DAPI (Ex: 350 nm; Em: 479 nm with dichroic mirror at 400 nm) (Molecular Devices, Sunnyvale, CA).

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The images were quantified and analyzed using MetaXpress software (Molecular Devices). “Translocation Enhanced” module was used for the NF- κ B translocation analysis. The nucleus was defined by Hoechst 33342 staining. The levels of NF- κ B translocation were calculated and expressed as the difference between average fluorescence intensity in nucleus and in cytoplasm (Nuc – Cyt). After stimulating with TNF α , the inhibitory effect of test compounds on TNF α induced NF- κ B translocation was expressed as % of fluorescence intensity difference (Nuc-Cyt) in the control wells (TNF α only) after subtracting background (no TNF α treatment). Data shown are average values from triplicate samples.

Western Blotting – Cells were lysed in NP-40 buffer (1.0% NP-40, 10 mM Hepes, pH 7.4, 150 mM NaCl, 5 mM NaF, 2 mM Na₃VO₄, 5 mM Na₄P₂O₇, 10 μ g/ml aprotinin, 10 μ g/ml leupeptin, 1 mM PMSF). Equal volumes of cell lysate were subject to electrophoresis on SDS-PAGE (12.5%). Proteins were then electrotransferred to a nitrocellulose membrane (GE water and Process Technologies, Trevose, PA) as described previously (Zhang et al., 1999). Membranes were blocked in a solution of 5% non-fat dry milk in TBS-T buffer (20 mM Tris pH 7.6, 500 mM NaCl, 0.5% Tween-20) for 30 min followed by incubation with primary antibody for at least two hours. The membrane was then washed and treated with the corresponding horseradish peroxidase-conjugated anti-mouse immunoglobulin [Ig] or anti-rabbit Ig as indicated. Immunodetection was performed using West Pico (Pierce, Rockford, IL) or West Dura (Pierce) followed by imaging on Kodak’s Image Station 2000R (New Haven, CT).

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Immunoprecipitation – Cells were seeded in 150 mm plates to approximately 70% confluency one day before treatment. Cells were treated with increasing doses of EF24 or curcumin with or without TNF α as indicated and lysed in 1% NP-40 lysis buffer at 4°C. Lysates were clarified by centrifugation (14,000 rpm, 10 min, 4°C). Cytoplasmic extracts were immunoprecipitated with an anti-IKK α antibody (Imgenex, San Diego, CA) at 4°C overnight. Protein G Sepharose beads (50% slurry; Pharmacia) in lysis buffer were added to each reaction the following day and incubated for an additional two hours. Protein G beads were then gently spun down and washed two times with NP-40 lysis buffer and one final time with the kinase assay buffer (50 mM Hepes, pH 7.4; 20 mM MgCl₂; 2 mM DTT). The immunocomplexes were used for IKK kinase assays.

Kinase assays – (a) IKK immunocomplex assay. Immunoprecipitated complexes were added to kinase reaction buffer containing [γ -³²P]ATP (20 μ Ci with 10 μ M unlabeled ATP), and glutathione S-transferase (GST)-I- κ B α (residues 1-54; 5 μ g) in a total volume of 25 μ l and incubated at 30°C for 30 min. Reactions were stopped by boiling the kinase solution in a 6 X SDS sample buffer for 5 min. The samples were resolved on SDS-PAGE (12.5%). The top portion of the gel was transferred and immunoblotted with anti-IKK β antibody while the bottom portion was stained with Coomassie Blue dye (0.05%). Radiolabeled phosphate incorporation into GST- I- κ B α was assessed by the PhosphoImager and quantified with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). (b) *In vitro* recombinant IKK β assay. Activated recombinant IKK β in MOPS buffer (8 mM MOPS-NaOH, pH 7.0, 200 μ M EDTA, 15 mM MgCl₂) (Upstate Cell Signaling Solutions, Lake Placid, NY;) was used to assess the direct effect of EF24

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or curcumin on the kinase. The test compounds were incubated in the presence of 40 ng of IKK β for 15 minutes at room temperature. The addition of Mg-ATP cocktail (15 mM MgCl₂, 100 μ M ATP, 8 mM MOPS-NaOH, pH 7.0, 5 mM β -glycero-phosphate, 1 mM EGTA, 200 nM sodium orthovanadate, 200 nM DTT) purified GST- I- κ B α (5 μ g) and [γ -³²P]ATP (0.5 μ Ci) in a final volume of 25 μ l started the reaction which was allowed to proceed at 30°C for 15 min. Reactions were terminated and processed as described in (a). In addition, the radiolabeled GST-I- κ B α protein bands were excised for quantification with a scintillation counter (Beckman LS 6500, Beckman Coulter, Fullerton, CA). For competition assays, total ATP was varied while the ratio of [γ -³²P]ATP to cold ATP remaining constant in the reaction. Test compounds and ATP were incubated in the presence of IKK β (20 ng) at room temperature. At various time-points within the linear range of the enzyme, the reaction was terminated by spotting assay mixture (5 μ l) onto P81 phosphocellulose paper (Whatman). The filters were washed three times with phosphoric acid (0.75%) and once with acetone. Radioactivity was determined by liquid scintillation counting.

