Molecular Pharmacology Fast Forward. Published on June 24, 2008 as doi:10.1124/mol.108.046201
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MOL #46201

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

Andrea L Kasinski, Yuhong Du, Shala L Thomas, Jing Zhao, Shi-Yong Sun, Fadlo R. Khuri, Cun-Yu Wang, Mamoru Shoji, Aiming Sun, James P. Snyder, Dennis Liotta, and Haian Fu

Graduate Programs in Genetics and Molecular Biology (A.L.K), and in Molecular and Systems Pharmacology (S.L.T), Departments of Pharmacology (Y.D., J.Z., H.F.), Hematology & Oncology (S.Y.S, F.R.K, M.S.), and Chemistry (A.S., J.P.S., D.L.), and Emory Chemical Biology Discovery Center (Y.D, A.S., J.P.S., D.L., H.F.), Emory University, 1510 Clifton Road, Atlanta, GA 30322; Division of Oral Biology and Medicine (C.Y.W.), UCLA Dentistry, Los Angeles, CA 90095

Molecular Pharmacology Fast Forward. Published on June 24, 2008 as DOI: 10.1124/mol.108.046201
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a. Running title: Mechanism of action by EF24

b. Corresponding author:

Dr. Haian Fu

Department of Pharmacology

Emory University School of Medicine

1510 Clifton Road

Atlanta, GA 30322

E-mail: hfu@emory.edu

Telephone: 404-727-1335, Fax: 404-727-0365.

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-κB, inhibitor of kappa B; IKK, inhibitor of kappa B kinase; NF-κB, 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-κB) 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-κB 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-κB signaling pathway through direct action on the I-κB 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-κB with an IC_{50} of 1.3 μM compared with curcumin with an IC_{50} of 13 μM, (iii) EF24 effectively inhibits TNFα-induced I-κB 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 TNFα-induced NF-κB signaling by EF24 extends the therapeutic application of EF24 to other NF-κB-dependent diseases, including inflammatory diseases such as rheumatoid arthritis.

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Introduction

Curcumin, isolated from the rhizomes of the plant *Curcma 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-kB transcription factor.

NF-κB is maintained in an inactive state in the cytoplasm by the inhibitor of kappa B (Iκ-B), which masks the nuclear localization signal of NF-κB. Phosphorylation of Iκ-B by the inhibitor of kappa B kinase (IKK), via the canonical NF-κB pathway, results in subsequent ubiquitination of Iκ-B and proteasomal degradation. It is this degradation of Iκ-B that liberates NF-κB, allowing its localization to the nucleus and the transcriptional activation of its target genes. On the other hand, aberrant activation of NF-κB 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-κB effect is in part due to the upregulation of NF-κB -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-κB, resulting in reduced therapeutic efficacy (Nakanishi and Toi, 2005). Thus, agents that effectively impair the NF-κB 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-κB 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-flurobenzylidene)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. TNFα (Sigma-Aldrich Chemicals, St Louis, MO) was resuspended in water to a final concentration of 10 μg/ml. Glutathione S-transferase-I-κBα (1-54) was purified from expression plasmid as previously described (Mercurio et al., 1997). Antibodies against pS32-I-κBα and I-κBα were purchased from Cell Signaling (Beverly, MA). Antibodies against IKKα and IKKβ 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% CO_2. 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 Na_3VO_4, 5 mM $Na_4P_2O_7$, 10 μg/ml aprotonin, 10 μg /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 IC_{50} 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 IC_{50} 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-kB 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-κB translocation analysis. The nucleus was defined by Hoechst 33342 staining. The levels of NF-κB translocation were calculated and expressed as the difference between average fluorescence intensity in nucleus and in cytoplasm (Nuc – Cyt). After stimulating with TNFα, the inhibitory effect of test compounds on TNFα induced NF-κB translocation was expressed as % of fluorescence intensity difference (Nuc-Cyt) in the control wells (TNFα only) after subtracting background (no TNFα 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 Na_3VO_4, 5 mM $Na_4P_2O_7$, 10 µg/ml aprotinin, 10 µg/ml leupeptin, 1 mM PMSF). Equal volumes of cell lysate were subject to electrophoresis on SDS-PAGE (12.5%). Proteins were then electrotransfered 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 TNFα 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-IKKα 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 $MgCl_2$; 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-^{32}P]ATP (20 μCi with 10 μM unlabeled ATP), and glutathione S-transferase (GST)-I-κBα (residues 1-54; 5 μg) in a total volume of 25 μl 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-IKKβ antibody while the bottom portion was stained with Coomassie Blue dye (0.05%). Radiolabeled phosphate incorporation into GST- I-κBα was assessed by the PhosphoImager and quantified with the ImageQuant software (Molecular Dynamics, Sunnyvale, CA). (***b***) *In vitro* recombinant IKKβ assay. Activated recombinant IKKβ in MOPS buffer (8 mM MOPS-NaOH, pH 7.0, 200 μM EDTA, 15 mM $MgCl_2$) (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 IKKβ for 15 minutes at room temperature. The addition of Mg-ATP cocktail (15 mM $MgCl_2$, 100 μM 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-κBα (5 μg) and [γ-^{32}P]ATP (0.5 μCi) in a final volume of 25 μl 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-κBa 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-^{32}P]ATP to cold ATP remaining constant in the reaction. Test compounds and ATP were incubated in the presence of IKKβ (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 μl) 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 μM 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 μM. 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-κB

