3-Formylchromone Interacts with Cysteine 38 in p65 Protein and with Cysteine 179 in IκBα Kinase, Leading to Down-regulation of Nuclear Factor-κB (NF-κB)-regulated Gene Products and Sensitization of Tumor Cells*

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3-Formylchromone (3-FC) has been associated with antitumor potential through a mechanism yet to be elucidated. Because of the critical role of NF-κB in tumorigenesis, we investigated the effect of this agent on the NF-κB activation pathway. Whether activated by inflammatory agents (such as TNF-α and endotoxin) or tumor promoters (such as phorbol ester and okadaic acid), 3-FC suppressed NF-κB activation. It also inhibited constitutive NF-κB expression correlated with sequential phosphorylation, IκBα phosphorylation, p65 nuclear translocation, and reporter gene expression of anti-apoptotic gene products, and potentiation of apoptosis in tumor cells.

We hypothesized that 3-FC modulates the NF-κB pathway in inflammation and tumorigenesis, and 3-FC mediates this inhibition. Under normal conditions, NF-κB is nuclear, but inhibition of proteasome-mediated degradation of IκBα leads to NF-κB activation.

This article has been retracted by the publisher. An investigation at MD Anderson determined that images were used to represent different experimental conditions. Specifically, the IKKα immunoblot from Fig. 2G was flipped horizontally and reused as the IKKβ immunoblot in Fig. 2H, and the IKKβ immunoblot in Fig. 2G was flipped horizontally and reused as the IKKα immunoblot in Fig. 2H.
cific proteins in the pathway, leading to suppression of NF-κB-regulated gene products and chemosensitization of tumor cells.

MATERIALS AND METHODS

Reagents—Bacterially derived human recombinant TNF-α, purified to homogeneity with a specific activity of 5 × 10⁷ units/mg, was provided by Genentech (South San Francisco, CA). Penicillin, streptomycin, RPMI 1640 medium, Iscove’s modified Dulbecco’s medium, DMEM, and FBS were obtained from Invitrogen. 3-FC (see Fig. 1A), phorbol 12-myristate 13-acetate, LPS, okadaic acid, and antibodies against FLAG and β-actin were obtained from Sigma. Phospho-specific anti-IkBα (Ser32/36) and anti-p65 (Ser517) antibodies were purchased from Cell Signaling (Danvers, MA). An antibody against p65 for use in immunocytochemistry was obtained from Abcam (Cambridge, MA). Anti-VEGF antibody was purchased from NeoMarkers (Fremont, CA). Anti-IkBα, anti-IKKα, and anti-IKKβ antibodies were obtained from Imgenex Corp. (San Diego, CA). Plasmids (pcDNA3.1 and pcDNA expression vectors) p65 and mouse p65(C38S)) were kindly provided by Dr. T. D. dong Wu (University of North Carolina, Chapel Hill, NC).

Cell Lines—The cell lines HL-60 (human promyelocytic leukemia), Jurkat (human T-cell leukemia), A293 (human embryonic kidney), K562 (human chronic myelogenous leukemia), U266 and RPMI-8226 (human multiple myeloma), and HCT116, Caco-2, and HT-29 (colon cancer) were obtained from American Type Culture Collection (Manassas, VA). The human cell line KMB-5 (chronic myeloid leukemia) was provided by Dr. Nicholas J. Donato (University of Michigan Comprehensive Cancer Center, Ann Arbor, MI). KMB-5 cells were cultured in Iscove’s modified Dulbecco’s medium with 15% FBS; K562, HL-60, Jurkat, RPMI-8226, and U266 cells were cultured in RPMI 1640 with 10% FBS; and A293, HCT116, Caco-2, and HT-29 cells were cultured in DMEM supplemented with 10% FBS. All culture media were supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin.