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Results

EF24 exhibits a more potent cytotoxic effect than curcumin

To evaluate the potency of EF24 in comparison to its parent compound curcumin, we carried out cell viability tests with the SRB assay on a panel of lung cancer cells (Fig. 2). Treatment of cells with both EF24 and curcumin led to a significantly decreased viability. Dose-response studies revealed that the IC_{50} of EF24 was in the range of 0.7 to 1.3 μ M for various cell lines as summarized in Table 1 (Fig. 2). On the other hand, under the same treatment condition, the IC_{50} for curcumin ranged from 15 – 20 μ M. These data indicate a more potent cytotoxic effect of EF24 over curcumin for lung cancer cells. This study was extended to include ovarian, breast, prostate, and cervical cancer cells. A similar trend was observed with EF24 exhibiting an IC_{50} at least 20 fold lower than curcumin for each cell line, respectively, with the exception of PC3 (10-fold lower) (Table 1). These results are consistent with previous reports and demonstrate that this monoketone analog of curcumin, EF24, has a much-improved cytotoxic activity over the parent compound (Adams et al., 2005; Adams et al., 2004).

High content analysis (HCA) revealed an effective EF24 action in blocking nuclear translocation of NF- κ B

In order to understand the mechanism of action of EF24 for its improved bioactivity, we examined its effect on the NF- κ B signaling pathway, which is suggested to be targeted by curcumin (Lin, 2007; Singh and Khar, 2006). The NF- κ B transcription factor mediates a critical survival mechanism in lung cancer cells. To monitor the activation status of NF- κ B, we employed a HCA approach to visualize the dynamic movement of the NF- κ B p65

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subunit between the cytoplasm and nucleus under various experimental conditions. The p65 subunit in A549 cells was detected with an immunofluorescent probe and its movement was captured through an automated fluorescence microscopy (Fig. 3). To validate the HCA assay and to establish experimental conditions for testing the EF24 effect, we initially utilized a known NF- κ B activator, TNF α , to manipulate the NF- κ B movement (Rothe et al., 1995). While the majority of the p65 subunit was detected in the cytoplasm, the addition of TNF α (10 ng/ml) resulted in complete translocation of the p65 protein into the nucleus as indicated by overlapping p65 green fluorescent signal with DAPI stained blue nuclear signal (Fig. 3a). Time course and dose-response experiments were carried out to establish an EC₅₀ for TNF α (1.5 ng/ml), which was used to set the subsequent experimental conditions (Fig. 3b). To examine whether EF24 has any effect on the NF- κ B pathway, A549 cells were pretreated with EF24 before TNF α was added to cause predominant nuclear translocation of NF- κ B. Strikingly, EF24 pretreatment retained the NF- κ B in the cytoplasm even when the amount of TNF α used completely relocated the p65 subunit to the nucleus. Curcumin showed a similar effect albeit with much higher concentration in order to achieve an effect as EF24 (Fig. 3). Quantification of captured images under various experimental conditions led to the establishment of dose-response curves, which gave rise to an IC₅₀ of 1.3 μ M and 13 μ M for EF24 and curcumin, respectively (Fig. 3c). The ten fold difference in blocking the NF- κ B translocation activity between EF24 and curcumin as revealed by the quantitative HCA is consistent with their difference in cytotoxicity against lung cancer cells. It is likely that EF24 induces cell death in part through interfering with the NF- κ B-mediated survival signaling.

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EF24 inhibits TNF α -induced I- κ B phosphorylation and subsequent degradation

When in the cytoplasm, NF- κ B is associated with I- κ B as an inactive protein complex. Nuclear translocation of NF- κ B requires dissociation of I- κ B, which is controlled by phosphorylation of I- κ B at S32 and S36 and the subsequent degradation induced by various extracellular signals including TNF α (Beg et al., 1993). It is possible that EF24's effect on nuclear translocation of NF- κ B is through its action on I- κ B phosphorylation and/or degradation. To test this model, we first established conditions to monitor the status of I- κ B phosphorylation and degradation that takes into account their transient nature. A549 cells were treated with TNF α . Phosphorylation of I- κ B was visible within three minutes of treatment and reached a high level in approximately seven minutes. It was sustained for an additional six minutes (Fig. 4a). I- κ B phosphorylation was followed by its degradation. Degradation was most notably detected after 20 minutes of treatment with TNF α while the control protein, Raf-1, remained stable during the entire course of the test. Based on these observations, cells were treated with TNF α for 10 min for detecting I- κ B phosphorylation and for 20 min for monitoring I- κ B stability in subsequent experiments.

To examine the effect of EF24 on I- κ B stability, A549 cells were pretreated with EF24 (5 μ M) or curcumin (5 or 50 μ M) followed by stimulation with TNF α . As shown in Fig. 4b, TNF α alone rapidly induced I- κ B degradation while pretreatment with EF24 (5 μ M) for only 10 min was able to effectively block the TNF α effect, showing accumulated I- κ B even in the presence of TNF α . On the other hand, curcumin required a

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much higher concentration (50 μ M) and a longer period of time (60 min) in order to achieve a level of inhibition similar to EF24. As a control, Raf-1 remained stable during these experimental conditions, suggesting a specific effect of TNF α on I- κ B.

