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Inhibition of transcription factor NF-κB signaling proteins IKKβ and p65 through specific cysteine residues by epoxyquinone A monomer: Correlation with its anti-cancer cell growth activity

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Ac-DEVD-AMC, N-acetyl-Asp-Glu-Val-Asp-AMC (7-amino-4-methylcoumarin)
DHMEQ, dehydroxymethylepoxyquinomicin
DISC, death-inducing signaling complex
DMEM, Dulbecco's modified Eagle's medium

ABSTRACT

Transcription factor NF-κB is constitutively active in many human chronic inflammatory diseases and cancers. Epoxyquinone A monomer (EqM), a synthetic derivative of the natural product epoxyquinol A, has previously been shown to be a potent inhibitor of tumor necrosis factor-α (TNF-α)-induced activation of NF-κB, but the mechanism by which EqM inhibits NF-κB activation was not known. In this report, we show that EqM blocks activation of NF-κB by inhibiting two molecular targets: IκB kinase IKKβ and NF-κB subunit p65. EqM inhibits TNF-α-induced IκBα phosphorylation and degradation by targeting IKKβ, and an alanine substitution for Cys179 in the activation loop of IKKβ makes it resistant to EqM-mediated inhibition. EqM also directly inhibits DNA binding by p65, but not p50; moreover, replacement of Cys38 in p65 with Ser abolishes EqM-mediated inhibition of DNA binding. Pretreatment of cells with reducing agent dithiothreitol dose-dependently reduces EqM-mediated inhibition of NF-κB, further suggesting that EqM directly modifies the thiol group of Cys residues in protein targets. Modifications of the exocyclic alkene of EqM substantially reduce EqM's ability to inhibit NF-κB activation. In the human SUDHL-4 lymphoma cell line, EqM inhibits both proliferation and NF-κB DNA binding, and activates caspase-3 activity. EqM also effectively inhibits the growth of human leukemia, kidney, and colon cancer cell lines in the NCI's tumor cell panel. Among six colon cancer cell lines, those with low amounts of constitutive NF-κB DNA-binding activity are generally more sensitive to growth inhibition by EqM. Taken together, these results suggest that EqM inhibits growth and induces cell death in tumor cells through a mechanism that involves inhibition of NF-κB activity at multiple steps in the signaling pathway.

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DTT, dithiothreitol
EMSA, electrophoretic mobility shift assay
EqM, epoxyquinone A monomer
FBS, fetal bovine serum
FLAG, flu antigen
GI_{50}, dose for 50% cell growth inhibition
GST, glutathione S-transferase
ID_{50}, dose for 50% inhibition
IKKβ, IκB kinase β
JD, jesterone dimer
NCI, National Cancer Institute
NF-κB, nuclear factor-κB
PARP, poly(ADP-ribose) polymerase
PMSF, phenylmethylsulfonyl fluoride
TNF-α, tumor necrosis factor-α
IκBα, inhibitor of κBα

1. Introduction

The nuclear factor κB (NF-κB) family of eukaryotic transcription factors influences a number of important cellular and organismal processes, including cellular growth control, apoptosis, immune and inflammatory responses, and cellular stress responses [1]. The mammalian NF-κB family includes five related proteins (p50, p52, p65, c-Rel, and RelB) that form various combinations of homodimers and heterodimers to control the activity of numerous genes (see www.nf-kb.org). In many cell types, NF-κB is located in the cytoplasm in a latent, inactive form primarily bound to the inhibitor protein IκBα. NF-κB can be activated by a multi-component signal transduction pathway. Namely, in response to a variety of inducers (e.g., cytokines, growth factors), an IκB kinase (IKK) is rapidly activated that then phosphorylates two serine residues (Ser32 and Ser36) in IκBα [2]. Phosphorylated IκBα then undergoes polyubiquitination and is subsequently proteolytically degraded by the 26S proteasome [3]. The freed NF-κB complex can then enter the nucleus to regulate target gene expression.

Several reports have shown that NF-κB is constitutively active and present in the nucleus in a variety of human tumor cell types and cell lines [4]. Moreover, inhibition of this chronic NF-κB activity can, in many cases, slow the growth of these tumor cell lines or induce cell death. For example, the blocking of NF-κB signaling by arsenic-mediated inhibition of IKK sensitizes Hodgkin/Reed-Sternberg cell lines to apoptosis [5].

Some fungus-derived epoxyquinoids have been shown to inhibit NF-κB activation and to kill tumor cells [6]. For example, dehydroxymethylepoxyquinomicin (DHMEQ), an inhibitor of NF-κB nuclear transport, has been shown to have anti-inflammatory and anti-tumor effects in nude mice [7–10], and panepoxydone and isopanepoxydone have anti-NF-κB activity [11]. We have also synthesized and characterized the bioactivities of several natural and synthetic epoxyquinoids, including torreyanic acid, jesterone, jesterone dimer, cycloepoxydon, epoxyquinol A, and epoxyquinone A monomer (EqM) [12–16]. Relevant to this study, we found that the synthetic derivative EqM (Fig. 1A) is a more potent inhibitor of tumor necrosis factor (TNF-α)-induced activation of NF-κB DNA binding (ID_{50} approximately 2.3 μM) than the natural compound epoxyquinol A [15,17]. Because EqM was the most potent inhibitor of NF-κB activation among several epoxyquinol A derivatives [15], we were interested in determining the step(s) at which EqM inhibited the NF-κB pathway. In this paper, we demonstrate that EqM blocks NF-κB activation by targeting cysteine residues required for the activity of both IKKβ and p65. Moreover, we show that EqM effectively inhibits NF-κB DNA binding and induces apoptosis in a human lymphoma cell line and that EqM is especially toxic in leukemia, kidney, and colon cancer cell lines, among a panel of tumor cell lines. Thus, EqM may represent a useful model compound for the development of additional research or therapeutic compounds that target the NF-κB pathway at multiple levels.

2. Materials and methods

2.1. *Synthesis of epoxyquinone A monomer (EqM) and other epoxyquinoid compounds*

The syntheses of EqM, epoxyquinol A, and jesterone dimer have been described previously [13,15]. The synthesis of *ent*-EqM was achieved by employing a modification of the procedure reported for EqM [15] using D-di-isopropyl tartrate.