Electrophoretic Mobility Shift Assay—To assess NF-κB p65 Localization

NF-κB p65 Localization—To determine the effect of 3-FC on the nuclear translocation of NF-κB p65, immunocytochemical analysis as described previously (17). Briefly, cells were plated on a poly-l-lysine–coated slide with a Cytospin 4 centrifuge (ThermoShandon, Pittsburgh, PA), air-dried, and fixed with 4% paraformaldehyde. The slides were washed with PBS, blocked with 5% normal goat serum for 1 h, and then incubated with rabbit anti-p65 polyclonal antibody at a 1:200 dilution overnight at 4 °C. The slides were washed, incubated with Alexa Fluor 594-conjugated goat anti-rabbit IgG (Invitrogen) at a 1:200 dilution for 5 min. Finally, the protein was resolved by 10% SDS-PAGE, with NF-κB binding sites shown in boldface type) for 30 min at 37 °C. The resulting protein-DNA complex was separated from free oligonucleotides on 6.6% native polyacrylamide gels. The dried gels were visualized, and radioactive bands were quantified using a PhosphorImager imaging device (Molecular Dynamics, Sunnyvale, CA) and ImageQuant software. The EMSA for Oct-1 was performed as described for NF-κB using 32P end-labeled double-stranded oligonucleotides.

Transfection—The p65−/− cells were plated in 6-well plates and transiently transfected with FuGENE 6 (Roche Applied Science) with pcDNA3.1 or pcDNA expression vectors for mouse p65 or mouse p65(C38S) for 48 h (15). To measure the COX-2 reporter activity, we used plasmids containing the COX-2 promoter ligated with luciferase constructs. A293 cells were transfected with full-length (pGL3COX-2-luc, −891/+9) or mutated (pGL2COX-2-luc(−449/+225)(κB1/κB2)) COX-2 plasmids. The cells were cotransfected with pSV-β-galactosidase plasmid. After 24 h, cells were treated with 3-FC for 12 h and then stimulated with TNF-α (1 nm) for 24 h. Cells were lysed, and luciferase activity was measured as luciferase count/β-galactosidase count.
ASSAY FOR NF-κB-DEPENDENT REPORTER GENE EXPRESSION—To determine the effect of 3-FC on induction of NF-κB-dependent reporter gene transcription by TNF-α, TNFR1, TNF receptor-associated death domain (TRADD), TRAF2, NF-κB-inducing kinase (NIK), TAK1/TAB1 (TAK1-binding protein-1), IKKβ, and p65, we performed the secretory alkaline phosphatase (SEAP) assay as described previously (18).

ChIP Assay—The effect of 3-FC on binding of NF-κB to the COX-2 promoter was analyzed by ChIP assay (Millipore, Temecula, CA) following the manufacturer’s instructions. In brief, KBM-5 cells were incubated for 10 min with 1% formaldehyde, harvested, centrifuged, and resuspended in lysis buffer. After lysis and sonication, the lysate was collected by centrifugation and mixed with ChIP dilution buffer. The protein-DNA complex was immunoprecipitated using anti-p65 antibody. The complex was then washed, incubated with 5 μl NaCl, and heated at 65 °C for 4 h in the presence of proteinase K to reverse the cross-links of the protein-DNA complex. The DNA was recovered by extraction with phenol/chloroform and precipitation with ethanol. The DNA was then amplified by PCR using human COX-2 promoter primer sequences (5’-AAAGACATCTGGCAGGAACCTT-3’ (forward) and 5’-AGGAAGCTGGCCCAAATTTG-3’ (reverse)). These primers correspond to sequences −434/−414 and −319/−337 of the human COX-2 promoter.

Apoptosis Assay—To measure apoptosis, we used the Live/Dead® assay kit (Invitrogen). We stained the cells according to the manufacturer’s instructions. In this assay, a tetramethyl ester, a non-fluorescent polyamine, is converted into a non-fluorescent polyamine by live cells, in which it produces intracellular alkaline phosphatase, which is then cleaved by bacterial alkaline phosphatase into all-inactive, green fluorescent product and binds to nucleic acids, thereby staining the live cells. The dead cells remain unfluorescent in dead cells. Live and dead cells were counted under the Labophot-2 fluorescence microscope.

An early indicator of apoptosis is the rapid translocation and accumulation of the membrane phosphatidylserine (PS) from the cytoplasmic interface of the membrane to the extracellular surface. We examined PS externalization using FITC-conjugated annexin V. Briefly, 5 × 10⁵ cells were pretreated with 3-FC (50 μM) for 12 h, treated with TNF-α for 24 h, and analyzed for annexin V-positive cells following the manufacturer’s instructions (Santa Cruz Biotechnology).