In order to understand the mechanism of action of EF24 for its improved bioactivity, we examined its effect on the NF-κB signaling pathway, which is suggested to be targeted by curcumin (Lin, 2007; Singh and Khar, 2006). The NF-κB transcription factor mediates a critical survival mechanism in lung cancer cells. To monitor the activation status of NF-κB, we employed a HCA approach to visualize the dynamic movement of the NF-κB 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-κB activator, TNFα, to manipulate the NF-κB movement (Rothe et al., 1995). While the majority of the p65 subunit was detected in the cytoplasm, the addition of TNFα (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 EC_{50} for TNFα (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-κB pathway, A549 cells were pretreated with EF24 before TNFα was added to cause predominant nuclear translocation of NF-κB. Strikingly, EF24 pretreatment retained the NF-κB in the cytoplasm even when the amount of TNFα 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 IC_{50} of 1.3 μM and 13 μM for EF24 and curcumin, respectively (Fig. 3c). The ten fold difference in blocking the NF-κB 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-κB-mediated survival signaling.

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EF24 inhibits TNFα-induced I-κB phosphorylation and subsequent degradation

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

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

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much higher concentration (50 μM) 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 TNFα on I-κB.

Because phosphorylation of I-κB proceeds its degradation, we next examined the effect of EF24 on TNFα-induced phosphorylation of I-κB. A549 cells were pretreated with EF24 or curcumin for 30 min before the addition of TNFα (10 min). Cells were harvested for probing the status of I-κB phosphorylation with a phosphor-specific antibody, pS32- I-κB (Fig. 4c). EF24 inhibited TNFα-induced I-κB phosphorylation in a dose dependent manner with an IC_{50} about ten fold less than that of curcumin (Fig. 4d). These results suggest that EF24 antagonizes the nuclear translocation of NF-κB through its inhibitory action on the phosphorylation of I-κB and its subsequent degradation. In support of a selective role of EF24 in inhibiting the I-κB kinase signaling, further experiments showed that EF24 is unable to inhibit TNFα induced activation of JNK and ERK (Fig. S1).

EF24 directly inhibits the IKKβ kinase activity

Because phosphorylation of I-κB is catalyzed by IKK in the canonical NF-κB 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-κB 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 TNFα (10 ng/ml, 10 min). Cells were lysed and heterotrimeric

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IKK complexes were immunopreciritated with an antibody to IKKα. The kinase activity of the immunoprecipitated IKK complex was determined by its ability to phosphorylate recombinant GST-I-κB. While pretreatment of cells with EF24 was able to completely neutralize TNFα-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 IKKβ activity, a well defined kinase for I-κB. To test this notion, a reconstituted *in vitro* kinase assay was employed with recombinant IKKβ and other defined components in the reaction. Increasing concentrations of EF24 or curcumin were incubated with active recombinant IKKβ for 15 minutes prior to the addition of substrate, GST-I-κB, and [γ-^{32}P]ATP. Incorporation of radiolabeled ^{32}P to GST-I-κB was detected by radiography and quantified by scintillation counting of excised GST-I-κB protein bands (Fig. 6). It is striking that EF24 effectively inhibited the ability of IKKβ to phosphorylate its physiological substrate I-κB, with an estimated IC_{50} of 1.9 μM. Interestingly, EF24 also impaired the autokinase activity of IKKβ with a similar potency (data not shown). These data strongly support a direct role of EF24 in the inhibition of IKKβ. On the other hand, curcumin shows a much weaker effect on IKKβ in the *in vitro* kinase assay with an apparent IC_{50} of above 20 μM (Fig. 6). It is clear that the structural change of curcumin to EF24 has drastically enhanced its inhibitory effect on IKKβ catalytic activity. Thus, this *in vitro* kinase assay with recombinant IKKβ reveals IKKβ 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 IKKβ kinase assay, however Linweaver-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 IKKβ.