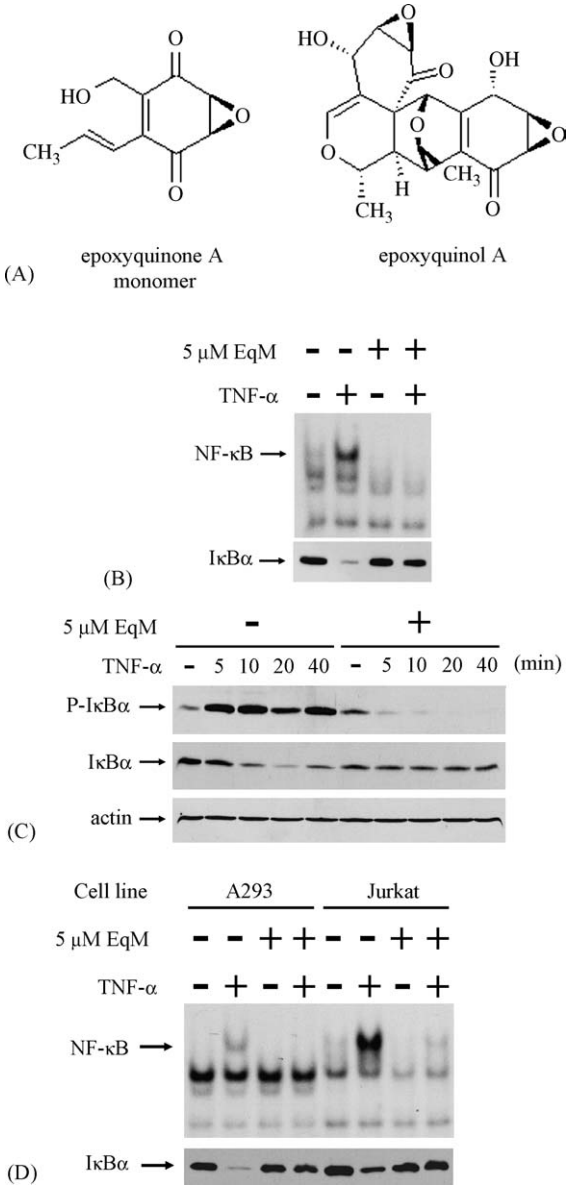

Fig. 1 – Epoxyquinone A monomer (EqM) is an effective inhibitor of TNF-α-induced activation of NF-κB DNA binding and phosphorylation and degradation of IκBα in a variety of cell lines. (A) Structures of epoxyquinone A monomer and epoxyquinol A. (B) Mouse 3T3 were pre-incubated with or without 5 μM EqM for 2 h prior to a 20 min induction with 5 ng/ml TNF-α. Extracts were subjected to an EMSA using a κB site probe (upper panel) or Western blotting using antiserum against IκBα (lower panel). The position of the NF-κB–DNA complex is indicated. (C) 3T3 cells were pretreated for 2 h with or without 5 μM EqM and then stimulated with 2 ng/ml TNF-α for the indicated times. Cell extracts were subjected to Western blotting using antiserum against phospho-Ser32 IκBα, IκBα, or actin. (D) Human A293 or Jurkat cells were analyzed for EqM-mediated inhibtion of TNF-α-induced NF-κB DNA binding and degradation of IκBα as described for (B).

EqM–thiophenol adduct (5:1 mixture of diastereomers) was synthesized by reaction of EqM with thiophenol in acetic acid (room temperature, 6 h). All compounds were dissolved in 100% methanol prior to use in cell culture experiments.

2.2. *Site-directed mutagenesis*

The mouse mutant p65C38S was generated by overlapping polymerase chain reaction (PCR)-based mutagenesis using pcDNA-p65 as the template and two synthetic complementary oligonucleotide primers containing the relevant (underlined) point mutation (sense, 5′-GCGATTCCGCTATAAATCCGAGG-GGCGCTCAGCGGG -3′; antisense, 5′-CCCGCTGAGCGCCCCT-CGGATTTATAGCGGAATCGC-3′) and two flanking primers. The final PCR product was digested with *Hin*dIII and *Kpn*I and was used to replace the corresponding *Hin*dIII/*Kpn*I fragment in pcDNA-mouse p65. The mutation was then confirmed by DNA sequencing.

2.3. *Cell culture, chemical treatment, and transfection*

All cells were grown in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Biologos, Naperville, IL) as described [16].

Treatment of cells with chemical compounds was performed as described previously [16]. Briefly, 24 h before treatment, the medium on 3T3, A293, or Jurkat cells was changed from DMEM containing 10% FBS to DMEM containing 0.5% FBS. After this 24-h period, cells were incubated for 2 h with the indicated concentrations of compounds or the control solvent methanol. After the 2-h incubation, cells were stimulated with the indicated concentrations of recombinant human TNF-α (R&D Systems, Minneapolis, MN) for the indicated times. For SUDHL-4 cells, transfected 3T3 and A293 cells and colon cancer cell lines, cultures were not serum starved prior to incubation with compounds.

Transfections were performed using the SuperFect Transfection Reagent, according to the manufacturer's instructions (QIAGEN, Valencia, CA). For IKKβ kinase assays, 100-mm plates of approximately 70% confluent 3T3 cells were transfected with 15 μg of a pcDNA expression plasmid for a FLAG-tagged version of wild-type or C179A mutant IKKβ. For electrophoretic mobility shift assays, A293 cells were transfected with pcDNA3.1 or with pcDNA expression vectors for human p50, mouse p65, or mouse p65C38S.

2.4. *Electrophoretic mobility shift assay (EMSA)*

EMSAs were performed using whole-cell extracts prepared in AT buffer (20 mM HEPES, pH 7.9, 1%, w/v, Triton X-100, 20%, w/v, glycerol, 1 mM EDTA, 1 mM EGTA, 20 mM NaF, 1 mM $Na_4P_2O_7$, 1 mM DTT, 1 mM Na_3VO_4, 1 μg/ml PMSF, 1 μg/ml leupeptin, 1 μg/ml pepstatin) as described previously [16]. Briefly, in a final reaction volume of 50 μl, equal amounts of cell protein (20–30 μg) were incubated with 2 μg poly(dI-dC), a 26-base pair [^{32}P]-labeled κB site probe (κB site: 5′-GGG-AAATTCC-3′; 35,000–70,000 cpm) in binding buffer (25 mM Tris–HCl, pH 7.4, 100 mM KCl, 6.25 mM $MgCl_2$, 0.5 mM EDTA, 0.5 mM DTT, 10%, w/v, glycerol). After incubation at 30 °C for

30 min, the reaction mixtures were resolved on 5% non-denaturing polyacrylamide gels. Gels were dried and the protein–DNA complexes were detected by autoradiography or phosphorimaging (Bio-Rad, Hercules, CA).