We also analyzed sub-G₁ fraction of the cell cycle using propidium iodide staining. This method is based on the fact that cells undergoing apoptosis will lose part of their DNA (due to DNA fragmentation), and those cells may be detected as a sub-G₁ population after propidium iodide staining.

Cell Proliferation Assay—We used a clonogenic assay to measure tumor growth because it produces results that are considered closer to the in vivo situation. Treated and untreated cells were seeded in 100-mm Petri dishes, allowed to form colonies for 14 days, and then stained as described previously (19).

Western Blot Analysis—Cytoplasmic, nuclear, and whole-cell extracts of untreated and treated cells were used in Western blot analysis. The protein extracts were resolved by SDS-PAGE. After electrophoresis, proteins were electrotransferred to nitrocellulose membranes, blotted with the relevant antibody, and detected using an enhanced chemiluminescence reagent (GE Healthcare).

Enzyme-linked Immunosorbent Assay—Human ELISA system kits (eBioscience, San Diego, CA) were used for the detection of IL-6 and TNF-α. The U266 cells were treated with different concentrations of 3-FC, and cell-free supernatants were collected after 12 h. The cytokine level was determined following the manufacturer’s protocol.

3-FC Molecular Docking with p65-DNA Complex—The crystal structure of the p65 domain was retrieved from the Protein Data Bank (code 1VKX). The docking program Glide (20, 21) was used to study the interactions of 3-FC with p65. The protein was prepared using the default parameters of the protein preparation workflow in the Schrödinger suite. The docking grid was centered around Arg³³, Arg³⁵, Tyr³⁶, Glu³⁸, and Arg¹⁸⁷, as they were deemed critical for ligand and DNA binding (22).

Glide docking software in the standard precision mode was used to generate a set of receptor-ligand complexes. The top scoring complex was selected as the starting point to generate an ensemble of 3-FC-ligand complexes to simulate induced fit effects upon binding. The exact modeling protocol was also applied to the generation of C38S mutant protein. The simulations were carried out with our in-house tool ORELI, which uses Ligand Information,4 the Rosetta suite of programs designed to perform the analysis of 100 complexes was generated and analyzed. For every iteration, the complex was randomly translated and rotated (1.0 Å and 1.5°) from the initial starting position, and the receptor residues within 0.5 Å of the ligand were optimized using low energy side chain rotamers. The resulting complex was scored using the all-atom Rosetta ligand scoring function and retained/rejected based on the Metropolis criterion. On the basis of our modeling and experimental data, we selected five top-ranked complexes.

3-FC was then redocked using Glide in the extra precision mode (20, 21) into the optimized binding sites, and the best scored pose was retained.

Statistical Analysis—Different parameters were monitored in normal and treated cells. Experiments were repeated a minimum of three times. Data are given as the mean ± S.D. Statistical analysis was carried out using Student’s two-tailed unpaired t test. A value of p < 0.05 was considered statistically significant.

RESULTS

3-FC Inhibits NF-κB Activation Induced by Carcinogens and Inflammatory Agents—Numerous agents, including tumor promoters (e.g. okadaic acid and phorbol 12-myristate 13-acetate) and inflammatory agents (such as TNF-α and LPS), are known to activate NF-κB. Various studies show that the mechanisms by which these agents activate NF-κB differ significantly (25). When we investigated whether 3-FC affects NF-κB activation induced by these agents, we found that all of them activated NF-κB in human myeloid KBM-5 cells and that pretreatment with 3-FC suppressed the activation (Fig. 1B), suggesting

4 S. S. Phatak and S. Zhang, unpublished data.
that 3-FC acts at a step in the NF-κB activation pathway that is common to all of these agents.

3-FC Suppresses TNF-α-induced NF-κB Activation in Dose- and Time-dependent Manner—We first examined the dose and time required to suppress TNF-α-induced NF-κB activation by 3-FC. EMSA results revealed that 3-FC inhibited TNF-α-mediated NF-κB activation in a dose-dependent (Fig. 1C, left panel) and time-dependent (right panel) manner.

3-FC Inhibits NF-κB Activation in Different Types of Cancer Cells—Whether 3-FC modulates TNF-α-induced NF-κB activation in tumor cells other than KBM-5 was examined. We found that the effect of 3-FC was not limited to KBM-5 cells only but was also observed in colon cancer (Caco-2 and HT-29), chronic myeloid leukemia (K562), T-cell leukemia (Jurkat), and promyelocytic leukemia (HL-60) cells (Fig. 1D).

