

Inactivation of NF- κ B Components by Covalent Binding of (–)-Dehydroxymethylepoxyquinomicin to Specific Cysteine Residues

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Received May 26, 2008

Previously, we designed and synthesized a potent NF- κ B inhibitor, DHMEQ. Although DHMEQ showed potent anti-inflammatory and anticancer activities in animals, its molecular target has not been elucidated. In the present study, its target protein was found to be p65 and other Rel homology proteins. We found that (–)-DHMEQ bound to p65 covalently with a 1:1 stoichiometry by conducting SPR and MALDI-TOF MS analyses. MS analysis of the chymotrypsin-digested peptide suggested the binding of (–)-DHMEQ to a Cys residue. Formation of Cys/(–)-DHMEQ adduct in the protein was supported by chemical synthesis of the adduct. Substitution of specific Cys in p65 and other Rel homology proteins resulted in the loss of (–)-DHMEQ binding. (–)-DHMEQ is the first NF- κ B inhibitor that was proven to bind to the specific Cys by chemical methodology. These findings may explain the highly selective inhibition of NF- κ B and the low toxic effect of (–)-DHMEQ in cells and animals.

Introduction

NF- κ B^a is a family of transcription factors up-regulating the expression of inflammatory cytokines, adhesion molecules, and antiapoptosis proteins. NF- κ B is a homo- or heterodimer of Rel family proteins having the Rel homology domain (RHD). Rel family proteins include p65 (RelA), p50, RelB, c-Rel, and p52. p65, c-Rel, and RelB have a trans-activation domain (TAD), although p50 and p52 do not.¹ Therefore, the p50 or p52 homodimer cannot activate the transcription of genes having the κ B site in their promoter. Interestingly, these complexes competitively inhibit the activity of other NF- κ B component dimers.² NF- κ B members are categorized into the canonical and noncanonical pathways. The typical canonical pathway employs the p65/p50 heterodimer, which is regulated by the I κ B proteins. I κ B- α masks the nuclear localization signal (NLS) of NF- κ B proteins and inactivates their function by inhibiting the nuclear translocation.³ NF- κ B is not constitutively activated in most normal cells. However, various signals such as cytokines, growth factors, bacterial and viral components, radiation, and reactive oxygen species often activate TRAF-6 or TRAF-2, which leads to the phosphorylation of I κ B- α by the activated IKK- α /IKK- β /NEMO complex.⁴ The phosphorylated I κ B- α is then ubiquitinated and eventually degraded by proteasomes.⁵ Next, the activated NF- κ B binds to importin to be translocated into the nucleus.⁶ Once there, NF- κ B binds to the κ B enhancer motif in the promoter region of the target gene. On the other hand, the noncanonical pathway NF- κ B typically consists of RelB and p100 as the inactive form. In this heterodimer, RelB is inactivated by p100 instead of I κ B and is located in the

cytoplasm as RelB/p100. After stimulation, the activated IKK- α homodimer leads to the phosphorylation and proteasome-mediated processing of p100 to generate p52.⁷

Earlier we designed and synthesized a novel NF- κ B inhibitor, dehydroxymethylepoxyquinomicin, abbreviated as DHMEQ.⁸ DHMEQ is synthesized from 2,5-dihydroxyaniline in five steps as its racemic form. After the chiral separation, (–)-DHMEQ (Figure 1A) was shown to be 10 times more potent than (+)-DHMEQ.⁹ It was shown to inhibit the nuclear translocation of NF- κ B.¹⁰ In animals, DHMEQ shows potent anti-inflammatory effects on rheumatoid arthritis,¹¹ renal inflammation,¹² and ameliorates cancer cachexia¹³ and suppresses osteoclastogenesis.¹⁴ It also suppresses the growth of prostate carcinoma,¹⁵ thyroid carcinoma,¹⁶ breast carcinoma,¹⁷ pancreatic carcinoma,¹⁸ multiple myeloma,¹⁹ and adult T-cell leukemia²⁰ in nude or SCID mice without any toxicity.²¹ In solid cancer models, it is likely that DHMEQ rather suppresses the inflammation induced by inflammatory and cancer cells around the tumor site to inhibit the tumor growth.²¹ Although DHMEQ has been widely used in *in vivo* and *in vitro* studies, its molecular target has not been elucidated.

In the present study, we searched for the molecular target of (–)-DHMEQ. As a result, we found that (–)-DHMEQ directly bound to the Rel family proteins through a specific Cys residue.

Results and Discussion

1. Covalent Binding of (–)-DHMEQ with p65. In addition to the inhibition of nuclear translocation of NF- κ B, (–)-DHMEQ was suggested to inhibit DNA binding of NF- κ B in nonsmall cell lung carcinoma A549 cells (data not shown). We examined the DNA binding of commercially available full-length p65 in a reconstitution system solely consisting of κ B DNA, p65, and (–)-DHMEQ. (–)-DHMEQ did inhibit the protein binding completely (Figure 1B). We tried to prepare the full-length p65 in *Escherichia coli*, but the expression was not enough. Then we employed p65(1–325), which included the Rel homology domain and NLS. (–)-DHMEQ effectively inhibited the DNA binding of this protein, p65(1–325), and we found that the stoichiometry of 1:1 provided enough of the protein to inhibit

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^a Abbreviations: NF- κ B, nuclear factor κ B; DHMEQ, dehydroxymethylepoxyquinomicin; TRAF, tumor necrosis factor receptor-associated factor; I κ B, inhibitor of κ B; IKK, I κ B kinase; NEMO, NF- κ B essential modulator; SCID mouse, severe combined immunodeficiency disease; EMSA, electrophoresis mobility shift assay; GST, glutathione S-transferase; SPR, surface plasmon resonance; MALDI TOF-MS, matrix assisted laser desorption ionization-time-of-flight mass spectrum; CRM1, chromosome region maintenance protein 1.