2.5. Western blotting

Western blotting was performed essentially as described [16]. Whole cell extracts were prepared in AT buffer (see above), and samples containing equal amounts of protein were separated on 6.7% (PARP), 7.5% (phospho-IKKβ (Ser181), IKKβ, p65), or 12.5% (phospho-IκBα (Ser32), IκBα, or actin) SDS-polyacrylamide gels and transferred to nitrocellulose membranes (Micron Separation Inc., Westborough, MA). The following primary antisera were used at 500-fold dilution (except where noted): anti-IκBα antiserum directed against C-terminal sequences of IκBα (SC-371, Santa Cruz Biotechnology, Santa Cruz, CA); anti-phospho-IκBα (Ser32) (#9241S, Cell Signaling Technology, Beverly, MA); anti-IKKβ (#2684, Cell Signaling Technology); anti-phospho-IKKα (Ser180)/IKKβ (Ser181) (#2681, Cell Signaling Technology); anti-p65 (1:4000 dilution; a kind gift of Nancy Rice, National Cancer Institute); anti-poly(ADP-ribose) polymerase (PARP) (SC-7150, Santa Cruz Biotechnology); anti-actin (SC-1616, Santa Cruz Biotechnology). Filters were incubated with primary antiserum either for 1 h at room temperature or overnight at 4 °C. The appropriate horseradish peroxidase-labeled secondary antiserum was added and immunoreactive proteins were detected with the Supersignal Dura West chemiluminescence detection system (Pierce, Rockford, IL).

2.6. Immune complex kinase assay

IKK kinase assays were performed essentially as described [16]. Two days after transfection with the FLAG-IKKβ expression vector, 3T3 cells were treated with the indicated concentrations of EqM for 2 h and were then lysed in AT buffer containing 20 mM β-glycerophosphate. FLAG-IKKβ was isolated with anti-FLAG M2 affinity beads (Sigma Chemical Co., St. Louis, MO). The immunoprecipitates were then incubated with 2 μg GST-IκBα (aa 1-55) and 5 μCi [γ-^{32}P]ATP in kinase reaction buffer (25 mM Tris–HCl, pH 7.5, 10 mM $MgCl_2$, 2 mM DTT, 50 μM ATP) containing phosphatase inhibitors (10 mM NaF, 0.5 mM Na_3VO_4, and 20 mM β-glycerophosphate) for 30 min at 30 °C. Phosphorylated GST-IκBα substrate was then electrophoresed on a 12.5% SDS-polyacrylamide gel and was detected by autoradiography or phosphorimaging.

For detection of endogenous IKK activity, serum-starved 3T3 cells were left untreated or treated with 5 μM EqM for 2 h and then were stimulated with 20 ng/ml TNF-α for 7.5 min before harvesting. The IKK complex was then immunoprecipitated with a polyclonal antiserum against IKKα (#2682, Cell Signaling Technology) and protein A-Sepharose beads, and samples were subjected to an in vitro kinase assay as described above.

2.7. Measurement of caspase-3 activity and DNA ladder formation

Total caspase-3 activity in SUDHL-4 cells was determined as described previously [16]. Briefly, cells were lysed by three freeze-thaw cycles in 10 mM HEPES, pH 7.4, 2 mM EDTA, 0.1% CHAPS, 5 mM DTT, 350 μg/ml PMSF, 10 μg/ml pepstatin, 10 μg/ml aprotinin, and 20 μg/ml leupeptin. Caspase-3 activity in extracts was then measured by cleavage of the fluorogenic substrate Ac-DEVD-AMC (BIOMOL Research Laboratories, Plymouth Meeting, PA). The isolation of low-molecular-weight DNA and the analysis of ''DNA ladders'' were performed as described previously [18].

2.8. National Cancer Institute (NCI) human tumor cell line screening

EqM was tested in the NCI's human tumor 60-cell line screen and data calculations were performed as described elsewhere [19–22]. Fig. 6A is a composite prepared from graphical information typically provided in the standard NCI screening data report package and values are the averages of triplicate tests.

3. Results

3.1. Epoxyquinone A monomer inhibits tumor necrosis factor-α-induced activation of NF-κB in a variety of cell lines

We previously showed that epoxyquinone A monomer (EqM) (Fig. 1A), a synthetic derivative of the fungal metabolite epoxyquinol A (Fig. 1A), can inhibit TNF-α-induced DNA binding by NF-κB in mouse 3T3 cells [15]. As a first step in determining where in the NF-κB signaling pathway EqM acts, we pretreated mouse 3T3 cells with 5 μM EqM and analyzed TNF-α-induced degradation of IκBα by Western blotting. Under these conditions, EqM blocked TNF-α-induced degradation of IκBα (Fig. 1B, lower panel). As a control, we show that this dose of EqM blocked TNF-α-induced activation of NF-κB DNA-binding activity in this experiment (Fig. 1B, upper panel). Because phosphorylation of N-terminal Ser residues (Ser32 and Ser36) in IκBα is a prerequisite for TNF-α-induced degradation of IκBα [2], we determined whether EqM could inhibit Ser32 phosphorylation of IκBα by Western blotting with an anti-phospho-IκBα antiserum. As shown in Fig. 1C, pretreatment of mouse 3T3 with 5 μM EqM effectively blocked TNF-α-induced phosphorylation of Ser32 in IκBα. As a control, we show that IκBα was rapidly phosphorylated at Ser32 in TNF-α-induced cells pre-incubated with the solvent methanol (−EqM, Fig. 1C).

To determine whether EqM could block activation of NF-κB in cell types other than mouse 3T3 cells, we assessed the ability of EqM to block NF-κB activation in human kidney carcinoma A293 and Jurkat T-leukemia cells, which are known to have a pronounced TNF-α-induced NF-κB response. As with 3T3 cells, 5 μM EqM inhibited the ability of TNF-α to induce both NF-κB DNA-binding activity (Fig. 1D, upper panel) and degradation of IκBα (Fig. 1D, lower panel) effectively in both A293 and Jurkat cells.

Taken together, these results suggest that EqM targets a central and common component(s) of the NF-κB signaling pathway and indicate that EqM is blocking TNF-α-induced activation of NF-κB, at least in part, at or upstream of phosphorylation of IκBα.