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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 IC_{50} 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-κB 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-κB, to block I-κB 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 IKKβ. Thus, direct targeting of IKKβ and its mediated survival signaling may partially explain the improved therapeutic potency of EF24 over curcumin.

Although the NF-κB 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 IKKβ inhibitors derived from the natural product curcumin. However, whether EF24 serves as a irreversible inhibitor of IKKβ requires further investigation. With the recognition of a new IKKβ inhibitor, EF24 may serve as a lead compound for further chemical optimization in search for more

potent and efficacious IKKβ 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 TNFα signaling by monitoring the phosphorylation status of two additional downstream targets of the TNFα 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 IKKβ 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 IKKβ 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 IKKβ.

The NF-κB pathway has been found to be activated in lung cancer, with various chemotheraputic regiments adding to already elevated NF-κB 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-κB pathway for sustained survival. This mechanism may involve upregulated NF-κB controlled survival genes, such as Bcl-XL and IAP. Thus, inhibition of IKKβ and its mediated NF-κB 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-κB 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-κB-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).

Molecular Pharmacology Fast Forward. Published on June 24, 2008 as DOI: 10.1124/mol.108.046201
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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-κB 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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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 TNFα induced NF-κB nuclear translocation. A549 cells were grown in 96-well plates and treated with TNFα or control (DMSO) for 30 min before sample processing for the detection of NF-κB as described in Materials and Methods (Upper two rows of ***a***). The induction of NF-κB nuclear translocation by TNFα was quantified as shown in ***b*** with an apparent EC_{50} of 1.5 ng/ml. The effect of EF24 or cucumin treatment (30 min) on TNFα induced nuclear translocation of NF-κB was shown in the lower two rows in (***a***) and quantified as shown in (***c***).

Fig. 4. EF24 blocks TNFα-induced I-κB degradation and phosphorylation. (***a***) A549 cells were treated with TNFα (10 ng/ml). Whole cell lysates were prepared at indicated times and analyzed for the phosphorylation state of I-κB 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-κB with antiserum against I-κB (Middle panel). Raf-1 was used as a control (Lower panel). (***b***) A549 cells were pretreated for various times with EF24 (5 μM), curcumin (5 μM or 50 μM) prior to the addition of TNFα. Cells were cultured in the presence of TNFα (10 ng/ml) for an additional 20 minutes, lysed and analyzed for total I-κB 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. TNFα was added to induce I-κB phosphorylation. Cell lysates were prepared after 7 min of treatment and used for probing pS32-I-κB followed by probing total I-κB with Western blots. Intensity of cross-reacting material bands on Western blots was estimated with a Kodak imaging system. The phosphorylation levels of I-κB at S32 are normalized to total IκB 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 TNFα (10 ng/ml) stimulation for 10 min. Cell lysates were prepared for immunoprecipitation of the IKK complex with an antibody raised against IKKα. The isolated IKK complex was used in an in vitro kinase assay with GST-I-κB as a substrate. Samples were resolved on a SDS-PAGE and stained for total I-κB protein with Coomassie Blue (Middle panel, ***a***). The gel was dried to reveal radiolabeled I-κB with PhosphoImager (upper panel, ***a***). The amount of IKKβ 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 TNFα treatment is shown in left lane. Quantified result from ***a*** is plotted relative to vehicle control (***b***).