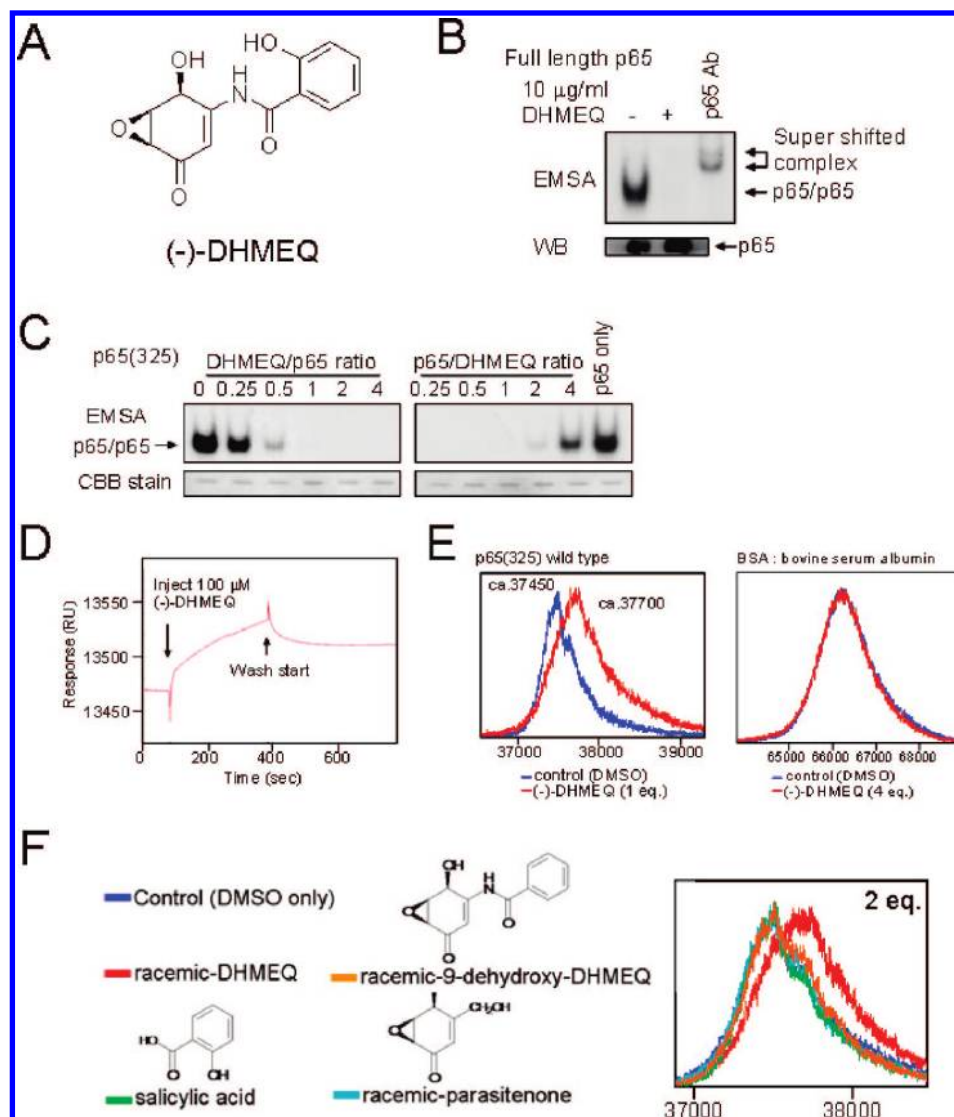


Figure 1. Direct interaction of (-)-DHMEQ with p65 in SPR and MALDI-TOF MS analysis. (A) Structure of (-)-DHMEQ. (B) In vitro binding assay with EMSA using full-length recombinant p65. The p65 protein was incubated with 10 μ g/mL (-)-DHMEQ before the addition of the labeled probe. (C) In vitro binding assay with EMSA using p65(1–325). The p65(1–325) protein was treated with the indicated equivalent molar amounts of (-)-DHMEQ (left; 20 μ M p65) or of p65(1–325) (right; 20 μ M (-)-DHMEQ). (D) SPR sensorgram showing (-)-DHMEQ binding to p65(1–325) on the CM5 sensor chip surface. (-)-DHMEQ (100 μ M) in PBS containing 5% DMSO was injected for 5 min. Data are given as the response units (RU). (E) MALDI-TOF MS analysis of p65(1–325). Recombinant p65(1–325) was untreated or treated with 1 equiv (-)-DHMEQ. BSA was incubated with or without 4 equiv of (-)-DHMEQ. (F) MALDI-TOF MS analysis of p65(1–325) with DHMEQ and its analogs. The p65(1–325) protein (20 μ M) was treated with 2 equiv of racemic DHMEQ or its analogs for 1 h. After incubation, the proteins were used for the MALDI TOF-MS analysis. We employed racemic DHMEQ instead of (-)-DHMEQ in this experiment because we only had racemic 9-dehydroxy-DHMEQ and parasitenone.

the binding completely, as shown in Figure 1C. Physical interaction of (-)-DHMEQ with p65(1–325) was demonstrated by the surface plasmon resonance analysis (Figure 1D). Next we studied the specificity and covalent binding to p65 by using MALDI-TOF MS analysis. We found that (-)-DHMEQ at a 1:1 stoichiometry shifted the peak of p65(1–325) with the increase equaling the approximate MW of DHMEQ (261), as shown in Figure 1E. On the other hand, it did not shift the peak of bovine serum albumin (BSA), even when 4 equiv were added (Figure 1E). We also studied the effect of (+)-DHMEQ on the p65/DNA binding activity and MALDI-TOF-MS peak shift. The (+)-enantiomer showed limited inhibition on the DNA binding even at 4 equiv. It also shifted the peak only weakly even at 2 equiv. Interestingly, (+)-DHMEQ did not shift the peak at all with the C38S mutant protein, suggesting that it could bind to Cys38, but only weakly. These results are consistent with our report that (+)-DHMEQ would be about 10 times weaker than

(-)-DHMEQ in inhibition of NF- κ B.⁹ As shown in Figure 1F, 9-dehydroxy-DHMEQ, parasitenone, and salicylic acid, all inactive structural analogs of DHMEQ, failed to shift the peak.

2. MALDI-TOF MS Analysis of Chymotrypsin-Treated p65 Peptide. To find the possible target peptide or amino acid in p65, we looked into the MALDI-TOF MS pattern after the protease digestion. The p65(1–325) protein was treated with (-)-DHMEQ and digested with chymotrypsin. This enzyme is known to hydrolyze peptide bonds on the C-terminal side of Tyr, Phe, and Trp residues. Chymotrypsin also weakly hydrolyzes after Leu. One of the expected fragments, peptide 37–66, contains a cysteine residue at 38 (Figure 2A). Fortunately, there was no Leu in the 37–66 residues of p65(1–325). As shown in Figure 2B, there was the peak of 3205 Da identical to 37–66. When reacted with (-)-DHMEQ, the peptide also gave a peak at 3448, which may be attributed to the DHMEQ-bound peptide