3-FC Inhibits Constitutive NF-κB Expression—Several tumor cell types are known to constitutively express NF-κB through a mechanism yet to be fully defined (13). Human multiple myeloma RPMI-8226 and U266 cells in particular are known to have constitutively active NF-κB. To determine whether 3-FC affects NF-κB expression in these cells, we exposed multiple myeloma cells to 3-FC and then analyzed the nuclear extracts using EMSA. As

![Figure 1](image-url)
shown in Fig. 1E, 3-FC completely suppressed constitutive NF-κB activation in these cells, indicating that 3-FC can suppress both inducible and constitutive NF-κB activation.

3-FC Does Not Inhibit Oct-1 Activation in Tumor Cells—Whether 3-FC inhibits activation of Oct-1 under the conditions it suppresses NF-κB was investigated. The results showed that 3-FC had no effect on constitutive Oct-1 (Fig. 1F).

3-FC Inhibits IkBα Degradation and Phosphorylation—The translocation of NF-κB to the nucleus is preceded by the phosphorylation and proteolytic degradation of IkBα. To determine whether the inhibition of TNF-α-induced NF-κB activation is due to the inhibition of IkBα degradation, we pretreated cells with 3-FC and then exposed the cells to TNF-α for various times. We examined the cells for NF-κB activation in the nucleus by EMSA and for IkBα degradation in the cytoplasm by Western blot analysis. EMSA revealed that although TNF-α activated NF-κB in control cells in a time-dependent manner, the cytokine had no effect on cells pretreated with 3-FC (Fig. 2A). Western blot analysis revealed that although TNF-α induced IkBα degradation in the control cells within 15 min, TNF-α had no effect on IkBα degradation in 3-FC-treated cells (Fig. 2B). These results indicate that 3-FC inhibited both TNF-α-induced NF-κB activation and IkBα degradation.

To determine whether the inhibition of TNF-α-induced IkBα degradation is due to the inhibition of IkBα phosphorylation, we blocked IkBα degradation with the proteasome inhibitor N-acetyl-leucyl-leucyl-norleucinal. Western blot analysis showed that the serine-phosphorylated, or Ser536 form of IkBα revealed that 3-FC suppressed TNF-α-induced IkBα phosphorylation (Fig. 2C). Western blot analysis examined whether 3-FC affects nuclear translocation of p65 in a time-dependent manner. When the cells were treated with 3-FC, TNF-α failed to induce nuclear translocation of p65 (Fig. 2D, left panel). When the cells were pretreated with 3-FC, TNF-α induced the phosphorylation of p65 at Ser536, which is required for its transcripational activity (11). Western blot analysis showed that TNF-α induced the phosphorylation of p65 and that 3-FC strongly suppressed it (Fig. 2D, left panel).

To confirm the results observed by Western blot analysis, we performed immunocytochemistry to localize p65 inside the cells. The results indicated that in untreated or 3-FC-treated KBM-5 cells, p65 was localized in the cytoplasm. Treatment with TNF-α induced p65 nuclear translocation, and 3-FC pretreatment suppressed TNF-α-induced nuclear translocation (Fig. 2D, right panel).

3-FC Inhibits TNF-α-induced IKK Activation—IKK is required for TNF-α-induced IkBα phosphorylation and p65 phosphorylation (26, 27). Because 3-FC inhibited the phosphorylation of IkBα and p65, we determined the effect of 3-FC on TNF-α-induced IKK activation in KBM-5 cells. An immune complex kinase assay showed that TNF-α activated IKK as early as 2 min after TNF-α treatment and that treatment with 3-FC strongly suppressed this activation (Fig. 2E). Neither TNF-α nor 3-FC affected the expression of the IKKα or IKKβ protein.

3-FC Directly Inhibits IKK Activation—To determine whether 3-FC suppresses IKK activity directly or indirectly by suppressing its activation, we incubated the immune complexes with 3-FC at various concentrations and then examined the kinase activity. The results showed that 3-FC directly inhibited the activity of IKK in a dose-dependent manner (Fig. 2F). This indicated that 3-FC can directly modulate TNF-α-induced IKK activation.