3.2. *EqM inhibits IKKβ activity and Cys179 in IKKβ is critical for EqM-mediated inhibition*

The IκB kinase β (IKKβ) subunit in the IKK complex is the primary kinase that phosphorylates Ser32 and Ser36 of IκBα in response to TNF-α stimulation [2]. We therefore sought to determine whether IKKβ was a molecular target for EqM by examining the effect of EqM on IKKβ kinase activity from cells that were either stimulated with TNF-α (i.e., endogenous IKKβ) or overexpressing IKKβ. As shown in Fig. 2A, 5 μM EqM blocked stimulated endogenous IKKβ kinase activity as judged by an immune complex kinase assay using extracts from TNF-α-induced 3T3 cells and GST-IκBα as a substrate. Similarly, 5 μM EqM treatment inhibited by approximately 36% the IKKβ immune complex kinase activity in lysates from cells over-expressing FLAG-IKKβ (Fig. 2B). The complete versus partial inhibition of endogenous versus exogenous IKKβ kinase activity, respectively, by 5 μM EqM is likely to reflect the higher levels of IKKβ in transfected cells. Indeed, 10 μM EqM more effectively blocked the kinase activity of over-expressed wild-type IKKβ (Fig. 2C). In contrast, the kinase activity of an IKKβ mutant in which Cys179 is replaced with Ala was not inhibited by 10 μM EqM, and its activity was (in repeated assays) slightly increased by pre-incubation of cells with EqM (Fig. 2C). Moreover, pretreatment of cells with EqM increased the levels of Ser181 phosphorylation of C179A IKKβ but not wild-type IKKβ (Fig. 2D); phosphorylation of Ser181 is known to activate IKKβ [2]. Taken together, these results indicate that EqM blocks phosphorylation of IκBα by inhibiting IKKβ through a mechanism that requires Cys179.

We have previously shown that treatment of cells with the epoxyquinoid jesterone dimer (JD), which can also inhibit IKKβ, converts over-expressed IKKβ to a high-molecular-weight form, which migrates on SDS-polyacrylamide gels where a dimer of IKKβ is predicted to migrate [16]. Like JD, treatment of transfected cells with EqM converts wild-type and C179A IKKβ proteins to covalently modified high-molecular-weight forms (Fig. 2E). (The phosphorylated forms of wild-type and C179A IKKβ are also seen in high-molecular-weight forms after treatment with EqM (Fig. 2D).)

3.3. *EqM can also directly block DNA binding by p65, but not p50*

The anti-inflammatory sesquiterpene lactone parthenolide has been shown to block NF-κB activation by inhibiting two steps in this pathway: IKKβ kinase activity and p65 DNA binding [23,24]. EqM and parthenolide share electrophilic groups (epoxy and α,β-unsaturated ketones) and both compounds inhibit IKKβ through Cys179. Therefore, we determined whether EqM could also directly block NF-κB DNA binding by performing EMSAs on extracts from A293 cells transfected with either p50 or p65 expression plasmids. As shown in Fig. 3A, EqM dose-dependently blocked the DNA-binding activity of p65, but not p50. Substitution of Cys38 with Ser in p65 abolished EqM-mediated inhibition of p65 DNA binding (Fig. 3B, upper panel). Of note, there was considerably more κB site-binding activity in extracts from p65C38S-transfected cells than wild-type p65-transfected cells, even though both proteins were expressed at similar levels (Fig. 3B,

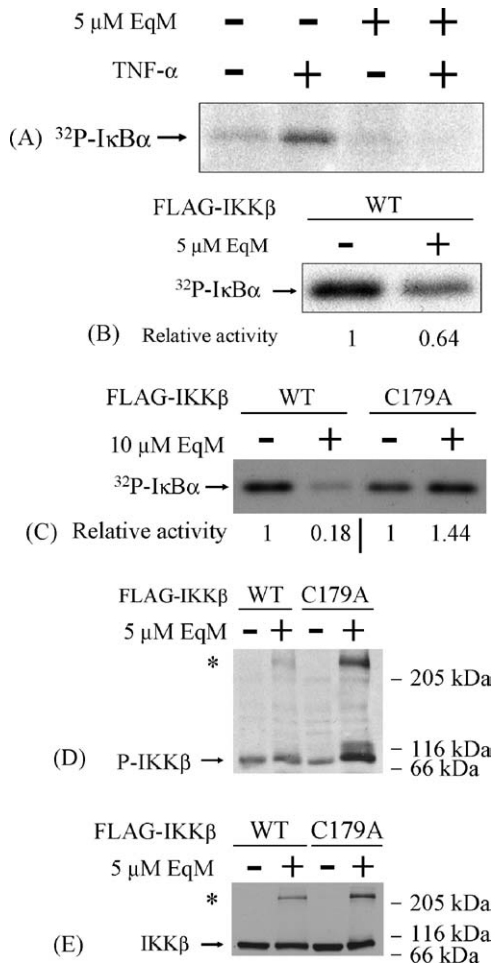

Fig. 2 – Epoxyquinone A monomer inhibits IKKβ kinase activity via targeting Cys179 of IKKβ. (A) 3T3 cells were pre-incubated for 2 h with or without 5 μM EqM, followed by stimulation with 20 ng/ml TNF-α for 7.5 min. The IKK complex was immunoprecipitated with a polyclonal anti-IKKα antibody and protein A Sepharose-beads. The in vitro IKK kinase activity was then analyzed in the immunocomplexes using GST-IκBα as a substrate. (B) 3T3 cells were transfected with an expression plasmid for FLAG-tagged wild-type IKKβ. A kinase assay (as in (A)) was then performed on anti-FLAG immunoprecipitates from transfected cells pretreated for 2 h with or without 5 μM EqM. The relative IKK activity is the average of three experiments. (C and D) Mouse 3T3 cells were transfected with FLAG-tagged wild-type IKKβ or mutant IKKβ C179A expression vector DNA and then left untreated or treated with the indicated concentrations of EqM for 2 h before harvesting. An in vitro kinase assay (C) or Western blotting using antiserum against phospho-IKKβ (Ser181) (D) was then performed. In (C), the kinase activity values in the presence of EqM are relative to the kinase activity for each FLAG-IKKβ protein in the absence of EqM. (E) Extracts from (D) were analyzed by Western blotting with anti-IKKβ antiserum. Arrows indicate the monomer forms and asterisks the "dimer" forms of IKKβ in (D) and (E).

lower panel). Increased DNA binding by the p65C38S mutant has been described previously [24].

3.4. *Pretreatment of cells with dithiothreitol (DTT) and two structural modifications of EqM reduce its ability to inhibit activation of NF-κB in mouse 3T3 cells*

DTT is a potent reducing agent and can protect thiol groups in proteins that have been oxidized. Because EqM appears to target specific cysteine residues in both IKKβ and p65, we were interested in determining whether DTT could protect cells from EqM-mediated inhibition of TNF-α-induced NF-κB activation. As shown in Fig. 4A, pretreatment of 3T3 cells with increasing concentrations of DTT dose-dependently abolished EqM-mediated inhibition of TNF-α-induced NF-κB DNA binding and IκBα degradation.