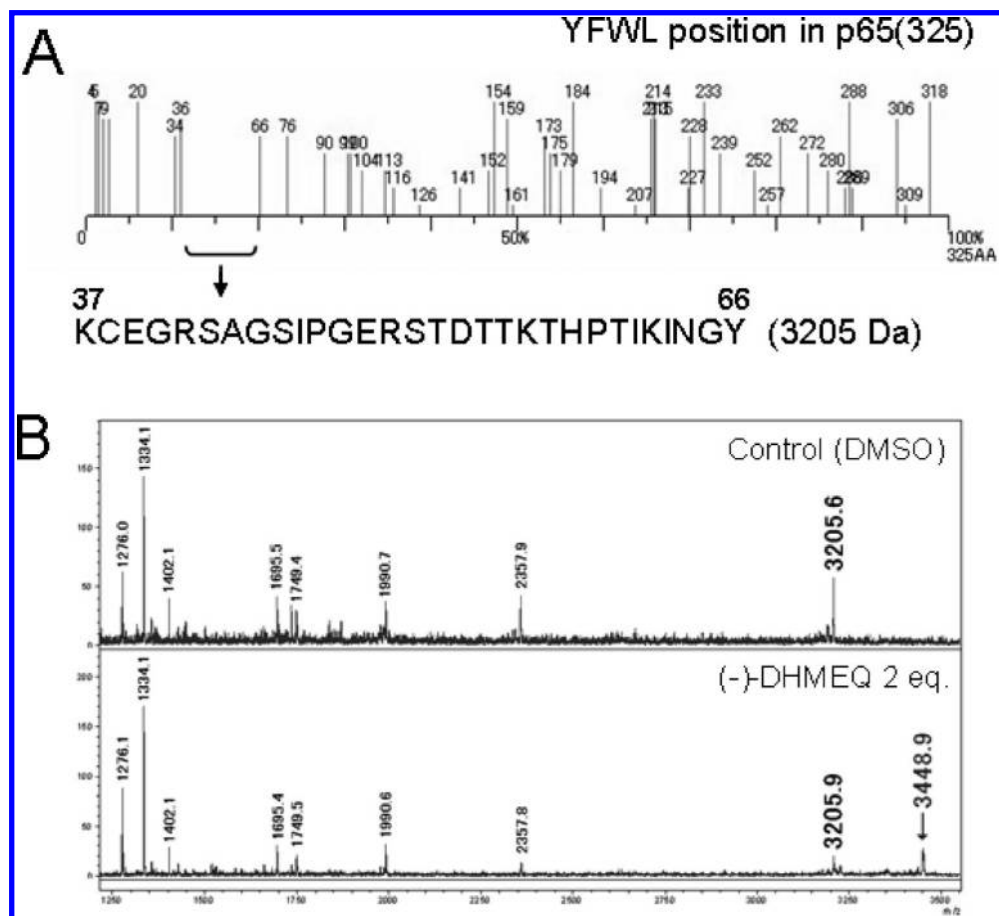


Figure 2. Peptide analysis of (-)-DHMEQ/p65(1-325) adduct after protease digestion. (A) Profile of chymotrypsin-digested peptides. Chymotrypsin hydrolyzes the peptide-bond C-terminal to tyrosine, phenylalanine, tryptophan, and leucine residues. The prospective peptide containing Cys38 is the peptide 37-66, with a MW of 3205 Da. (B) MALDI-TOF MS analysis of chymotrypsin-digested (-)-DHMEQ/p65(1-325) adduct. (upper panel) The fragment of mass 3205 corresponds to the 37-66 peptide. (lower panel) The p65(1-325) protein was treated with (-)-DHMEQ. The new peak of 3448 corresponds to the mass of 3205 + dehydrated DHMEQ.

(Figure 2B). The difference in MW, which was 243, would explain the structure of dehydrated DHMEQ. Thus, (-)-DHMEQ was suggested to bind to Cys38 in p65.

3. Preparation of Cys/(-)-DHMEQ Adducts. To support the possible structure of DHMEQ attached to p65, we prepared the conjugate of (-)-DHMEQ with *N*-*t*-butyloxycarbonyl-L-Cys methyl ester (Figure 3). The adduct compound was obtained by the reaction in DMF and sodium phosphate buffer at room temperature. The mass and NMR spectra showed the epoxide ring-opening structure in the adduct molecule. It is likely the thiol group of Cys might make a nucleophilic attack on the epoxide of (-)-DHMEQ. The regiochemistry was determined by 2D NMR spectrum analysis, using ^1H - ^1H COSY, HMQC, and HMBC. The one proton doublet at $\delta = 3.38$ ppm was assigned to the proton on the C-4 of (-)-DHMEQ to which Cys bound and its corresponding carbon signal was observed at $\delta = 51.6$ ppm on the basis of HMQC. The carbon signal at $\delta = 51.6$ ppm showed long-range C-H correlation to H-2 at $\delta = 6.92$ ppm and, consequently, the signal was assigned to C-4, as shown in the HMBC spectrum. The proposed structure possesses diols at C-5 and C-6, which can form an isopropylidene structure. Then, to confirm this regiochemistry, we also prepared the *O*-isopropylidene derivative, as described in the Experimental Section, Synthesis 4. Thus, it is likely that (-)-DHMEQ would bind to Cys38 of p65, forming a new S-C covalent bond. The (-)-DHMEQ/Cys adduct molecule did not inhibit NF- κ B even at high concentrations (data not shown). For the experiment in Figure 2B, the digested peptide had been

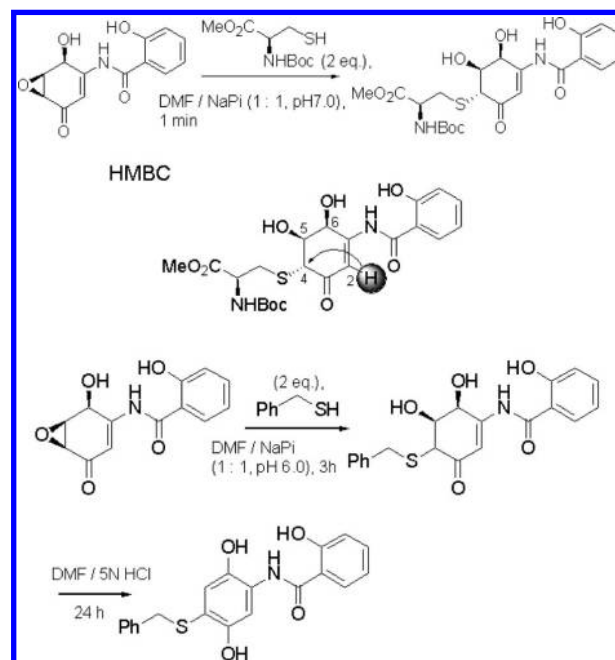


Figure 3. Preparation of (-)-DHMEQ/protected Cys adduct. Reaction of (-)-DHMEQ with *t*-Boc-L-cysteine methyl ester resulted in the epoxide opening to give a new C-S bond. To obtain dehydrated (-)-DHMEQ, we prepared benzylthio(-)-DHMEQ. Acid treatment of this compound gave the dehydrated product.