3-FC Interacts with Cys38 in p65 and Cys179 in IκBα Kinase

Because the IκKβ subunit of the IKK complex contains various cysteine residues, we hypothesized that 3-FC may inhibit IKK through direct modification of one or more of these cysteine residues. We used a reducing agent, DTT, to determine whether the modulation of IKK activity by 3-FC is caused by the oxidation of critical cysteine residues. The addition of DTT to the kinase reaction mixture reversed the 3-FC-mediated inhibition of TNF-α-induced IKK activity (Fig. 2G), suggesting that a cysteine residue is involved in the pathway.

IKKβ contains a cysteine at position 179 in its activation loop that is critical for its activity (28). To determine whether this residue is involved in 3-FC-mediated IKK inhibition, we transfected A293 cells with wild-type FLAG-IKKβ or FLAG-IKKβ with a C179A mutation, 3-FC inhibited wild-type IKKβ. In contrast, 3-FC had no apparent effect on the activity of the IKKβ mutant for various cysteine residues. These findings suggest that 3-FC inhibits IKKβ through oxidation of Cys179.

3-FC Induces TAK1 Activation—Because TNF-α-induced NF-κB activation is required for its transcriptional activity, we determined whether the inhibitory effect of 3-FC on TNF-α-induced NF-κB activation is mediated through TAK1. The results of the kinase assay revealed that 3-FC suppressed TNF-α-induced TAK1 activation (Fig. 2I).

3-FC Directly Blocks Binding of NF-κB p65 Subunit to DNA—Previous studies have shown that certain agents suppress NF-κB activation by directly blocking the binding of NF-κB to DNA (30, 31). We determined whether 3-FC mediates suppression of NF-κB activation through a similar mechanism using nuclear extracts from TNF-α-treated cells. The nuclear extracts were incubated with 3-FC for different times and then assayed for NF-κB binding to the DNA. EMSA showed that the chromone inhibited NF-κB binding to the DNA in a time-dependent manner (Fig. 3A).

It is possible that 3-FC inhibits the binding of NF-κB to the DNA through modification of NF-κB proteins (31). We found that co-incubation of nuclear extracts with 3-FC in the presence of DTT reversed the effect of 3-FC completely (Fig. 3B). It has been shown that the p65 subunit of NF-κB has a sensitive cysteine that is highly reactive (22). Thus, we transfected p65−/− cells with the p65 plasmid and examined whether DTT can reverse the inhibitory effect of 3-FC on p65-DNA binding. Fig. 3C shows that 3-FC inhibited p65 binding to the DNA and that DTT reversed the effect.

It has been shown that Cys38 in p65 is highly susceptible to various agents (15, 22). To determine whether Cys38 is a target for 3-FC, we transfected p65−/− cells with a wild-type or mutant (C38S) p65 plasmid. EMSA results showed that 3-FC inhibited the DNA binding of wild-type p65 but not mutant p65 (Fig. 3D). These results demonstrate that Cys38 in p65 is a target of 3-FC.
3-FC Represses TNF-α-induced NF-κB-dependent Reporter Gene Expression—Although EMSA results showed that 3-FC blocked NF-κB activation, DNA binding alone does not always correlate with NF-κB-dependent gene transcription, suggesting that there may be additional regulatory steps. When we investigated this question, we found that TNF-α induced the expression of the NF-κB-regulated SEAP reporter gene and that 3-FC suppressed the expression in a dose-dependent manner (Fig. 4A).
3-FC Interacts with Cys\(^{38}\) in p65 and Cys\(^{179}\) in I\(\kappa\)B\(\alpha\) Kinase

3-FC Suppresses NF-\(\kappa\)B-dependent Reporter Gene Expression—Because 3-FC inhibits NF-\(\kappa\)B activity, and COX-2 expression is enhanced by NF-\(\kappa\)B, we examined whether 3-FC can modulate the expression of NF-\(\kappa\)B-regulated gene products. We found that TNF-\(\alpha\) induces expression of cell survival and proliferative proteins (Bcl-2, Bcl-x\(_L\), survivin, and cIAP-1) in a time-dependent manner, whereas pretreatment with 3-FC down-regulated the expression (Fig. 5A).