As a small-scale investigation of the structural requirements for EqM activity, we also synthesized two compounds related to EqM: *ent*-EqM, which is the optical isomer (enantiomer) of EqM, and EqM–thiophenol adduct (Fig. 4B). We then assessed the abilities of these EqM derivatives to inhibit TNF-α-induced NF-κB DNA binding in mouse 3T3 cells at various concentrations, as compared to EqM (Fig. 4C). *ent*-EqM was less effective than EqM at blocking activation of NF-κB, indicating that the absolute stereochemistry of EqM is important, but not essential for its activity. On the other hand, EqM–thiophenol adduct did not show detectable inhibition of NF-κB DNA binding at up to 20 μM, showing that a structural modification of the exocyclic alkene moiety of EqM can significantly impair its ability to inhibit activation of NF-κB. In summary, these results suggest that EqM inhibits its molecular targets via thiol group modification and that the exocyclic alkene moiety of EqM is critical for its ability to inhibit NF-κB.

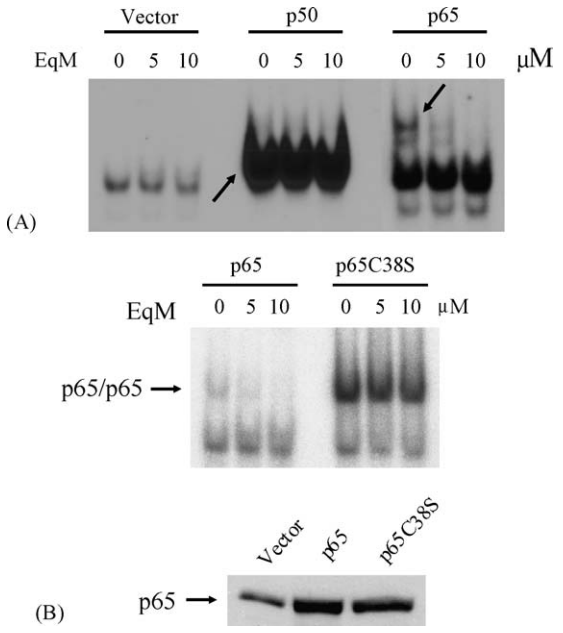

Fig. 3 – Epoxyquinone A monomer directly inhibits DNA binding of p65, but not p50, and Cys38 in p65 is critical for the inhibition by EqM. (A) A293 cells were transfected with empty vector, human p50, or mouse 65 expression vector for 2 days, followed by treatment with the indicated concentrations of EqM for 1 h. Extracts were then analyzed by an EMSA using a [^{32}P]-labeled κB-site oligonucleotide. The left arrow indicates the position of the p50 homodimer–DNA complex and the right arrow indicates the p65 homodimer–DNA complex. The p65 lanes were exposed longer than the p50 and vector lanes because the DNA-binding activity of p65 is weaker than p50. (B) A293 cells were transfected with an expression vector for mouse p65 or mutant p65C38S and then an EMSA was performed on extracts prepared from cells pretreated with indicated concentrations of EqM (as in (A)). At the bottom is shown an anti-p65 Western blot of cells transfected with a vector control or an expression vector for p65 or p65C38S.

3.5. *EqM inhibits constitutive NF-κB DNA binding and induces apoptosis in human SUDHL-4 lymphoma cells*

We and others [16,25] have previously shown that the SUDHL-4 lymphoma cell line has constitutive nuclear κB site-binding activity that consists primarily of p50/c-Rel heterodimers. Therefore, we sought to determine whether EqM has the ability to block κB-site DNA-binding activity and to induce cell killing in SUDHL-4 cells. In addition, we compared these activities of EqM in SUDHL-4 cells to epoxyquinol A, a natural dimeric epoxyquinoid that we have previously shown can also inhibit NF-κB activation, but less potently than EqM [15]. In these experiments, we first determined the concentrations of EqM and epoxyquinol A required to inhibit κB-site DNA binding in SUDHL-4 cells by incubating the cells with increasing concentrations of each compound for 3 h and then performing an EMSA. As shown in Fig. 5A, 0.5 μM EqM and 5 μM epoxyquinol A resulted in approximately 50% inhibition of κB-site DNA-binding activity. We then determined the effect of various concentrations of EqM and epoxyquinol A on the proliferation of SUDHL-4 cells. As shown in Fig. 5B, at 72 h after treatment, the proliferation of SUDHL-4 cells was inhibited by approximately 60% in 0.5 μM EqM and 70% in 5 μM epoxyquinol A. Taken together, these results show that there is a general correlation between the concentrations of EqM and epoxyquinol A required to block both NF-κB DNA binding and cell proliferation in human SUDHL-4 lymphoma cells. Furthermore, treatment of SUDHL-4 cells with EqM appears to be inhibiting their proliferation by inducing apoptosis because EqM-treated cells show increased caspase-3 activity (Fig. 5C) and dose-dependent cleavage of the cell-death caspase substrate PARP (Fig. 5D). As a control, we show that the epoxyquinoid jesterone dimer, which we have previously characterized [16], also induces caspase-3 activity and PARP cleavage in SUDHL-4 cells (Figs. 5C and D).

3.6. *EqM inhibits the growth of several human leukemia, kidney, and colon cancer cell lines in the NCI 60-cell line panel*

The effectiveness of EqM at inhibiting the growth of the NCI panel of 60 human tumor cell lines was evaluated. As shown in Fig. 6A, treatment of cells with EqM preferentially inhibited the growth of the human leukemia cell lines CCRF-CEM, HL-60(TB), RPMI-8226 and SR, colon cancer cell lines HCT-116 and