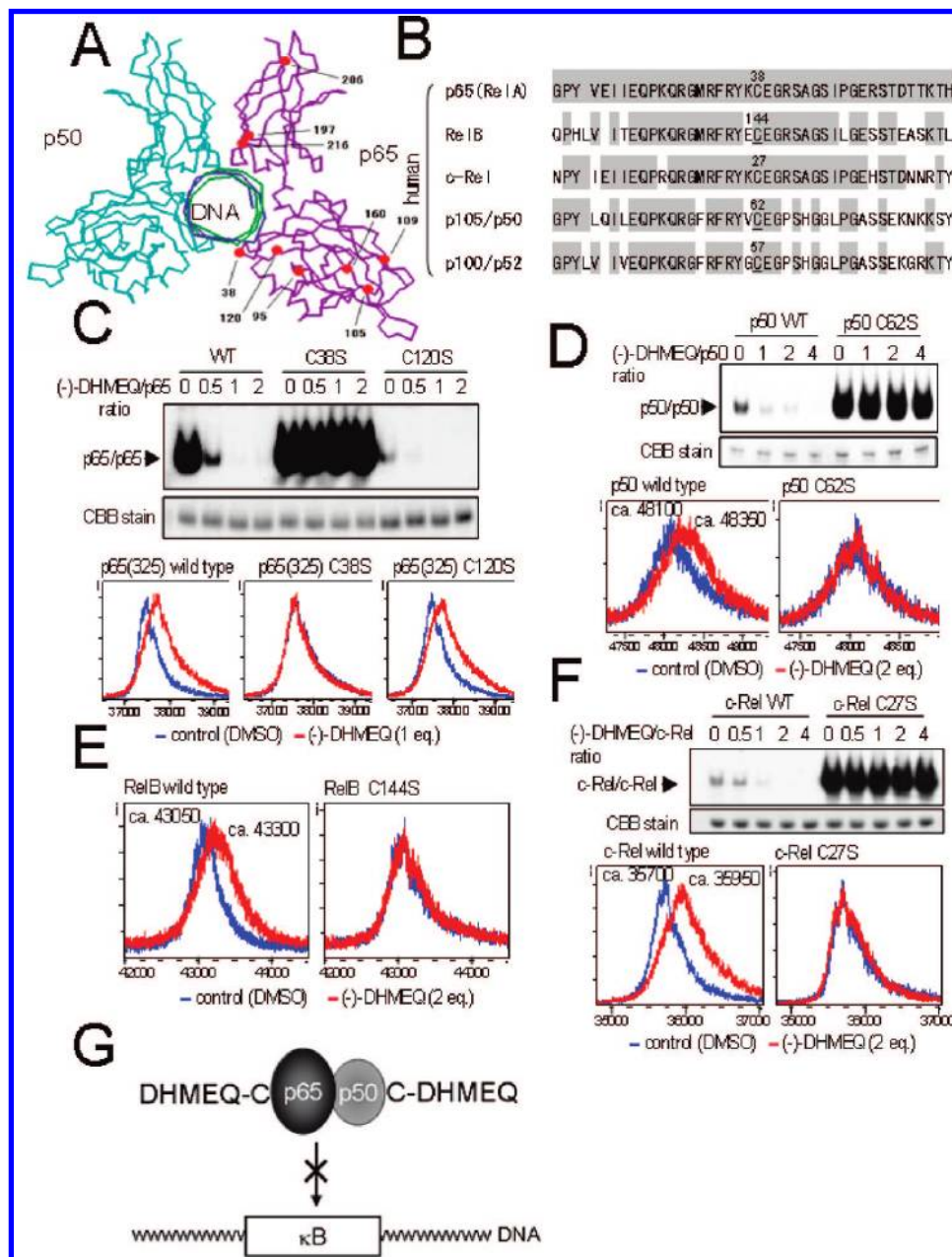


Figure 4. Binding of (-)-DHMEQ to specific Cys in Rel family proteins. (A) A schematic drawing of p65-p50-DNA complex (Chen et al. *Nature* **1998**, 391, 410-413). Both p65 Cys38 and Cys120 are located close to the DNA in the complex. (B) Sequence alignment of Rel family proteins around Cys38 of p65. All the Rel proteins have a cysteine residue corresponding to p65 Cys38. (C) In vitro binding assay with EMSA and MALDI-TOF MS using wild-type p65(1-325), p65(1-325) C38S, and p65(1-325) C120S. Each p65(1-325) of 20 μ M in PBS was treated with the indicated equivalents of (-)-DHMEQ. (D) In vitro DNA-binding assay and MALDI-TOF MS analysis of wild-type p50 and p50 C62S. (E) MALDI-TOF MS analysis of wild-type RelB(1-391) and RelB(1-391) C144S. (F) In vitro DNA-binding assay and MALDI TOF-MS analysis of wild-type c-Rel(1-307) and c-Rel(1-307) Cys27Ser (C27S).

treated with trifluoroacetic acid (TFA). Then we treated the adduct molecule with TFA, which was not successful, because the Boc protecting group of the (-)-DHMEQ/Cys adduct was eliminated under the acid condition. So, we prepared benzylthio (-)-DHMEQ and treated it with HCl. As a result, we obtained dehydrated benzyl-thio (-)-DHMEQ.

4. Covalent Binding of (-)-DHMEQ to Specific Cys. As shown in Figure 4A, the Cys38 and Cys120 residues of p65 are located close to the DNA in the p65/p50/DNA complex. In addition, the Rel family proteins include p65, p50, RelB, c-Rel, and p52. The sequence homology between them is shown in Figure 4B, with the alignment made so as to bring the positions equivalent to Cys38 in p65. The Cys38Ser (C38S) mutant of p65(1-325) bound to κ B DNA strongly, as reported previ-

ously,²² and this binding was not inhibited by (-)-DHMEQ at any (-)-DHMEQ/p65 ratio (Figure 4C). The C120S mutant weakly bound to κ B DNA, as reported,²² and this binding was effectively inhibited by (-)-DHMEQ. The same results were obtained by the MALDI-TOF MS analysis. The (-)-DHMEQ shifted the peak of C120S mutant but not that of C38S, as shown in Figure 4C. Then, the p50 protein was prepared, and the κ B binding of the p50 dimer was inhibited by (-)-DHMEQ almost to the same extent as the p65 one (Figure 4D). The DNA binding of the C62S mutant was not inhibited by (-)-DHMEQ (Figure 4D). The same results were obtained with p50 and p50(C62S) by the MALDI-TOF MS analysis, as shown in Figure 4D. Because it was difficult to prepare enough full-length RelB, we prepared RelB(1-391). The binding of (-)-DHMEQ to

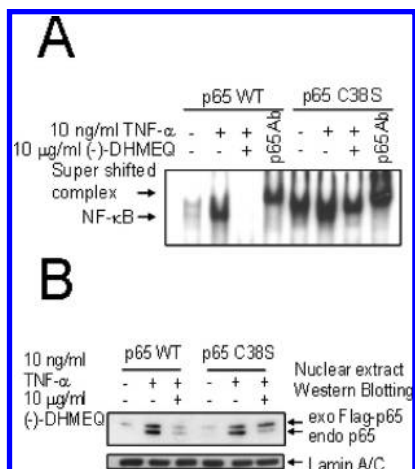


Figure 5. (–)-DHMEQ binds to p65 Cys38 in cultured cells. (A) Effect of (–)-DHMEQ on wild-type and C38S mutant p65 DNA binding activity in cultured Hela cells. The transfected cells were treated with 10 μg/mL of (–)-DHMEQ for 2 h and then stimulated with 10 ng/mL TNF-α for 30 min, and thereafter, their nuclear proteins were extracted and used for the EMSA. (B) Western blotting analysis of nuclear extracts of Hela cells for p65 transfected with wild-type or C38S p65. The cells were treated as in (A).