We also examined the effects of 3-FC on NF-\(\kappa\)B-regulated cell proliferative proteins. The results indicated that 3-FC inhibited the expression of cyclin D\(_1\) and COX-2 induced by TNF-\(\alpha\) (Fig. 5A). The results also indicated that TNF-\(\alpha\) induced the expression of invasive (MMP-9 and ICAM-1) and angiogenic (VEGF) proteins in a time-dependent manner, and 3-FC was found to suppress the expression of these proteins (Fig. 5A).

3-FC Inhibits TNF-\(\alpha\)-induced COX-2 Promoter Activity—TNF-\(\alpha\) induces the expression of the COX-2 gene, which has inhibitory effect of 3-FC on NF-\(\kappa\)B-DNA binding. Nuclear extracts of TNF-\(\alpha\)-treated KBM-5 cells were incubated with 100 \(\mu\)M 3-FC for 30 min and then assayed for DNA binding by EMSA. D, lack of effect of 3-FC on DNA binding of mutant p65 (C38S). The p65\(^{\text{wt}}\) cells were transfected with p65 plasmid, and nuclear extracts were incubated with 100 \(\mu\)M 3-FC for 30 min and then assayed for p65 binding to DNA by EMSA.

FIGURE 3. Direct effect of 3-FC on p65-DNA binding. A, 3-FC directly affects NF-\(\kappa\)B-DNA binding. Nuclear extracts (NE) were prepared from untreated cells or cells treated with TNF-\(\alpha\) (0.1 nM), incubated with 100 \(\mu\)M 3-FC for the indicated times, and then assayed for NF-\(\kappa\)B activation using EMSA. B, DTT reverses the inhibitory effect of 3-FC on NF-\(\kappa\)B-DNA binding. Nuclear extracts of TNF-\(\alpha\)-treated KBM-5 cells were incubated with 100 \(\mu\)M 3-FC for 30 min in the absence or presence of 100 \(\mu\)M DTT and then assayed for NF-\(\kappa\)B activation using EMSA. C, lack of effect of 3-FC on DNA binding of mutant p65 (C38S). The p65\(^{\text{wt}}\) cells were transfected with p65 plasmid, and nuclear extracts were incubated with 100 \(\mu\)M 3-FC for 30 min and then assayed for DNA binding by EMSA.

3-FC Down-regulates Proinflammatory Cytokine Production in Multiple Myeloma Cells—We investigated whether 3-FC has an effect on the production of IL-6 and TNF-\(\alpha\) in U266 cells...
that are regulated by NF-κB. The production of IL-6 and TNF-α in U266 cells was suppressed by 3-FC in a concentration-dependent manner (Fig. 5).

**3-FC Potentiates TNF-α-induced Apoptosis**—Next, we examined whether 3-FC potentiates TNF-α-induced apoptosis. As examined by intracellular esterase activity, 3-FC enhanced TNF-α-induced apoptosis in KBM-5 cells. Specifically, TNF-α-induced apoptosis was increased from 24 to 48% in the cells (Fig. 6A).

To confirm the results of esterase activity, the sub-G₁ fraction, an indicator of apoptosis, was analyzed. The results indicated that apoptosis was induced at 23.2% by TNF-α, at 11.2% by 3-FC, and at 39.6% by TNF-α plus 3-FC (Fig. 6B).

One of the early events of apoptosis is externalization of the membrane PS on the cell surface. Because of the affinity of annexin V for PS, annexin V staining can be used to detect early apoptotic cells. The effect of 3-FC on PS externalization induced by TNF-α was investigated. The number of annexin V-positive cells was significantly increased when cells were pre-treated with 3-FC before TNF-α treatment (Fig. 6C).

Whether 3-FC enhances the TNF-α-induced activation of caspase-3, caspase-8, and caspase-9 and cleavage of PARP was investigated. We found that TNF-α alone had little effect on PARP cleavage and caspase activation. However, pretreatment of the cells with 3-FC increased caspase activation and PARP cleavage (Fig. 6D). We also found that 3-FC treatment completely suppressed the colony-forming ability of tumor cells (Fig. 6E).