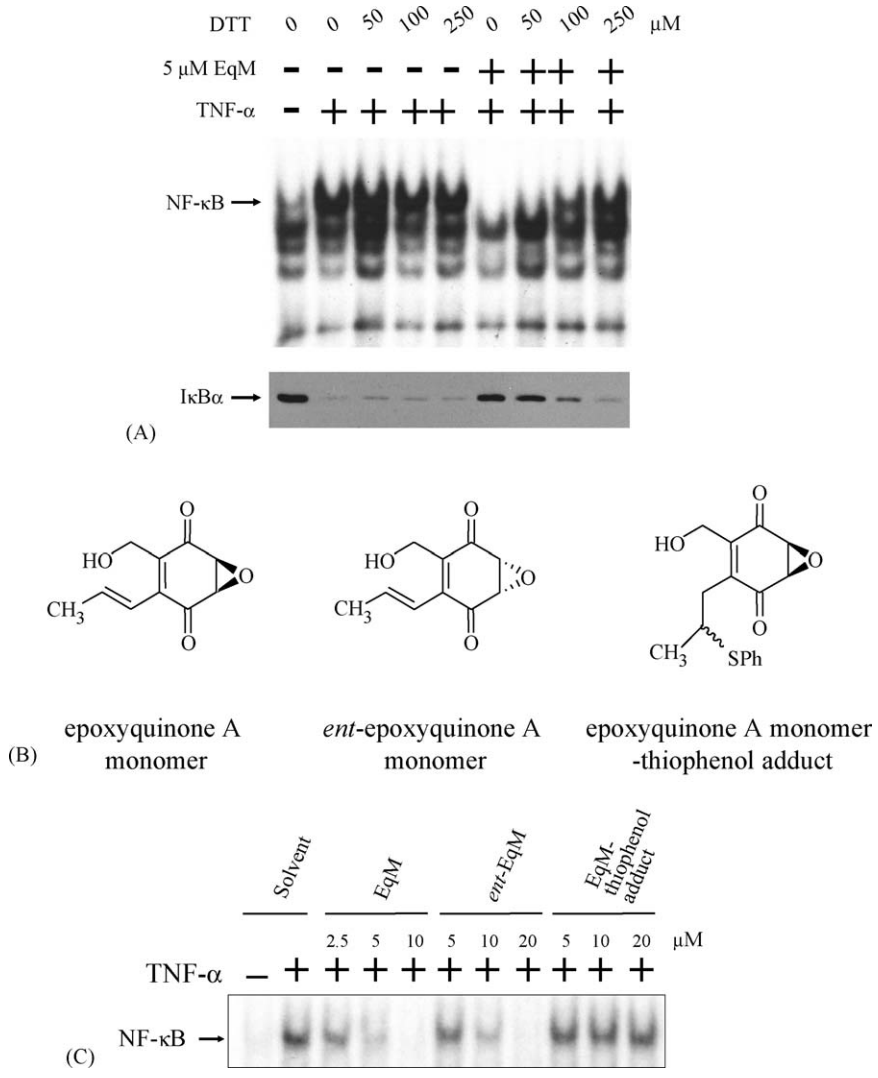

Fig. 4 – The effect of DTT pretreatment and EqM structural modifications on inhibition of NF-κB inhibition by EqM. (A) Mouse 3T3 cells were pre-incubated in the presence of increasing concentrations of DTT for 2 h prior to treatment with or without 5 μM EqM for an additional 2 h. Cells were then stimulated with 5 ng/ml TNF-α for 20 min before harvesting. Extracts were subjected to a κB site EMSA (upper panel) or Western blotting using antiserum against IκBα (lower panel). The position of the NF-κB–DNA complex is indicated. (B) Structures of epoxyquinone A monomer (EqM), *ent*-epoxyquinone A monomer (*ent*-EqM), and epoxyquinone A monomer–thiophenol adduct (EqM–thiophenol adduct). (C) 3T3 cells were pre-incubated with the indicated concentrations of the named compounds for 2 h and extracts from 2 ng/ml TNF-α-stimulated cells were subjected to an EMSA (as in (A)).

SW-620, and renal cancer cell line 786-0 (among others) with GI_{50} concentrations ranging from 0.04 to 0.72 μM. In contrast, the non-small cell lung cancer lines (e.g., NCI-H322M) and central nervous system cancer lines (e.g., SNB-75) were resistant to EqM, at concentrations as high as 34 μM. These patterns were also observed at the total growth inhibition (TGI) level of response (data not shown).

To determine whether these EqM-induced growth inhibitory responses were correlated with the amount of constitutive NF-κB DNA-binding activity in this panel of cells, we chose to focus on the six colon cancer cell lines, in that many of these cell lines have previously been shown to have constitutive NF-κB DNA-binding activity [26–29]. As shown in Fig. 6B, the amount of constitutive NF-κB DNA-binding activity varied among these cell lines. In general (with the exception of the HT29 cell line), the cells with the least amount of κB-site DNA-binding activity (HCT-116, HCT-15, and SW-620) were most sensitive to EqM-induced growth inhibition.

Lastly, colon cancer cell line KM12 (which has the greatest amount of constitutive NF-κB DNA-binding activity) and cell line SW-620 (which has the least constitutive NF-κB DNA-binding activity) were analyzed for the induction of apoptosis by EqM (as judged by N-terminal cleavage of the caspase substrate PARP and the formation of fragmented DNA ladders characteristic of apoptosis). In the KM12 cell line, EqM induced both caspase-directed cleavage of PARP and the formation of nucleosome-sized DNA ladders (Fig. 6C), both consistent with the induction of apoptosis. In contrast, in the SW-620 cell line,