Because phosphorylation of I- κ B proceeds its degradation, we next examined the effect of EF24 on TNF α -induced phosphorylation of I- κ B. A549 cells were pretreated with EF24 or curcumin for 30 min before the addition of TNF α (10 min). Cells were harvested for probing the status of I- κ B phosphorylation with a phosphor-specific antibody, pS32- I- κ B (Fig. 4c). EF24 inhibited TNF α -induced I- κ B phosphorylation in a dose dependent manner with an IC₅₀ about ten fold less than that of curcumin (Fig. 4d). These results suggest that EF24 antagonizes the nuclear translocation of NF- κ B through its inhibitory action on the phosphorylation of I- κ B and its subsequent degradation. In support of a selective role of EF24 in inhibiting the I- κ B kinase signaling, further experiments showed that EF24 is unable to inhibit TNF α induced activation of JNK and ERK (Fig. S1).

EF24 directly inhibits the IKK β kinase activity

Because phosphorylation of I- κ B is catalyzed by IKK in the canonical NF- κ B signaling pathway, the above results imply a more potent EF24 action over curcumin on the IKK protein complex. To examine whether EF24 and curcumin differentially target the cellular IKK complex for effective NF- κ B inhibition, we monitored the kinase activity of IKK upon compound treatment and compared the efficacy of EF24 and curcumin. A549 cells were pretreated with increasing doses of either a compound or vehicle for one hour before the addition of TNF α (10 ng/ml, 10 min). Cells were lysed and heterotrimeric

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IKK complexes were immunoprecipitated with an antibody to IKK α . The kinase activity of the immunoprecipitated IKK complex was determined by its ability to phosphorylate recombinant GST-I- κ B. While pretreatment of cells with EF24 was able to completely neutralize TNF α -activated IKK complex activity, pretreatment with curcumin required a much higher dose to achieve a similar effect (Fig. 5). Although this cell-based immunocomplex kinase assay allows the detection of permeabilized compound effect on intracellular IKK complex activity, it is unable to distinguish a direct effect of compound on a specific kinase from an indirect effect. It is possible that EF24 directly targets the IKK β activity, a well defined kinase for I- κ B. To test this notion, a reconstituted *in vitro* kinase assay was employed with recombinant IKK β and other defined components in the reaction. Increasing concentrations of EF24 or curcumin were incubated with active recombinant IKK β for 15 minutes prior to the addition of substrate, GST-I- κ B, and [γ - 32 P]ATP. Incorporation of radiolabeled 32 P to GST-I- κ B was detected by radiography and quantified by scintillation counting of excised GST-I- κ B protein bands (Fig. 6). It is striking that EF24 effectively inhibited the ability of IKK β to phosphorylate its physiological substrate I- κ B, with an estimated IC₅₀ of 1.9 μ M. Interestingly, EF24 also impaired the autokinase activity of IKK β with a similar potency (data not shown). These data strongly support a direct role of EF24 in the inhibition of IKK β . On the other hand, curcumin shows a much weaker effect on IKK β in the *in vitro* kinase assay with an apparent IC₅₀ of above 20 μ M (Fig. 6). It is clear that the structural change of curcumin to EF24 has drastically enhanced its inhibitory effect on IKK β catalytic activity. Thus, this *in vitro* kinase assay with recombinant IKK β reveals IKK β as a direct target of EF24.

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Further kinetics studies showed both a decrease in K_m and V_{max} when EF24 was present in the IKK β kinase assay, however Lineweaver-Burk plots are not parallel (Fig. S2 and data not shown). This suggests that EF24 may be acting as a mixed-type inhibitor with respect to ATP. These data further strengthen our conclusion that EF24 directly acts on and inhibits IKK β .

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Discussion

The monoketone analog of curcumin, EF24, has been shown to induce cell cycle arrest and apoptosis in a number of cancer cell lines with potency much higher than curcumin. Consistent with previous reports, EF24 exhibited IC₅₀ values 10-20 times lower than that of curcumin in a panel of non-small cell lung cancer cells with different genetic background as well as in ovarian, cervical, breast, and prostate cancer cells. However, the molecular mechanism that underlying the enhanced therapeutic efficacy remains to be defined. Here we present evidence that supports a direct action of EF24 on the NF- κ B survival signaling pathway. The amount of EF24 required for suppression of lung cancer A549 cell growth has been correlated with its ability to prevent the nuclear translocation of p65 subunit of NF- κ B, to block I- κ B phosphorylation and its subsequent degradation, and to inhibit the catalytic activity of the IKK protein complex (Fig. 7). Curcumin, on the other hand, exhibited at least ten times lower potency in all of the above assays. Importantly, in an *in vitro* reconstituted kinase assay, EF24 has been shown to inhibit the kinase activity of IKK β . Thus, direct targeting of IKK β and its mediated survival signaling may partially explain the improved therapeutic potency of EF24 over curcumin.