RelB(1–391) was slightly weaker than that to p65(1–325). But (–)-DHMEQ at 2 equiv shifted the peak of RelB(1–391) clearly, whereas it did not shift the peak with the C144S mutant (Figure 4E). RelB does not form homodimers,²³ so the effect of (–)-DHMEQ on RelB DNA-binding activity was not measured. We also prepared cRel(1–307) instead of full-length cRel. As shown in Figure 4F, the DNA binding of the cRel(1–307) dimer was effectively inhibited by (–)-DHMEQ, but that of the cRel(1–307) C27S mutant was not inhibited by (–)-DHMEQ. The same results were obtained with cRel and cRel(C27S) by the MALDI-TOF MS analysis (Figure 4F). Interestingly, (–)-DHMEQ did not inhibit the DNA binding of p52. Only at a higher concentration did it shift the peak of p52 in the MALDI-TOF MS analysis, but the binding appeared to be rather nonspecific. Thus, (–)-DHMEQ was shown to covalently bind to the specific Cys residue in Rel family proteins except p52 to inhibit the function.

5. (–)-DHMEQ Binds to p65 Cys38 in Cultured Cells.

We transfected Hela cells with wild-type or C38S full-length p65. As shown in Figure 5A, TNF-α enhanced the NF-κB activity in wild-type p65 overexpressing cells. (–)-DHMEQ inhibited this activation completely. On the other hand, when C38S p65 was overexpressed, the basal NF-κB activity in EMSA was markedly increased, but this increase was not prominently inhibited by (–)-DHMEQ (Figure 5A). Moreover, both endogenous and transfected p65 in the nucleus were increased by TNF-α in the Western blot analysis. On the other hand, (–)-DHMEQ lowered only endogenous p65 and transfected wild-type p65, not the transfected p65 C38S (Figure 5B). Thus, it is likely that (–)-DHMEQ would bind to Cys38 of p65 in cultured cells to inhibit NF-κB.

6. Discussion.

Several chemical ligands, including NF-κB inhibitors, have been reported to bind to Cys 38 of p65, but there has been no evidence of chemical binding. NO induces nitration on Tyr66 and Tyr152 of p65,²⁴ and glutaredoxin induces S-glutathionylation of p65 to inhibit NF-κB activity.²⁵ 15-Deoxy-Δ12,14-prostaglandin J₂, a naturally occurring analogue of prostaglandin D₂, inhibits IKK activity through its binding to IKK Cys179, and it also inhibits NF-κB DNA binding through the binding of p65 Cys38 and p50 Cys62.²⁶ Sesquiterpene lactones, including parthenolide, helenalin, 4β,15-epoxy-

millier-9E-enolide, ethacrynic acid, epoxyquinol A, and epoxyquinol A monomer were reported to require Cys38 of p65 for the inhibition of NF-κB activity.^{22,27–29} Parthenolide inhibits both DNA binding of NF-κB and IKK.²⁷ Epoxyquinol A monomer inhibits NF-κB/DNA binding possibly by its binding to p65 Cys38,²⁹ and it also inhibits IκB-α degradation possibly through the binding to IKK Cys179.²⁹ On the other hand, (–)-DHMEQ specifically inhibited p65 and other Rel family proteins such as p50 (Figure 4D), RelB (Figure 4E), and cRel (Figure 4F) without inhibiting IKK.¹⁰ So (–)-DHMEQ should be more specific to inhibit NF-κB than other known inhibitors. (–)-DHMEQ is the first ligand that was shown to bind to the specific Cys residues in Rel homology proteins.

(–)-DHMEQ binds to p50 Cys62 but not p52 Cys57. The amino acid sequence of p50 is similar to that of p52. However, Val61 in p50 is replaced into Gly56 in p52, which may cause the difference.

The p65 Cys38 and the corresponding Cys residue of other Rel family proteins have been reported to be essential for NF-κB DNA binding. According to the crystal structures of the p50/p65 heterodimer,³⁰ p50/RelB heterodimer,³¹ and p52/p52 homodimer,³² the Cys residues corresponding to p65 Cys38 (RelB Cys144, p50 Cys62, p52 Cys57) in the L1 loop are involved in the hydrogen bonding to the phosphate group of κB DNA. Adduct formation by these cysteines should cause loss of hydrogen bonding.

The C38S mutant of p65(1–325) bound to κB DNA more strongly than the C120S mutant. These results are consistent with a previous report.²² They also reported that, although the DNA binding of C38S was enhanced, the trans-activation potency of the mutants was rather decreased. Therefore, the DNA-binding ability of p65 mutants does not correlate with the trans-activation potency.

As expected, (–)-DHMEQ was attacked by the thiol group of blocked Cys with the epoxide opening (Figure 3). This structure was supported by our observation that the p65-derived peptide fragment with Cys38 showed the additional peak MW of dehydrated (–)-DHMEQ. In the process of the peptide/MS analysis, the peptide was treated with trifluoroacetic acid to assist ionization. Actually, as shown in Figure 3, we demonstrated that the Cys-(–)-DHMEQ adduct can be dehydrated (–18 Da) by the acid treatment to give a new aromatic ring.

There is much evidence that DHMEQ inhibits nuclear translocation, such as in lymphoma cells.³³ Therefore, we examined whether (–)-DHMEQ would decrease binding to importin or enhance the CRM1/exportin-dependent export. First, we employed GST pull-down analysis using GST-tagged importin α3, which is important for the nuclear translocation of p65 and other Rel family proteins.⁶ As a result, although the stimulant-dependent binding between GST-importin α3 and p65 was detected, (–)-DHMEQ did not inhibit this interaction. Next, we studied the effect of (–)-DHMEQ on the CRM1/exportin system. Rel family proteins³⁴ and IκB-α³⁵ have CRM1/exportin-dependent nuclear export signal (NES). We examined involvement of the CRM1/exportin system on the DHMEQ-induced inhibition of p65 nuclear localization by using leptomycin B, which inhibits the CRM1/exportin system.³⁶ As a result, leptomycin B increased p65 nuclear localization but did not inhibit the (–)-DHMEQ-dependent decrease in p65 nuclear localization. Thus, it is possible that the inhibition of p65 nuclear localization by DHMEQ may be mediated by other p65-interacting proteins. It may also be possible that (–)-DHMEQ

would change the equilibrium between nuclear and cytoplasmic NF- κ B by the change in DNA affinity.