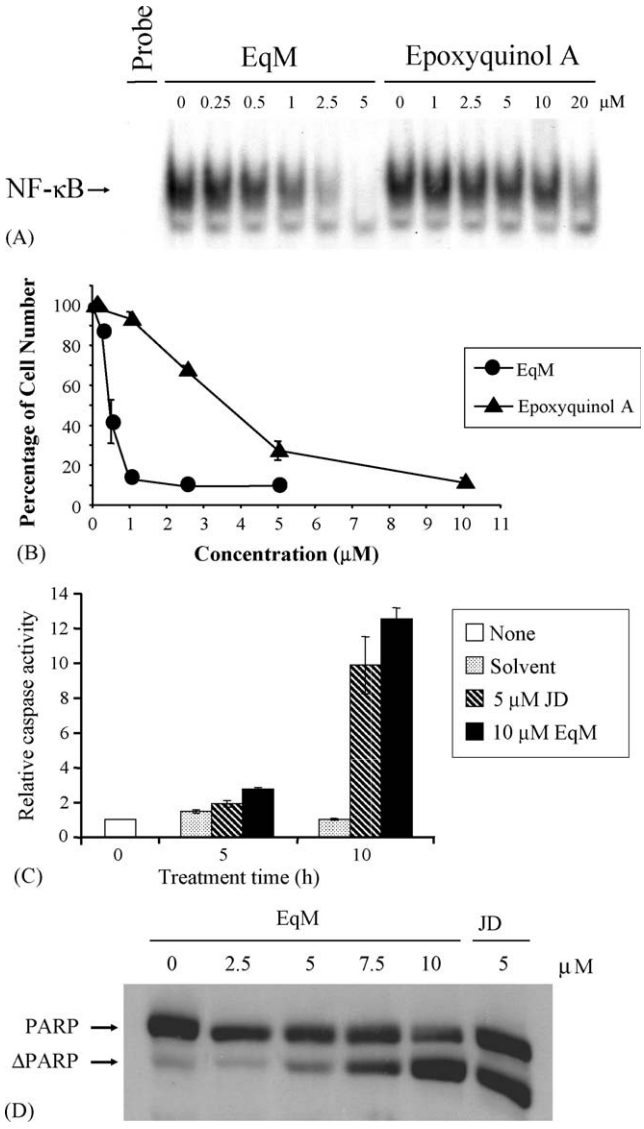

Fig. 5 – Epoxyquinone A monomer inhibits NF-κB DNA binding and cell growth and induces caspase-3 activity in SUDHL-4 diffuse large B-cell lymphoma cells. (A) SUDHL-4 cells were incubated with various concentrations of EqM or epoxyquinol A for 3 h as indicated. An EMSA using a κB site probe was then performed on extracts from SUDHL-4 cells. The position of the NF-κB DNA-binding complex is indicated. (B) 10^5 SUDHL-4 cells were plated in 16-mm wells in 0.5 ml of DMEM supplemented with 10% fetal bovine serum for 6 h prior to treatment. SUDHL-4 cells were incubated with the indicated concentrations of EqM or epoxyquinol A. Cells in triplicate wells for each treatment were then counted 3 days later. (C) SUDHL-4 cells were incubated for the indicated times with no addition (None), solvent, jesterone dimer (JD; 5 μM), or EqM (10 μM). Total caspase-3 activity in extracts was measured as described in Section 2. (D) SUDHL-4 cells were incubated for 16 h with the indicated concentrations of EqM or JD before harvesting. PARP cleavage was monitored by anti-PARP Western blotting. The upper band indicates the position of full-length PARP, and the lower band (ΔPARP) indicates the position of the caspase-cleaved form of PARP.

EqM treatment induced neither DNA laddering nor caspase-directed cleavage of PARP (Fig. 6C), even though these cells were obviously dying (as judged by cell rounding and detachment from the plate). Moreover, both PARP and actin were non-specifically degraded in SW-620 cells after treatment with EqM. The lack of DNA ladders and the complete degradation of PARP and actin suggest that EqM induces cell death in the SW-620 cell line by necrosis, which is consistent with the previous finding that aspirin (which, like EqM, can also inhibit IKKβ [30]) induces necrosis in the SW-620 cell line [31].

4. Discussion

In this report, we show that the synthetic epoxyquinoid EqM blocks TNF-α-induced activation of NF-κB by inhibiting both IKKβ activity and NF-κB DNA binding, and both inhibitions appear to require specific reactive Cys residues (in IKKβ and p65). In addition, EqM treatment of a human lymphoma cell line with constitutive nuclear κB site-binding activity inhibits both NF-κB DNA binding and cell growth, and induces apoptosis. Furthermore, EqM selectively inhibits the growth of several leukemia, colon, and kidney cancer cell lines. These results suggest that EqM, a monomeric derivative of the fungal metabolite epoxyquinol A, may provide insight into methods of intervening in diseases that require activation of the NF-κB pathway for their pathophysiology.

Interestingly, EqM is an oxidized variant of a related molecule, ECH ((2R, 3R, 4S)-2,3-epoxy-4-hydroxy-5-hydroxymethyl-6-(1E)-propenyl-cyclohex-5-en-1-one), which has been shown to inhibit Fas-mediated apoptosis by interacting with and blocking self-activation of pro-caspase-8 in the death-inducing signaling complex (DISC) [32,33]. On the other hand, EqM (RKTS-32) did not block Fas-induced apoptosis, but showed significant cell toxicity [34]. These findings suggest that EqM and ECH have different cellular targets and bioactivities.

Our results show that specific Cys residues in both IKKβ (Cys179) and p65 (Cys38) are required for EqM to inhibit their activity. In addition, pretreatment of cells with dithiothreitol, which may reduce reactive Cys residues in target proteins and/or inactivate the Cys-reactivity of EqM, blocks the ability of EqM to inhibit TNF-α-induced NF-κB DNA binding. Thus, the simplest model to explain these results is that EqM reacts directly with Cys179 and Cys38 in IKKβ and p65, respectively, to inhibit their activities. However, these need not be the only Cys residues that EqM reacts with in each of these proteins; for example, EqM and the epoxyquinoid jesterone dimer (JD) can both cross-link an IKKβ C179A mutant to a covalently modified high-molecular-weight form [16] (Fig. 2E), even though EqM and JD do not inhibit the kinase activity of IKKβ C179A (Fig. 2C; data not shown). Moreover, the IKKβ C179A mutant shows slightly increased kinase activity and increased phosphorylation of Ser181 (a modification that increases IKKβ activity [2]) when cells are treated with EqM (see Fig. 2C and D), suggesting that EqM interacts with the IKKβ C179A mutant through Cys residues other than Cys179 to increase its activity (which is not inhibited due to the lack of a reactive Cys residue at position 179). A number of other