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

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

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

Cell Line	IC_{50} (μM)	
	EF24	***Curcumin***
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

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

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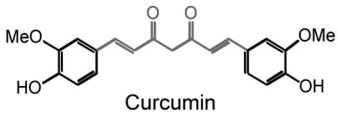
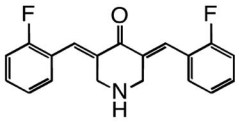
Figure 1



Curcumin



EF24

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Figure 2

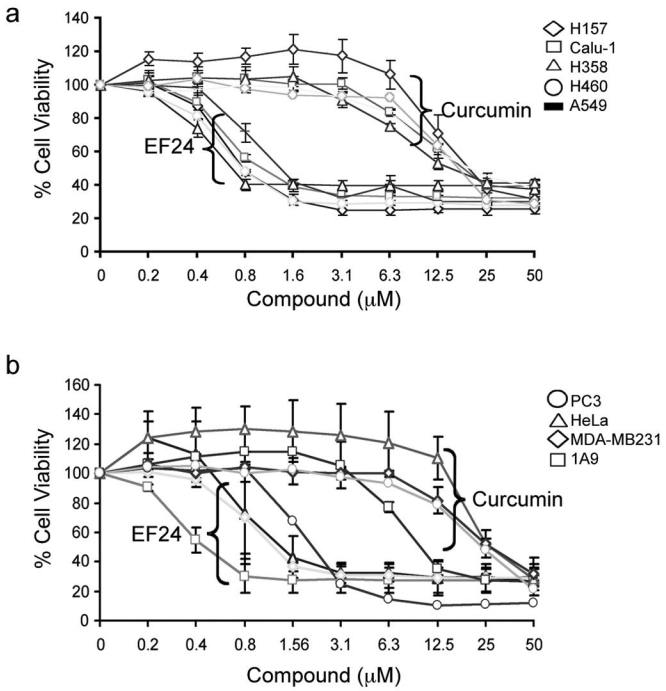

Figure 3

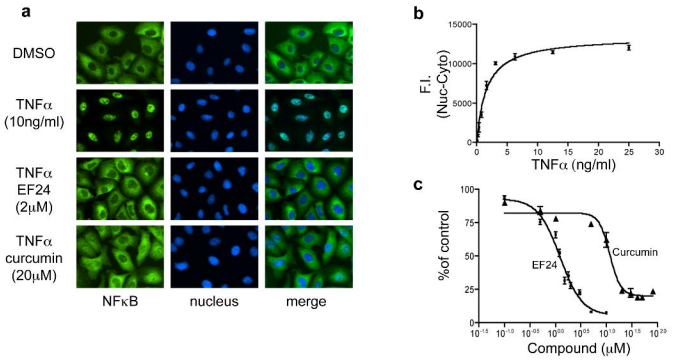
a
DMSO
TNFα (10ng/ml)
TNFα EF24 (2μM)
TNFα curcumin (20μM)
NFκB
nucleus
merge
b
F.I. (Nuc-Cyto)
15000
10000
5000
0
0
5
10
15
20
25
30
TNFα (ng/ml)
c
%of control
100
75
50
25
0
EF24
Curcumin
Compound (μM)