Conclusions

Earlier we designed and synthesized a novel NF- κ B inhibitor, DHMEQ. (–)-DHMEQ was shown to be more potent than (+)-DHMEQ. DHMEQ was reported to show potent anti-inflammatory and anticancer effects in animals without any toxicity, and it is being developed as a chemotherapeutic agent. Although DHMEQ has been widely used in vivo and in vitro studies, its molecular target has not been elucidated. In the present study its target protein was found to be p65 and other Rel homology proteins. (–)-DHMEQ was shown to covalently bind to the specific Cys residues in p65 at a 1:1 stoichiometry. Formation of Cys(–)-DHMEQ adduct in the protein was supported by the chemical synthesis of the adduct molecule. (–)-DHMEQ is the first ligand that was proved to covalently bind to the specific Cys residues in Rel family proteins. These findings may explain its highly selective inhibition of NF- κ B and the low toxic effect of (–)-DHMEQ in cells and animals and should be useful for further molecular design of NF- κ B inhibitors.

Experimental Section

Synthesis. 1. Synthesis of (–)-DHMEQ/Protected L-Cysteine Adduct. To a solution of *N*-(*tert*-butoxycarbonyl)-L-cysteine methyl ester (19.2 mg, 81.6 μ mol) in DMF (0.92 mL) and 0.1 M sodium phosphate buffer (1.9 mL, pH 7.0) was added (–)-DHMEQ (10.0 mg, 38.3 μ mol) in DMF (1 mL). The mixture was stirred at RT for 1 min. Then the mixture was diluted with EtOAc (20 mL) and washed with H₂O (10 mL \times 2). The organic layer was dried and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 2:1 \rightarrow 5:1) to provide 10.5 mg (56%) of adduct as a colorless oil.

Physicochemical data: TLC R_f = 0.58 (toluene/EtOH = 3:1); ¹H NMR (DMSO-*d*₆; 300 MHz) δ 1.39 (s, 9H), 2.97 (m, 2H), 3.38 (d, 1H, J = 2.1 Hz), 3.65 (s, 3H), 4.12–4.17 (m, 2H), 4.56 (ddd, 1H, J = 7.8, 2.1, 2.1 Hz), 5.74 (d, 1H, J = 3.9 Hz), 6.16 (d, J = 7.8 Hz), 6.92 (br s, 1H), 6.96 (d, 1H, J = 8.1 Hz), 6.99 (dd, J = 8.1 Hz), 7.41 (d, 1H, J = 8.1 Hz), 7.44 (ddd, J = 8.1, 8.1, 1.7 Hz), 7.94 (dd, 1H, J = 8.1, 1.7 Hz), 11.16 (br s, 1H), 11.82 (br s, 1H); ¹³C NMR (DMSO-*d*₆; 75 MHz) δ 28.1 \times 3, 32.2, 51.6, 52.1, 53.5, 65.7, 72.4, 78.6, 107.2, 117.0, 118.4, 119.8, 131.2, 134.2, 152.5, 156.2, 156.9, 164.8, 171.3, 193.8; HRMS (FAB) calcd for C₂₂H₂₈N₂O₉S (M⁺, ¹H) m/z 497; found, 497.

2. Preparation of Benzylthiol/(–)-DHMEQ Adduct. Benzylthiol/(–)-DHMEQ adduct was prepared as follows: To a solution of (–)-DHMEQ (0.1 g, 0.383 mmol) in DMF (19.1 mL) and 0.1 M sodium phosphate buffer (19 mL, pH 6.0) was added benzyl mercaptan (90 μ L, 0.77 mmol). The mixture was stirred at RT for 3 h. Then the mixture was diluted with EtOAc (100 mL) and washed with H₂O (50 mL \times 2). The organic layer was dried and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOH/toluene, 1:8) to obtain 0.132 g (89%) of adduct as a colorless oil.

Physicochemical data: TLC R_f = 0.38 (toluene/EtOH = 3:1); ¹H NMR (DMSO-*d*₆; 300 MHz) δ 3.38 (d, 1H, J = 1.8 Hz), 4.04, 4.06 (2d, 2H, J = 3.3 Hz), 3.89–4.07 (m, 1H), 4.56 (ddd, 1H, J = 7.8, 1.8, 1.8 Hz), 5.67 (d, 1H, J = 3.3 Hz), 6.14 (d, 1H, J = 7.8 Hz), 6.95 (d, 1H, J = 1.8 Hz), 6.98 (dd, 1H, J = 8.0 Hz), 7.01 (d, 1H, J = 8.0 Hz), 7.23 (m, 6H), 7.95 (dd, 1H, J = 8.0, 1.8 Hz), 11.13 (s, 1H), 11.78 (br s, 1H).

3. Acidic Dehydration of Benzylthiol/(–)-DHMEQ Adduct. The adduct (12.0 mg, 31.1 μ mol) was dissolved in DMF (1.0 mL) and 5 N aq HCl (1.0 mL) and then stirred at RT for 24 h. Then the mixture was diluted with EtOAc (20 mL) and washed with H₂O (10 mL \times 2). The organic layer was dried and concentrated in vacuo. The residue was purified by column chromatography on

silica gel (EtOAc/hexane, 1:2) to yield 4.9 mg (43%) of product as a yellow oil.

Physicochemical data: TLC R_f = 0.47 (toluene/EtOH = 3:1); ¹H NMR (DMSO-*d*₆; 300 MHz) δ 4.05 (s, 2H), 6.69 (s, 1H), 6.94–7.00 (m, 2H), 7.19–7.43 (m, 6H), 8.01 (dd, 1H, J = 8.1, 1.8 Hz), 8.08 (s, 1H), 9.28 (s, 1H), 9.43 (s, 1H), 10.75 (s, 1H), 11.62 (br s, 1H).

4. Preparation of *O*-Isopropylidene Derivative of Cys/(–)-DHMEQ Adduct. To a stirred solution of (–)-DHMEQ/Cys adduct (33.2 mg, 66.9 μ mol) in acetone/Me₂C(OMe)₂ (2 mL, 1:1), a microspatula amount of *p*-TsOH was added. The mixture was stirred RT for 30 min. Then the mixture was diluted with EtOAc (20 mL) and washed with H₂O (10 mL \times 2). The organic layer was dried and concentrated in vacuo. The residue was purified by column chromatography on silica gel (EtOAc/hexane, 1:1) to provide 26.5 mg (74%) of *O*-IP (–)-DHMEQ/Cys adduct as colorless oil.