Although the NF- κ B signaling pathway has been implicated as one of the curcumin targets, a direct inhibitory effect of curcumin on the catalytic activity of a purified IKK protein has not been shown (Bharti et al., 2003; Deeb et al., 2004). Our research identifies EF24 as a new chemical class of IKK β inhibitors derived from the natural product curcumin. However, whether EF24 serves as a irreversible inhibitor of IKK β requires further investigation. With the recognition of a new IKK β inhibitor, EF24 may serve as a lead compound for further chemical optimization in search for more

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potent and efficacious IKK β inhibitors (Karin, 2006). It should be noted that curcumin has been reported to be an inhibitor of a number of other kinases as well, in particular protein kinase C, epidermal growth factor receptor tyrosine kinase and mTOR serine/threonine kinase (Lin, 2007). It remains to be determined whether EF24 may be active against other kinases. At this point, we have ruled out the possibility that EF24 acts as a general inhibitor of TNF α signaling by monitoring the phosphorylation status of two additional downstream targets of the TNF α pathway, JNK and ERK1/2. Neither of these kinases were found to be suppressed when EF24 was present (Figure, S1).

Why is EF24 a more effective inhibitor of IKK β than curcumin? This question cannot be answered definitively in the present work, but a possible explanation can be outlined based on structural differences and the commonly accepted mechanism of kinase inhibition. Curcumin exists in the enol form both in the solid state and in solution (Mague et al., 2004; Payton et al., 2007). In addition, in the crystal lattice, the molecule adopts an extended planar form that spans 19 Å in the longest direction (i.e. H---H). The X-ray structure of EF24, on the other hand, depicts a nonplanar molecule with a propeller arrangement of the terminal phenyl rings that incorporates a reasonable degree of flexibility and a long dimension of 15 Å (Snyder, J. P. and Sun, A. unpublished). Assuming that both curcumin and EF24 occupy at least partially the ATP binding site of IKK β kinase as the origin of their inhibition, the shorter and more flexible EF24 would appear to be more adaptable to the globular binding pocket. For comparison, we refer to roscovitine, a compound that potently blocks a number of cyclin-dependent kinases (Meijer and Raymond, 2003). Roscovitine is a nonplanar flexible molecule with a long dimension of 14 Å in a low energy conformation. The properties match those of EF24

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and suggest that a compact and potentially mobile kinase ligand is best suited to the ATP site. The mixed-type competitive inhibitor of EF24 with respect to ATP partially supports this notion. Ultimately, this hypothesis can be tested by determination of the X-ray structures of curcumin and EF24 bound to IKK β .

The NF- κ B pathway has been found to be activated in lung cancer, with various chemotherapeutic regimens adding to already elevated NF- κ B activation in tumors and representative cell lines (Mukhopadhyay et al., 1995; Wang et al., 1996). Lung cancer A549 cells may have developed certain dependency on upregulated NF- κ B pathway for sustained survival. This mechanism may involve upregulated NF- κ B controlled survival genes, such as Bcl-XL and IAP. Thus, inhibition of IKK β and its mediated NF- κ B signaling by EF24 likely leads to the suppression of survival mechanism and the induction of cells death. On the other hand, it has been reported that upregulated NF- κ B also has a transcription independent function through suppression of tumor suppressor gene PTEN expression (Vasudevan et al., 2004). Activated p65 in nucleus has been shown to sequester the transcriptional coactivators CBP/p300, leading to the suppression of PTEN expression. PTEN is a negative regulator of Akt, a major survival kinase in A549 cells (Stambolic et al., 1998). In addition to inhibiting NF- κ B-controlled expression of survival genes, EF24-mediated IKK inhibition may result in the release of p65-sequestered CBP/p300 and the subsequent activation of PTEN. In support of our model, Selvendiran et al demonstrated that EF24 induces the expression of PTEN in ovarian cancer cells, which mediates EF24-triggered G2/M cell cycle arrest and apoptosis (Selvendiran et al., 2007).

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Because the IKK protein complex plays a vital role in cellular responses to many environmental signals under both physiological and pathological conditions, IKK inhibitors are known to have significant therapeutic values (Karin, 2006). It is expected that the EF24 class of agents may not only have a role as potential cancer therapeutics, but may also have important applications in various IKK/NF- κ B dysregulated autoimmune and inflammatory diseases, including rheumatoid arthritis.

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Acknowledgments

We thank members of the Fu, Khuri, Sun and Liotta laboratories for assistance and enlightening discussions.

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Reference

Adams BK, Cai J, Armstrong J, Herold M, Lu YJ, Sun A, Snyder JP, Liotta DC, Jones DP and Shoji M (2005) EF24, a novel synthetic curcumin analog, induces apoptosis in cancer cells via a redox-dependent mechanism. *Anticancer Drugs* **16**(3):263-275.

Adams BK, Ferstl EM, Davis MC, Herold M, Kurtkaya S, Camalier RF, Hollingshead MG, Kaur G, Sausville EA, Rickles FR, Snyder JP, Liotta DC and Shoji M (2004) Synthesis and biological evaluation of novel curcumin analogs as anti-cancer and anti-angiogenesis agents. *Bioorg Med Chem* **12**(14):3871-3883.

Aggarwal S, Ichikawa H, Takada Y, Sandur SK, Shishodia S and Aggarwal BB (2005) Curcumin (Diferuloylmethane) Downregulates Expression of Cell Proliferation, Antiapoptotic and Metastatic Gene Products Through Suppression of I{kappa}B{alpha} Kinase and AKT Activation. *Mol Pharmacol*.

Anand P, Kunnumakkara AB, Newman RA and Aggarwal BB (2007) Bioavailability of curcumin: problems and promises. *Mol Pharm* **4**(6):807-818.