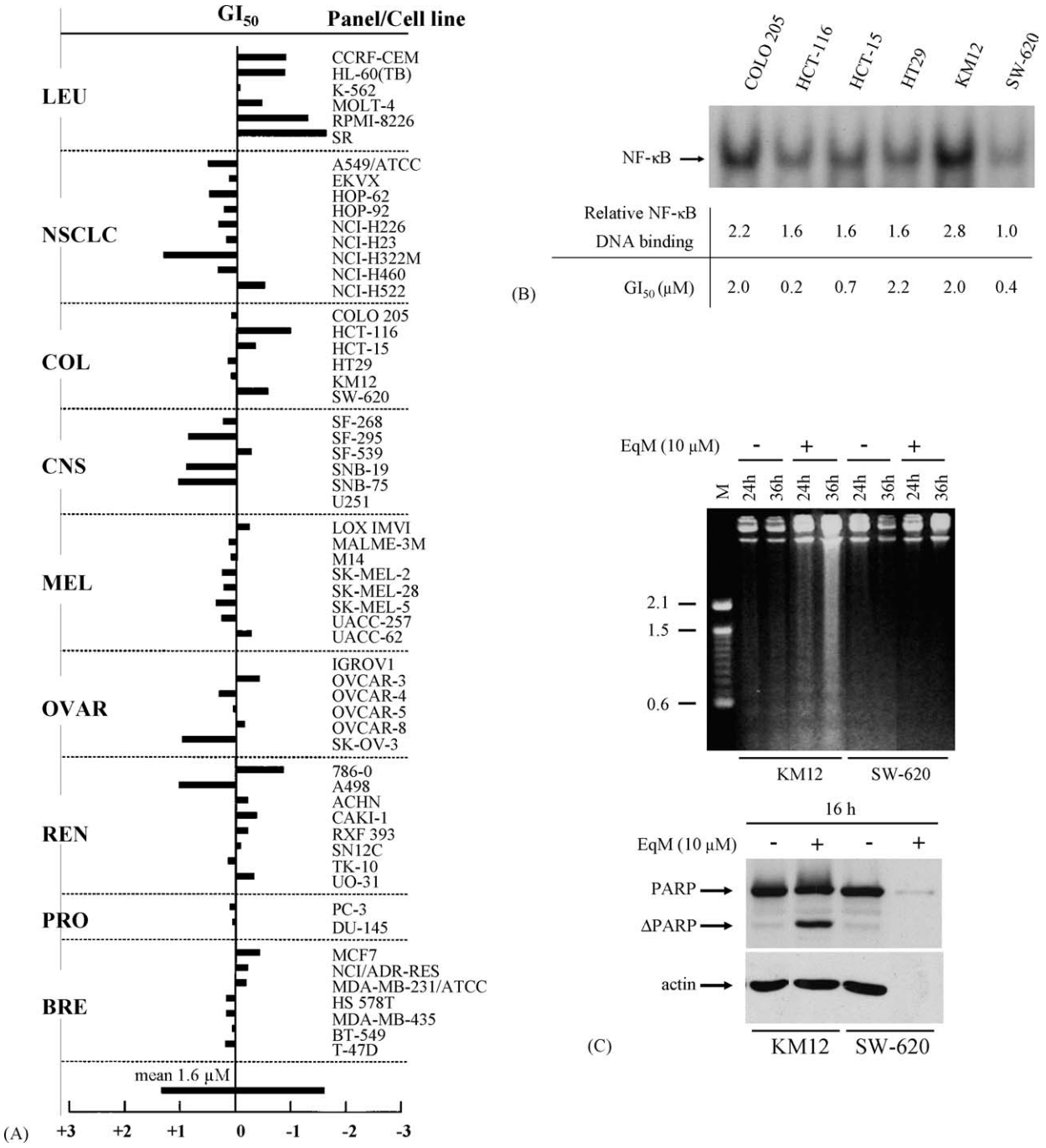

Fig. 6 – Epoxyquinone A monomer exhibits growth inhibition in leukemia, colon, and renal cancer lines from the NCI's human tumor cell-based screen. (A) Growth inhibition at 50% (GI_{50}) was assessed for tumor cell lines from the indicated types of cancers. LEU, leukemia; NSCLC, non-small cell lung cancer; COL, colon; CNS, central nervous system; MEL, melanoma; OVAR, ovarian; REN, renal; PRO, prostate; BRE, breast. (B) Comparison of constitutive DNA-binding activity and EqM-induced growth inhibition in six colon cancer cell lines. A κB-site EMSA was performed using equal amounts of protein from the indicated colon cancer cell lines (see (A)). The relative amount of NF-κB DNA binding (an average of three experiments) and the GI_{50} (from (A)) are indicated below each lane. (C) Cell lines KM12 and SW-620 were treated with the solvent methanol (−) or 10 μM EqM (+) for the indicated times and cell lysates were analyzed for DNA-laddering (upper panel) or PARP cleavage (lower panel) as described for Fig. 5D. DNA size markers. (M; upper panel) are expressed in kilobase pairs.

reactive compounds, including the sesquiterpene lactones parthenolide, helenalin, and 4β,15-epoxy-miller-9*E*-enolide [23,24,35,36], cyclopentenone prostaglandins prostaglandin A_1 and 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 [37–39], arsenite [40], and the gold compound auranofin [41], have been shown to require Cys179 for inhibition of IKKβ activity and/or Cys38 for inhibition of NF-κB DNA binding. Moreover, like EqM, some compounds, such as the avicins [42], 15-deoxy-$\Delta^{12,14}$-prostaglandin J_2 [38,39], and parthenolide [23,24], have been shown to block two steps in NF-κB activation.

Natural products containing epoxides or α,β-unsaturated ketones are known to react with nucleophilic functionalities such as thiols that are present in biomolecules [43–47]. There is also precedent for modified biological activity of natural products containing an α,β unsaturated ketone moiety after reaction with nucleophiles such as thiophenol or glutathione [48–51]. It is possible that the exocyclic alkene side chain of EqM is a key point of covalent attachment to Cys residues in its biologically relevant protein target(s), which may account for the disruption of EqM's inhibitory activity by modification of that moiety in EqM–thiophenol adduct (Fig. 4B and C). However, given that EqM has at least two reactive points of attachment, EqM could modify single protein targets or could cross-link two (and less likely, three) protein targets. The identification of the complete profile of molecular targets of EqM will likely shed light on its molecular mechanism(s) of action.

In previous work, we identified the synthetic epoxyquinoid jesterone dimer as an IKK inhibitor [16], and showed that, like EqM, JD is an effective blocker of NF-κB DNA-binding activity and an inducer of apoptosis in the SUDHL-4 cell line [16]. DHMEQ, a derivative of the antibiotic epoxyquinomicin C, is also an effective inhibitor of activation of NF-κB [7] and has been shown to suppress the growth of several cancer cell types, including T-cell leukemia, multiple myeloma, breast, prostate, and thyroid cancer, in mouse models [9,10,52–54]. In this report, we have assessed the growth inhibitory activity of EqM against a panel of human tumor cell lines: among these cell lines, EqM appeared to be most potent against leukemia, colon, and kidney cancer cell lines. Interestingly, a number of studies have shown that these three tumor cell types often have constitutive nuclear NF-κB DNA-binding activity [27,28,55–57]. Among the six colon cancer cell lines in the NCI panel, we found that, in general, the cell lines with the lowest amount of constitutive NF-κB DNA-binding activity (such as HCT-116, HCT-15, and SW-620) were most sensitive to growth inhibition by EqM (see Fig. 6B). These results suggest that reduction of NF-κB DNA binding in these colon cancer cells to below a threshold level, which might be reached more readily in cells with lower amounts of constitutive DNA-binding activity, is at least one requirement for growth inhibition by EqM. The one exception to the correlation between levels of NF-κB DNA-binding activity and sensitivity to EqM-induced growth inhibition is colon cancer cell line HT29, which has somewhat low levels of NF-κB DNA-binding activity and yet required the highest dose (among the six cell lines) for growth inhibition by EqM. Interestingly, proteasome inhibitors, which generally block NF-κB induction by inhibiting degradation of IκB, have been shown to *activate* the NF-κB pathway in HT29 cells [58]. Thus, the effects of EqM on NF-κB activity (and consequently cell growth) in HT29 cells may be different than in the other five colon cancer cell lines studied herein, further suggesting that one cannot always predict the effects of specific pathway inhibitors in all tumor cell lines. In addition, we have found that EqM induces apoptosis in the colon cancer cell line (KM12) with the highest level of NF-κB DNA-binding activity, but necrosis in the cell line (SW-620) with the least amount of NF-κB DNA-binding activity (Fig. 6C).

In summary, we have described a molecule, epoxyquinone A monomer, that inhibits two steps in the NF-κB signaling pathway and can efficiently inhibit tumor cell viability. Recently, we have shown that EqM and JD can induce apoptosis in lymphoma cell lines with and without IκBα, suggesting that these epoxyquinoids can use multiple NF-κB signaling targets to kill tumor cells [59]. Further development and characterization of compounds, like EqM, with multiple targets within single signal transduction pathways may lead to more effective anti-tumor and anti-inflammatory therapeutics.

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