Physicochemical data: TLC R_f (EtOAc/hexane = 3:1); ¹H NMR (DMSO-*d*₆; 270 MHz) δ 1.29 (s, 3H), 1.38 (2s, 3H, 9H), 2.97 (dd, 1H, J = 13.7, 9.2 Hz), 3.09 (dd, 1H, J = 13.7, 5.1 Hz), 3.64 (s, 3H), 3.69 (d, 1H, J = 2.9 Hz), 4.17 (m, 1H), 4.68 (dd, 1H, J = 4.4, 2.9 Hz), 4.98 (d, 1H, J = 4.4 Hz), 6.93 (s, 1H), 6.96 (d, 1H, J = 8.1 Hz), 6.99 (dd, J = 8.1 Hz), 7.41 (d, 1H, J = 8.1 Hz), 7.44 (ddd, J = 8.1, 8.1, 1.7 Hz), 7.92 (dd, 1H, J = 8.8, 1.5 Hz), 10.65 (s, 1H), 11.88 (s, 1H); ¹³C NMR (DMSO-*d*₆; 101 MHz) δ 26.6, 27.5, 28.1 \times 3, 33.1, 46.6, 52.1, 53.7, 70.6, 76.1, 78.6, 108.4, 110.5, 117.0, 118.1, 119.5, 130.9, 134.4, 150.0, 155.3, 156.3, 165.2, 171.2, 192.7.

Protein Chemistry. 1. Materials. (–)-DHMEQ and its inactive analogue were synthesized in our laboratory as described before.⁹ Recombinant human TNF- α was purchased from Peprotech Inc. (London, U.K.). Mouse monoclonal anti-p65 antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Full-length p65 protein was purchased from Active Motif (Carlsbad, CA). Mouse monoclonal anti-p65 NF- κ B and anti-p50 NF- κ B antibody were from Santa Cruz Biotechnology (Santa Cruz, CA). Rabbit monoclonal anti-Lamin A/C antibody was purchased from Cell Signaling (Beverly, U.S.A.). Mouse monoclonal anti-FLAG antibody and anti- α -tubulin antibody came from Sigma (St. Louis, MO).

2. Electrophoretic Mobility Shift Assay (EMSA). The binding reaction mixture contained nuclear extract (5 μ g of protein), 2 μ g poly(dI-dC), and 10000 cpm a ³²P-labeled probe (oligonucleotide containing NF κ B or Oct-1 binding site) in binding buffer (75 mM NaCl, 1.5 mM EDTA, 1.5 mM DTT, 7.5% glycerol, 1.5% NP-40, 15 mM Tris-HCl; pH 7.0). Using the recombinant protein, we incubated the reaction mixture containing 20 μ M recombinant protein and 5% DMSO with or without (–)-DHMEQ in phosphate-buffered saline (PBS) for 1 h at 4 °C. To determine the DNA-binding activity of recombinant protein, we used 20 ng of recombinant protein from the reaction mixture for the EMSA. Samples were incubated for 20 min at room temperature (RT) in this mixture. DNA/protein complexes were separated from free DNA on 4% native polyacrylamide gel in 0.25 mM TBE buffer. The DNA probes used for NF- κ B binding were purchased from Promega (Madison, WI). The following sequences were used as NF- κ B and Oct-1 binding probes: 5'-AGT TGA GGG GAC TTT CCC AGG C and 5'-GCC TGG GAA AGT CCC CTC AAC T for NF- κ B binding and 5'-TGT CGA ATG CAA ATC AAT AGA A and 5'-TTC TAG TGA TTT GCA TTC GAG A for Oct-1 binding. These oligonucleotides were labeled with [γ -³²P]-ATP (3000 Ci/mmol; GE Healthcare, Little Chalfont, U.K.) by use of T4 polynucleotide kinase (Takara, Ohtsu, Japan), and purified by passage through a Nick column (GE Healthcare).

3. Surface Plasmon Resonance Analysis. Sensorgrams were recorded on a Biacore T100 instrument using the CM5 sensor chip of research grade (GE Healthcare). For the immobilization of p65(1–325), we employed the amine coupling method. The p65 protein (1 mg/mL) was reacted to the activated carboxyl group in the chip in the presence of 100 mM *N*-hydroxysuccinimide (NHS) and 400 mM *N*-ethyl-*N'*-(3-dimethylaminopropyl)

carbodiimide hydrochloride (EDC) in 10 mM sodium acetate buffer (pH 4.5). After immobilization, unreacted NHS groups were blocked by ethanolamine. Sensorgrams were run in the automatic subtraction mode using flow cell 1 (FC 1) as an unmodified reference. Data were collected for FC 2, which is immobilized about 14000 RU of p65 at 25 °C. Using the low molecular weight (LMW) kinetics mode, we injected 100 μ M (–)-DHMEQ in phosphate-buffered saline (PBS) containing 5% DMSO for 5 min. The running buffer used for the binding experiments was phosphate-buffered saline (PBS) containing 5% DMSO. Bulk correction was carried out by using phosphate-buffered saline (PBS) containing 4–6% DMSO.

4. MALDI-TOF MS Analysis. The adduct of (–)-DHMEQ and protein was prepared typically by incubation of 20 μ M recombinant p65 with each equivalent of (–)-DHMEQ in phosphate-buffered saline (PBS) for 1 h at 4 °C. The molecular weight of the recombinant NF- κ B components incubated with or without (–)-DHMEQ was determined by using a reflex MALDI-TOF MS spectrometer (Bruker Daltonics, Billerica, MA), operating in a linear mode.

5. Digestion of (–)-DHMEQ/p65 Adduct with Chymotrypsin. The reaction mixture of 20 μ L containing 20 μ M recombinant protein and 5% DMSO with or without (–)-DHMEQ in phosphate-buffered saline (PBS) was incubated for 1 h at 4 °C. After incubation, 80 μ L of acetone was added to the reaction mixture, which was then kept for 1 h at –80 °C. The proteins were pelleted by centrifugation for 15 min at 14000 rpm, resuspended, and digested with 0.75 μ g of chymotrypsin in 20 μ L of chymotrypsin digestion buffer (100 mM Tris-HCl [pH 7.5], 10 mM CaCl₂) for 2 h at 25 °C. Digested peptides were desalinated and condensed by using a ZipTip C18 column (Millipore), as described by the manufacturer. The molecular weights of the chymotrypsin-digested peptides were determined with a reflex MALDI-TOF mass spectrometer (Bruker Daltonics), operating in the reflection mode.