Beg AA, Finco TS, Nantermet PV and Baldwin AS, Jr. (1993) Tumor necrosis factor and interleukin-1 lead to phosphorylation and loss of I kappa B alpha: a mechanism for NF-kappa B activation. *Mol Cell Biol* **13**(6):3301-3310.

Bharti AC, Donato N, Singh S and Aggarwal BB (2003) Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. *Blood* **101**(3):1053-1062.

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Cheng AL, Hsu CH, Lin JK, Hsu MM, Ho YF, Shen TS, Ko JY, Lin JT, Lin BR, Ming-Shiang W, Yu HS, Jee SH, Chen GS, Chen TM, Chen CA, Lai MK, Pu YS, Pan MH, Wang YJ, Tsai CC and Hsieh CY (2001) Phase I clinical trial of curcumin, a chemopreventive agent, in patients with high-risk or pre-malignant lesions. *Anticancer Res* **21**(4B):2895-2900.

Commandeur JN and Vermeulen NP (1996) Cytotoxicity and cytoprotective activities of natural compounds. The case of curcumin. *Xenobiotica* **26**(7):667-680.

Deeb D, Jiang H, Gao X, Hafner MS, Wong H, Divine G, Chapman RA, Dulchavsky SA and Gautam SC (2004) Curcumin sensitizes prostate cancer cells to tumor necrosis factor-related apoptosis-inducing ligand/Apo2L by inhibiting nuclear factor-kappaB through suppression of IkappaBalpha phosphorylation. *Mol Cancer Ther* **3**(7):803-812.

Dhillon N, Wolff RA, Abbruzzese JL, Hong DS, Camacho LH, Li L, Braithe FS and R K (2006) Phase II clinical trial of curcumin in patients with advanced pancreatic cancer. *Journal of Clinical Oncology, 2006 ASCO Annual Meeting Proceedings* **24**(18S):14151.

Karin M (2006) NF-kappaB and cancer: Mechanisms and targets. *Mol Carcinog* **45**(6):355-361.

Levi MS, Borne RF and Williamson JS (2001) A review of cancer chemopreventive agents. *Curr Med Chem* **8**(11):1349-1362.

Lin JK (2007) Molecular targets of curcumin. *Adv Exp Med Biol* **595**:227-243.
Mague JT, Alworth WL and Payton FL (2004) Curcumin and derivatives. *Acta Crystallogr C* **60**(Pt 8):o608-610.

MOL #46201

Meijer L and Raymond E (2003) Roscovitine and other purines as kinase inhibitors. From starfish oocytes to clinical trials. *Acc Chem Res* **36**(6):417-425.

Mercurio F, Zhu H, Murray BW, Shevchenko A, Bennett BL, Li J, Young DB, Barbosa M, Mann M, Manning A and Rao A (1997) IKK-1 and IKK-2: cytokine-activated IkappaB kinases essential for NF-kappaB activation. *Science* **278**(5339):860-866.

Mukhopadhyay T, Roth JA and Maxwell SA (1995) Altered expression of the p50 subunit of the NF-kappa B transcription factor complex in non-small cell lung carcinoma. *Oncogene* **11**(5):999-1003.

Nakanishi C and Toi M (2005) Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. *Nat Rev Cancer* **5**(4):297-309.

Osawa T SY, Inayoshi M, Kawakashi S (1995) Antioxidative activity of tetrahydrocurcuminoids. *Biosci Biotechnol Biochem* **59**:1609-1612.

Payton F, Sandusky P and Alworth WL (2007) NMR study of the solution structure of curcumin. *J Nat Prod* **70**(2):143-146.

Rayet B and Gelinas C (1999) Aberrant rel/nfkb genes and activity in human cancer. *Oncogene* **18**(49):6938-6947.

Richmond A (2002) Nf-kappa B, chemokine gene transcription and tumour growth. *Nat Rev Immunol* **2**(9):664-674.

Rothe M, Sarma V, Dixit VM and Goeddel DV (1995) TRAF2-mediated activation of NF-kappa B by TNF receptor 2 and CD40. *Science* **269**(5229):1424-1427.

Rubinstein LV, Shoemaker RH, Paull KD, Simon RM, Tosini S, Skehan P, Scudiero DA, Monks A and Boyd MR (1990) Comparison of in vitro anticancer-drug-

MOL #46201

screening data generated with a tetrazolium assay versus a protein assay against a diverse panel of human tumor cell lines. *J Natl Cancer Inst* **82**(13):1113-1118.

Satoskar RR, Shah SJ and Shenoy SG (1986) Evaluation of anti-inflammatory property of curcumin (diferuloyl methane) in patients with postoperative inflammation. *Int J Clin Pharmacol Ther Toxicol* **24**(12):651-654.

Selvendiran K, Tong L, Vishwanath S, Bratasz A, Trigg NJ, Kutala VK, Hideg K and Kuppusamy P (2007) EF24 induces G2/M arrest and apoptosis in cisplatin-resistant human ovarian cancer cells by increasing PTEN expression. *J Biol Chem* **282**(39):28609-28618.

Shoba G, Joy D, Joseph T, Majeed M, Rajendran R and Srinivas PS (1998) Influence of piperine on the pharmacokinetics of curcumin in animals and human volunteers. *Planta Med* **64**(4):353-356.