**3-FC Docking Studies**—Finally, we employed molecular docking studies to confirm the experimental observations of 3-FC binding to p65. 3-FC was docked just below the L1 DNA-binding loop consisting of Arg₃₃, Cys₃₈, and Arg₄₁. Upon optimization with ORELI, the above loop region was moved upwards slightly compared with the crystal structure (Fig. 7). The distance between the Cys₃₈ and Cys₁₂₀ changed from 7.7 Å in the crystal structure to 10.2 Å in the model. This altered the χ₁ angle of Tyr₃₆ from −66.3° (in the crystal structure) to −67.5° such that the phenol moiety in the residue is oriented toward the DNA-binding region (Fig. 7). We believe that this conformational change presents steric hindrance to DNA, thus preventing its binding to p65. However, for the C38S mutant protein, the loop region seems to maintain a conformation similar to the crystal structure of p65 (Fig. 7), maintaining p65-DNA binding. These modeling results are in agreement with experimental observations.
DISCUSSION

This is the first report to suggest that a chromone can suppress the NF-κB activation pathway and expression of NF-κB-regulated gene products. In this study, 3-FC inhibited constitutive NF-κB activation, a critical element in the survival and proliferation of various tumor cell types (35). The inhibition of NF-κB activation by 3-FC was specific, as it failed to inhibit Oct-1 activation. We found that 3-FC acted at two different steps in the NF-κB signaling pathway. First, it interacted directly with the p65 subunit of NF-κB, and second, it suppressed TNF-α-induced IKK activation.

We found that 3-FC could inhibit the binding of reconstituted p65 to the DNA in vitro, which suggests that p65 is the direct target of 3-FC. These results are consistent with findings that 3-FC also suppressed the p65-induced NF-κB reporter activity. The reversal of the effects of 3-FC by a reducing agent suggests that a cysteine residue in p65 is modified by this agent. These results are consistent with those reported previously from our laboratory with caffeic acid phenethyl ester (31) and with those reported by another laboratory with sesquiterpene lactone parthenolide (22). A cysteine residue (Cys\textsuperscript{38}) has been identified in p65 subunits of NF-κB that is crucial for DNA binding (22). The results showed that when Cys\textsuperscript{38} was replaced with serine in p65, 3-FC failed to inhibit the DNA-binding ability of p65. This result suggests that 3-FC modifies Cys\textsuperscript{38}, leading to NF-κB suppression.

3-FC Interacts with Cys\textsuperscript{38} in p65 and Cys\textsuperscript{179} in IkBα Kinase

FIGURE 5. 3-FC inhibits expression of NF-κB-regulated gene products. A, 3-FC inhibits the expression of TNF-α-induced anti-apoptotic (Bcl-2, Bcl-x\textsubscript{L}, survivin, and cIAP-1), cell proliferative (cyclin D\textsubscript{1} and COX-2), metastatic (MMP-9 and ICAM-1), and angiogenic (VEGF) proteins. KBM-5 cells were incubated with 3-FC (100 μM) for 12 h and then treated with the indicated concentrations of 3-FC for 12 h. Cells were treated with 1 nM TNF-α for an additional 24 h, lysed, and subjected to a luciferase assay. Variations in transfection efficiency were normalized by measuring β-galactosidase activity. The luciferase activity was estimated as luciferase count/β-galactosidase count. The values are the mean ± S.D. for three independent replicates. * and #, significance of difference compared with the control and TNF-α-alone groups, respectively (p < 0.05). B, 3-FC inhibits the COX-2 promoter activity induced by TNF-α. Cells were transiently transfected with a COX-2 promoter linked to the luciferase reporter gene plasmid for 24 h and then treated with the indicated concentrations of TNF-α for an additional 24 h, and then lysed and subjected to a luciferase assay. The values are the mean ± S.D. for three independent replicates. * and #, significance of difference compared with the control and TNF-α-alone groups, respectively (p < 0.05). C, COX-2 promoter with mutant NF-κB-binding elements is resistant to 3-FC treatment. A293 cells were transfected with a luciferase expression construct ligated to the full-length (white bars) or mutant (black bars) COX-2 promoter. Cells were treated with 3-FC for 12 h, followed by TNF-α for an additional 24 h, and then lysed and subjected to a luciferase assay. The values are the mean ± S.D. for three independent replicates. * and #, significance of difference compared with the control and TNF-α-alone groups, respectively (p < 0.05). D, effect of 3-FC on binding of NF-κB to the COX-2 promoter. Cells were treated with 100 μM 3-FC for 12 h, followed by 1 μM TNF-α for the indicated times, and the proteins were cross-linked with DNA by formaldehyde and subjected to ChIP assay using anti-p65 antibody and the COX-2 primer. Reaction products were resolved by electrophoresis. IP, immunoprecipitate. E, 3-FC down-regulates IL-6 and TNF-α production in U266 cells. Cells were treated with the indicated concentrations of 3-FC, and cell free supernatants were harvested after 12 h. The levels of IL-6 and TNF-α were detected by ELISA. The values are the mean ± S.D. for three independent replicates. * and #, significance of difference compared with the control (p < 0.03).
We found that 3-FC targeted IKK to suppress the TNF-α-induced phosphorylation and degradation of IκBα that were concomitant with the inhibition of nuclear translocation and phosphorylation of p65. We showed that 3-FC directly inhibited TNF-α-activated IKK. Furthermore, the addition of a reducing agent reversed the effects of 3-FC on IKK activation, suggesting the involvement of cysteine residues. The mutation of Cys179 of IKKα to alanine abolished the inhibitory effect of 3-FC on IKK activation. In addition to direct effects of 3-FC on IKK, inhibition of TAK1 may also be responsible for the inhibition of IKK by 3-FC. However, how this chromone inhibits TAK1 remains to be elucidated.