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Figure 4

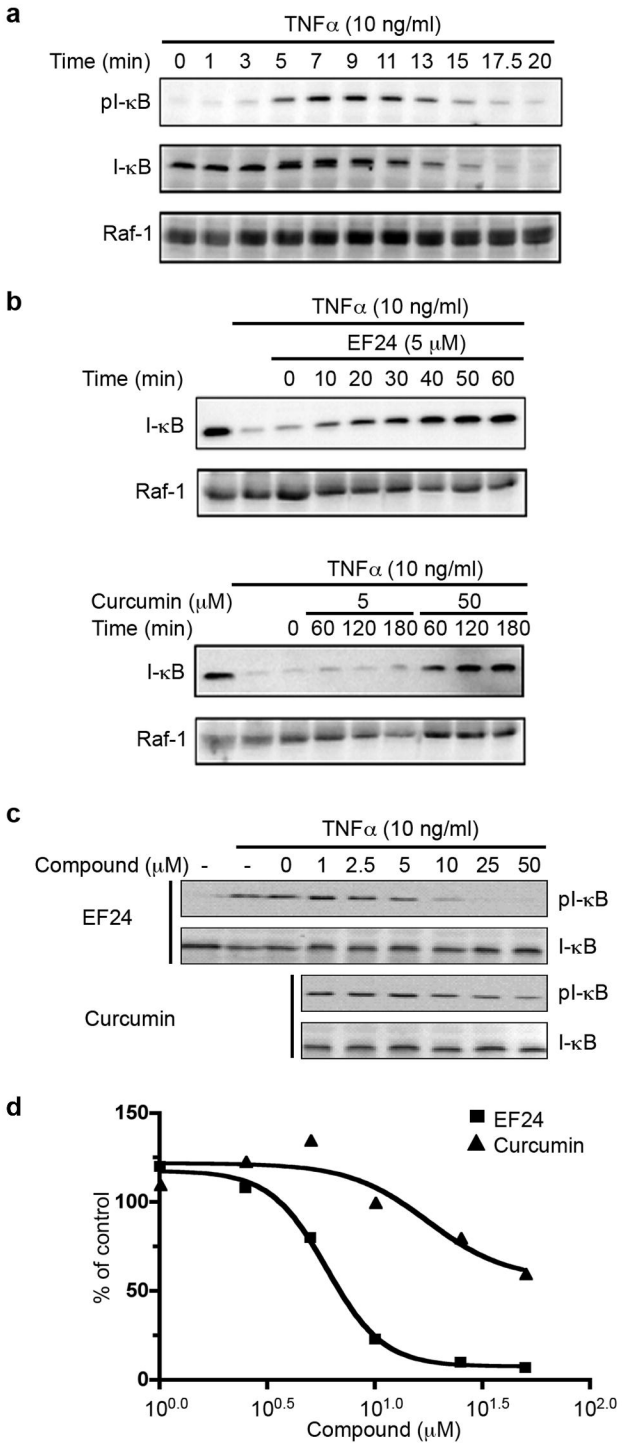

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Figure 5

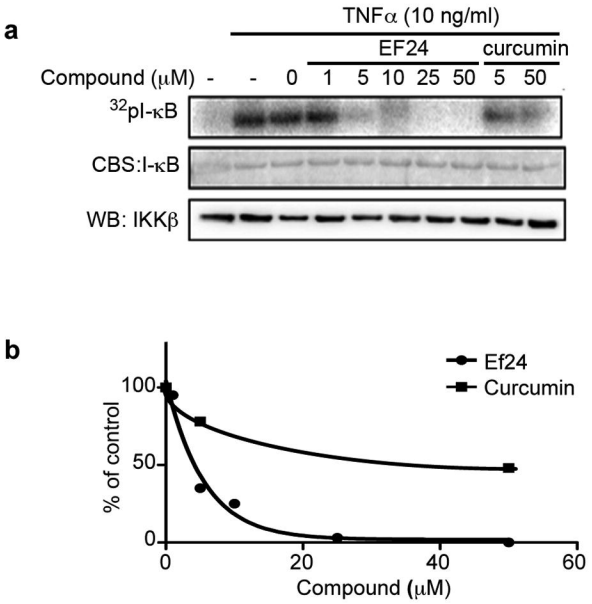

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Figure 6

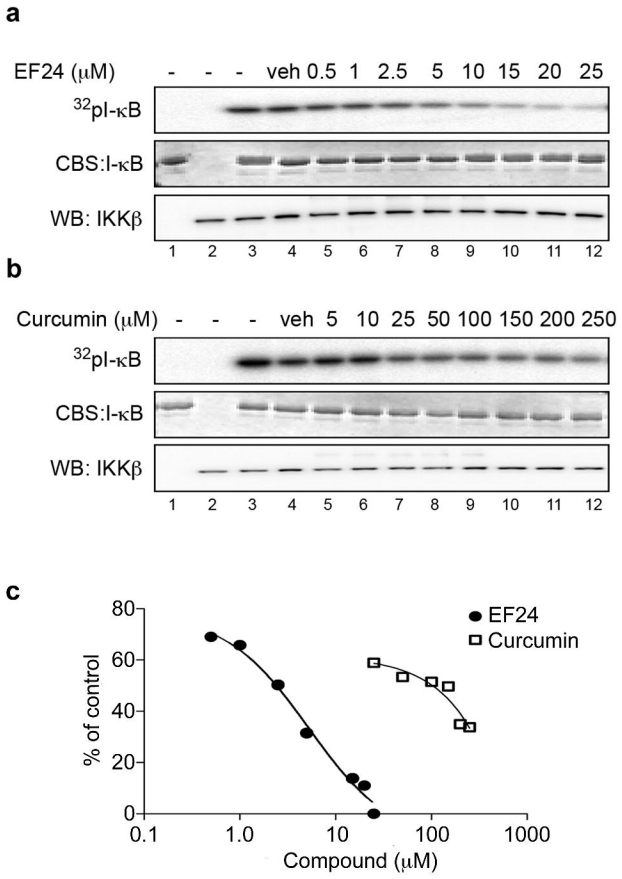

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Figure 7