Singh S and Khar A (2006) Biological effects of curcumin and its role in cancer chemoprevention and therapy. *Anticancer Agents Med Chem* **6**(3):259-270.

Skehan P, Storeng R, Scudiero D, Monks A, McMahon J, Vistica D, Warren JT, Bokesch H, Kenney S and Boyd MR (1990) New colorimetric cytotoxicity assay for anticancer-drug screening. *J Natl Cancer Inst* **82**(13):1107-1112.

Stambolic V, Suzuki A, de la Pompa JL, Brothers GM, Mirtsos C, Sasaki T, Ruland J, Penninger JM, Siderovski DP and Mak TW (1998) Negative regulation of PKB/Akt-dependent cell survival by the tumor suppressor PTEN. *Cell* **95**(1):29-39.

Vasudevan KM, Gurumurthy S and Rangnekar VM (2004) Suppression of PTEN expression by NF-kappa B prevents apoptosis. *Mol Cell Biol* **24**(3):1007-1021.

MOL #46201

Wang CY, Mayo MW and Baldwin AS, Jr. (1996) TNF- and cancer therapy-induced apoptosis: potentiation by inhibition of NF-kappaB. *Science* **274**(5288):784-787.

Zhang L, Chen J and Fu H (1999) Suppression of apoptosis signal-regulating kinase 1-induced cell death by 14-3-3 proteins. *Proc Natl Acad Sci U S A* **96**(15):8511-8515.

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Footnotes

This work was supported in part by grants from NIH (R01 GM53165 to HF., P01 CA116676 to FRK, HF, and SS) and Emory URC. FRK, SS, and HF are GCC Distinguished Scholars. HF is a GRA Distinguished Investigator. YD is Emory Drug Development and Pharmacogenomics Academy Fellow and recipient of Emory Head and Neck cancer SPORE career development award (P50 CA128613). SLT is a recipient of AAAS/Packard Fellowship and Emory FACE program fellow.

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Figure legends

Fig. 1. Structures of curcumin and its analog EF24

Fig. 2. EF24 shows more potent cytotoxic effect than curcumin on cancer cells. Cells were grown in 96 well plates and were treated with EF24 or curcumin as indicated for 48 hr. Cell viability was assessed by the SRB method and expressed as % control (DMSO). Results with a panel of lung cancer cells are shown in panel *a*: A549, H358 (lung adenocarcinoma); H460 (large cell carcinoma); H157 (squamous cell carcinoma); Calu-1 (lung epidermoid carcinoma). Panel *b* represents results with breast (MDA-MB231), cervical (HeLa), prostate (PC3), and ovarian (1A9) cancer cell lines.

Fig. 3. EF24 impairs TNF α induced NF- κ B nuclear translocation. A549 cells were grown in 96-well plates and treated with TNF α or control (DMSO) for 30 min before sample processing for the detection of NF- κ B as described in Materials and Methods (Upper two rows of *a*). The induction of NF- κ B nuclear translocation by TNF α was quantified as shown in *b* with an apparent EC₅₀ of 1.5 ng/ml. The effect of EF24 or curcumin treatment (30 min) on TNF α induced nuclear translocation of NF- κ B was shown in the lower two rows in (*a*) and quantified as shown in (*c*).

Fig. 4. EF24 blocks TNF α -induced I- κ B degradation and phosphorylation. (*a*) A549 cells were treated with TNF α (10 ng/ml). Whole cell lysates were prepared at indicated times and analyzed for the phosphorylation state of I- κ B with anti-pS32 antibody via

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Western blotting (Upper panel). Then, antibodies on the membrane were stripped and the membrane re-probed for total I- κ B with antiserum against I- κ B (Middle panel). Raf-1 was used as a control (Lower panel). (b) A549 cells were pretreated for various times with EF24 (5 μ M), curcumin (5 μ M or 50 μ M) prior to the addition of TNF α . Cells were cultured in the presence of TNF α (10 ng/ml) for an additional 20 minutes, lysed and analyzed for total I- κ B levels by Western blot (*Upper panels*). Raf-1 was used as a control (*Lower panels*). (c) A549 cells were pretreated with compounds (EF24 or curcumin) as indicated for 30 minutes. TNF α was added to induce I- κ B phosphorylation. Cell lysates were prepared after 7 min of treatment and used for probing pS32-I- κ B followed by probing total I- κ B with Western blots. Intensity of cross-reacting material bands on Western blots was estimated with a Kodak imaging system. The phosphorylation levels of I- κ B at S32 are normalized to total I κ B and expressed relative to control sample with DMSO treatment (d).

Fig. 5. EF24 inhibits the kinase activity of endogenous IKK complex A549 cells were pretreated with EF24 or curcumin at indicated doses for one hr followed by TNF α (10 ng/ml) stimulation for 10 min. Cell lysates were prepared for immunoprecipitation of the IKK complex with an antibody raised against IKK α . The isolated IKK complex was used in an in vitro kinase assay with GST-I- κ B as a substrate. Samples were resolved on a SDS-PAGE and stained for total I- κ B protein with Coomassie Blue (Middle panel, **a**). The gel was dried to reveal radiolabeled I- κ B with PhosphoImager (upper panel, **a**). The amount of IKK β in the immuno-complex used in each reaction was revealed by Western

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blot. As a negative control, the inactive IKK complex without TNF α treatment is shown in left lane. Quantified result from *a* is plotted relative to vehicle control (*b*).