There are two possibilities by which this chromone can modify cysteine residues in p65 and IKK: 1) by redox cycling and 2) by direct interaction. Redox cycling results in the generation of reactive oxygen species and depletes cellular glutathione levels. A previous study demonstrated that the depletion of cellular GSH by diethyl maleate prevented NF-κB induction in rat hepatocytes (36). Because 3-FC is a free radical scavenger (37), it is very unlikely that the effects of 3-FC are mediated through reactive oxygen species generation. It is also unlikely that reactive oxygen species are produced under the in vitro conditions used for the modification of IKK and p65 by 3-FC. This eliminates the first possibility. From molecular docking studies, we found that, in the presence 3-FC, Cys38 underwent alkylation and that the tyrosine molecule at position 36 moved from its position, which in turn inhibited p65 binding to DNA. However, the DNA binding of p65 in which
Cys^{38} had been mutated was not affected. These results suggest that 3-FC interacts with the cysteine residue. A similar mechanism has been reported for other NF-κB activators such as prostaglandins (38), arsenite (28), butyrate (39), nimbolide (40), and bharangin (41). However, other sophisticated techniques, such as circular dichroism, x-ray crystallography, and NMR spectroscopy, are needed to provide more conclusive information on such binding interactions.

We found that 3-FC inhibited NF-κB activation induced by inflammatory stimuli (TNF-α and LPS) and tumor promoters (phorbol ester and okadaic acid), suggesting that 3-FC must act at a step common to all these activators. The IKK complex is critical for the NF-κB activation by all these inducers (42). It is likely that the inhibition of IKK activity is the common step for the inhibition of NF-κB by 3-FC.

We found that 3-FC down-regulated the expression of NF-κB-regulated gene products, such as survivin, Bcl-xL, Bcl-2, and clAP-1, all of which are known to suppress apoptosis. We also found that 3-FC inhibited the expression of cyclin D1 and COX-2, involved in cell proliferation. The potentiation of TNF-α-induced apoptosis by 3-FC could be accounted for by the inhibition of anti-apoptotic proteins as described herein. Beside these, 3-FC abrogated TNF-α-induced proteins involved in invasion (ICAM-1 and MMP-9) and angiogenesis (e.g. VEGF). The down-regulation of the expression of ICAM-1 and VEGF by 3-FC suggests that this chromone may have a role in the inhibition of invasion and angiogenesis of tumor cells.

Overall, in this study, we have demonstrated that 3-FC is a potent inhibitor of NF-κB activation, which may explain its anti-inflammatory and anticancer effects. Further studies using animal models are needed to explore its therapeutic potential against cancer and other diseases.

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3-Formylchromone Interacts with Cysteine 38 in p65 Protein and with Cysteine 179 in IK B Kinase, Leading to Down-regulation of Nuclear Factor- B (NF-B)-regulated Gene Products and Sensitization of Tumor Cells
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