Fig. 6. EF24 inhibits IKK β kinase activity in a reconstituted system. Recombinant IKK β was incubated with increasing concentrations of EF24 (*a*) or curcumin (*b*) for 15 min. Addition of Mg/[γ -³²P] cocktail with purified GST-I- κ B started the reactions, which were continued for 15 min at 30°C. Proteins were separated by SDS-PAGE and processed for radiolabeled GST-I- κ B (*upper panels*), total GST-I- κ B (*middle panels*), and IKK β (*lower panels*) as described in legend to Fig. 5. Controls include reactions without IKK β (lane 1) or without I- κ B (lane 2).

Fig. 7. Working model for the EF24 mode of action. EF24 efficiently blocks the cytokine-induced nuclear translocation of NF- κ B, which is important for cell survival and inflammation signaling. This EF24 activity is at least in part due to its inhibitory effect on I- κ B degradation and possibly through a direct inhibitory effect on the kinase activity of IKK β .

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Table 1: Cytotoxicity of curcumin and EF24 against a panel of cancer cell lines

Cell Line	IC ₅₀ (μM)	
	<i>EF24</i>	<i>Curcumin</i>
1A9 (ovarian)	0.44 ± 0.09	9.85 ± 1.20
HeLa (cervical)	1.23 ± 0.88	26.9 ± 7.0
A549 (lung)	1.31 ± 0.01	19.75 ± 4.03
H358 (lung)	0.66 ± 0.09	14.9 ± 3.1
H460 (lung)	0.79	16.9
H157 (lung)	0.77 ± 0.06	19.55 ± 6.71
Calu-1 (lung)	1.03 ± 0.14	18.1 ± 2.46
MDA-MB231 (breast)	1.03 ± 0.40	26.55 ± 3.18
PC3 (prostate)	2.07	23.7

IC₅₀ values derived from Figure 1 are given as the average of at least two separate experiments. IC₅₀: concentration at which cell growth is inhibited by 50%.

Figure 1

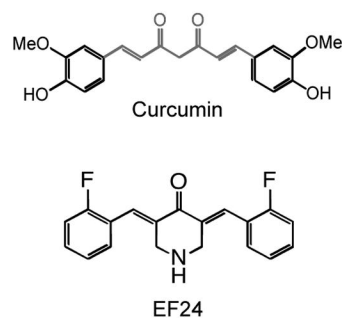


Figure 2

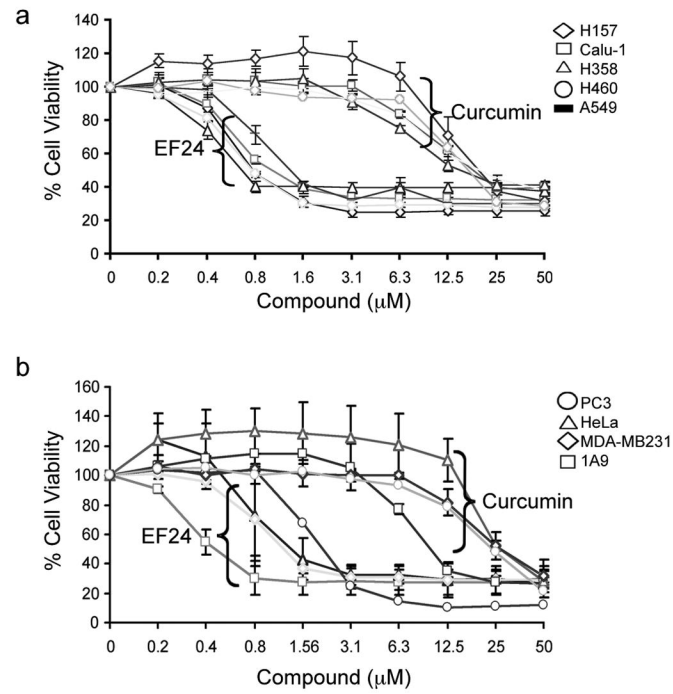


Figure 3

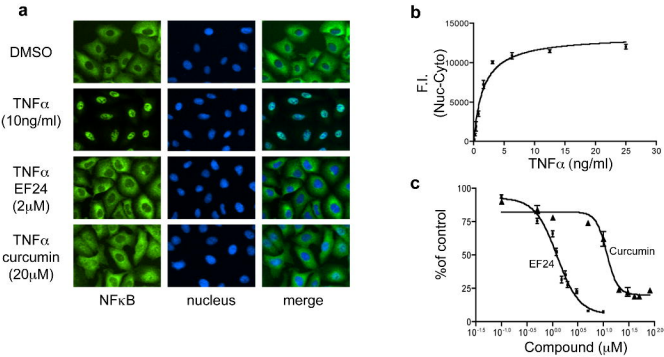


Figure 4

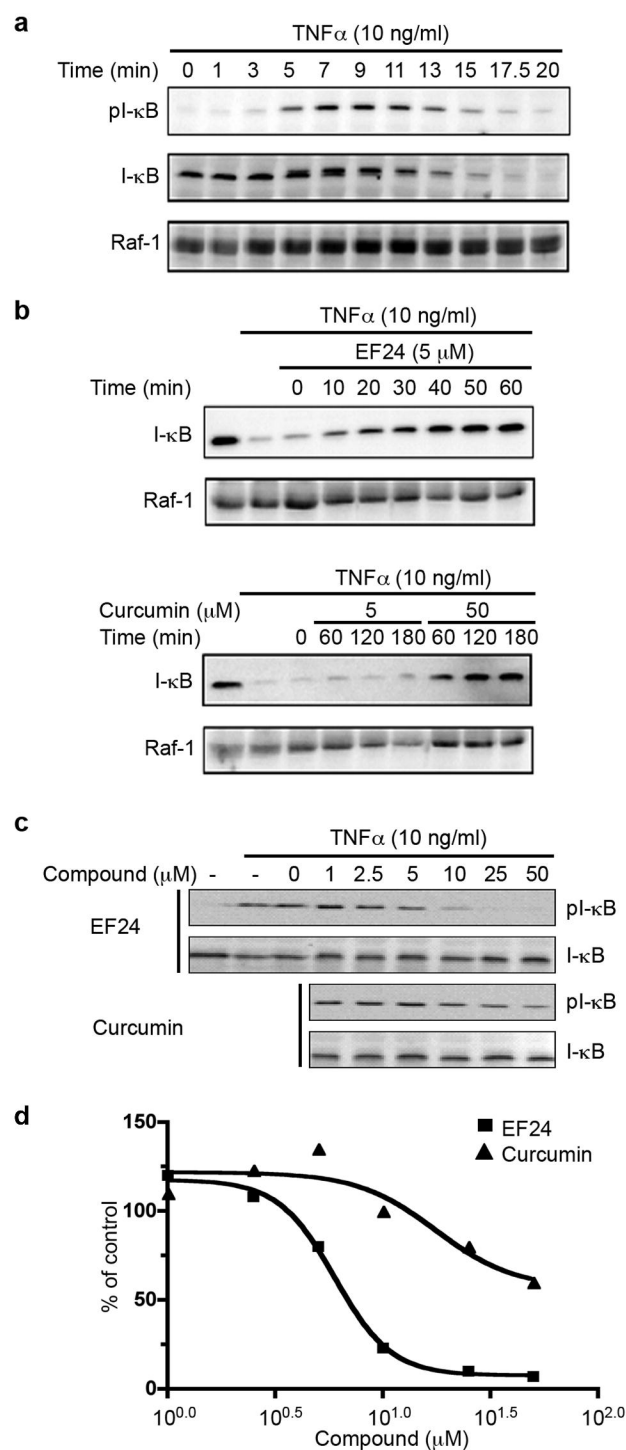


Figure 5

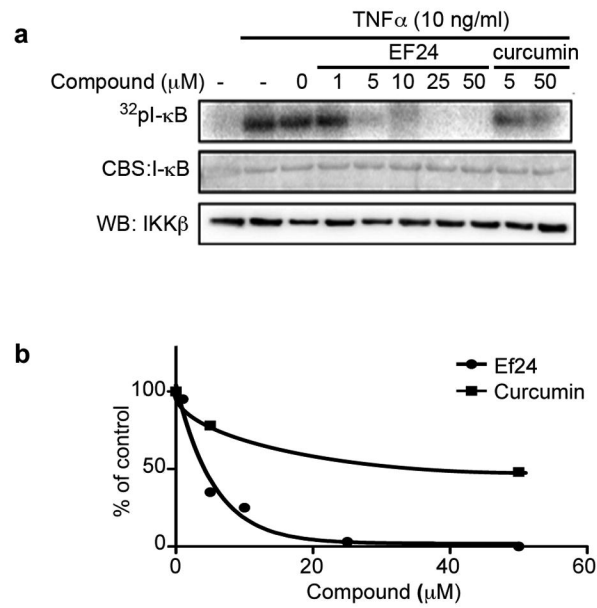
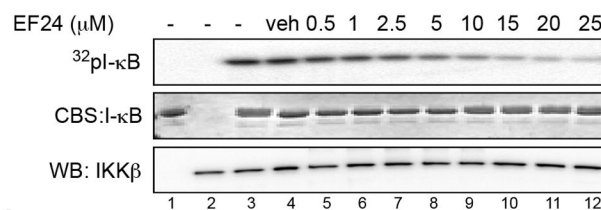
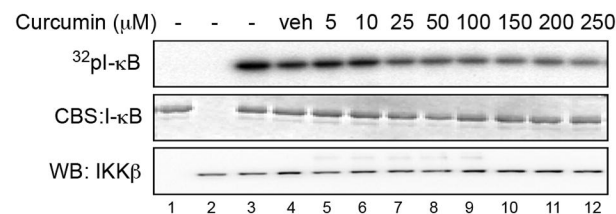


Figure 6

a



b



c

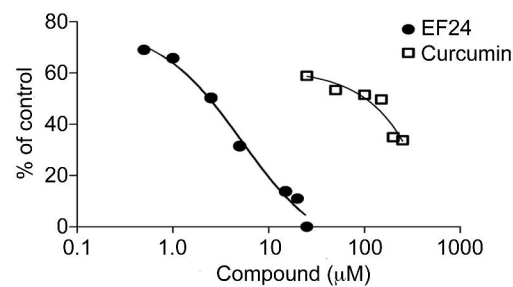


Figure 7