6. Plasmid Construction. Human p65 cDNA containing an N-terminal BamHI site and a C-terminal XhoI site was prepared from a human cultured cell cDNA pool by PCR. The primers used were 5'-TTTT **GGA TCC** ATG GAC GAA CTG TTC CCC CTC ATC (for p65, 5'-oligonucleotide, BamHI codons in bold face and initiation codons underlined), 5'-TTTT **CTC GAG** TTA CCG GGG GTC GGT GGG TCC GC (for shortened p65(325), 3'-oligonucleotide, XhoI codons in bold face and stop codons underlined), and 5'-TTTT **CTC GAG** TTA GGA GCT GAT CTG ACT CAG CAG (for full length p65, 3'-oligonucleotide, XhoI codons in bold face and stop codons underlined). Then p65 cDNA was cloned into the BamHI-XhoI site of pGEX-6P-1 vector (GE Healthcare) and pCMV-Tag2B vector (Stratagene, La Jolla, CA). Similarly, p50 corresponding to p105(1–405aa), RelB, p52 corresponding to p100(1–435aa), and c-Rel cDNA were prepared by PCR from the human cultured cell cDNA pool. The primers used were 5'-TTTTTT **AGA TCT** ATG GCA GAA GAT GAT CCA TAT TTG (for p50, 5'-oligonucleotide, BglII codons in bold face and initiation codons underlined) and 5'-TTTTTT **CTC GAG** CTA GGT TCC ATG CTT CAT CCC A (for p50, 3'-oligonucleotide, XhoI codons in bold face and stop codons underlined); 5'-TTTTTT **AGA TCT** ATG CTT CGG TCT GGG CCA GCC (for RelB, 5'-oligonucleotide, BglII codons in bold face and initiation codons underlined) and 5'-TTTTTT **CTC GAG** TTA CTC GCT GCA GAC CCC ATC GG (for shortened RelB(391), 3'-oligonucleotide, XhoI codons in bold face and stop codons underlined) or 5'-TTTTTT **CTC GAG** CTA CGT GGC TTC AGG CCC CG (for full-length RelB, 3'-oligonucleotide, XhoI codons in bold face and stop codons underlined); 5'-TTTTTT **GGA TCC** ATG GAG AGT TGC TAC AAC CCA GG (for p52, 5'-oligonucleotide, BamHI codons in bold face and initiation codons underlined) and 5'-TTTTTT **CTC GAG** TTA CGC CCC GCC CCC GCC TCC CG (for p52, 3'-oligonucleotide, XhoI codons in bold face and stop codons underlined); and 5'-TTTT **GGA TCC** ATG GCC TCC GGT GCG TAT AAC CCG (for cRel, 5'-oligonucleotide, BamHI codons in bold face and initiation codons underlined) and 5'-TTTT **CTC GAG** TTA GTG

A TC CTG GCA CAG TTT CTG (for shortened cRel(1–307), 3'-oligonucleotide, XhoI codons in bold face and stop codons underlined).

7. Site-Directed Mutagenesis. To obtain p65 Cys38Ser and Cys120Ser mutant clones, we employed inverse PCR³⁷ from the pGEX-6P-1-p65(1–325) plasmid as the template. The following primers were used for the construction of mutant proteins: 5'-CCG CTA CAA GTC CGA GGG GCG CTC CGC GG (5'-oligonucleotide, mutation point in bold face) and 5'-AAG CGC ATG CCC CGC TGC TTG GGC TGC TCA ATG AT (3'-oligonucleotide) for the p65 Cys38Ser mutant; 5'-CTG TGA AGA AGC GGG ACC TGG AGC AGG CTA TCA G (5'-oligonucleotide, mutation point in bold face) and 5'-ACT GGA TTC CCA GGT TCT GGA AAC TGT GGA TGC AG (3'-oligonucleotide) for the p65 Cys120Ser mutant. The PCR product was treated with DpnI for 120 min at 37 °C. The mutated pGEX-6P-1-p65(1–325) was separated on 1% agarose gel in TBE buffer and extracted from the gel by using a QIAEX II Gel Extraction Kit (QIAGEN, Valencia, CA). The mutated pGEX-6P-1-p65(1–325) was mixed with 1 mM ATP and T4-PNK (Takara) at 37 °C for 1 h and mixed with the ligation kit, Ligation High (Toyobo, Osaka, Japan), at 16 °C for 1 h. The plasmid was amplified in *E. coli* HB101 cells. Similarly, to obtain p50 Cys62Ser, RelB Cys144Ser, and cRel Cys27Ser, we employed inverse PCR using the plasmid containing each wild-type protein cDNA. The following primers were used for the construction of the mutant protein: 5'-CTG AAG GCC CAT CCC ATG GTG GAC TAC CTG GTG C (5'-oligonucleotide, mutation point in bold face) and 5'-ATA CAT AAC GGA AAC GAA ATC CTC TCT GTT TAG G (3'-oligonucleotide) for the p50 Cys62Ser mutant; 5'-CCG AGG GCC GCT CGG CCG GCA GCA TC (5'-oligonucleotide, mutation point in bold face) and 5'-ACT CGT AGC GGA AGC GCA TGC CGC GCT G (3'-oligonucleotide) for the cRel Cys144Ser mutant; 5'-CTG AAG GGC GAT CAG CAG GCA GCA TTC C (5'-oligonucleotide, mutation point in bold face) and 5'-ATT TGT ATC TAA AAC GCA TTC CCC TCT G (3'-oligonucleotide) for the cRel Cys27Ser mutant.

8. Recombinant Protein Purification. Recombinant proteins were expressed in *E. coli* BL21 cells as GST fusion proteins at 25 °C for 3 h by induction with 0.1 μ M isopropyl-1-thio- β -D-galactopyranoside. The bacteria were lysed in a sonication buffer (PBS, 0.1% NP-40, 1 mM DTT, 1% protease inhibitors (Nakarai Tesque, Kyoto, Japan)), sonicated for 10 min on ice, and centrifuged for 10 min at 14000 rpm at 4 °C. The supernatant was mixed with 100 μ L of equilibrated glutathione-sepharose 4B (GE Healthcare) at 4 °C for 60 min followed by washing five times with a sonication buffer. Glutathione-sepharose-immobilized GST fusion proteins of 100 μ g was mixed with 4 μ L of PreScission Protease (GE Healthcare) in a cleavage buffer (50 mM Tris-HCl [pH 7.5], 150 mM NaCl, 1 mM EDTA, 1 mM DTT) at 4 °C for 16 h and centrifuged for 10 min at 14000 rpm at 4 °C. The supernatant was used as the recombinant protein solution.

Biological Experiments. 1. Cell Culture. Hela cells were grown in Dulbecco's modified Eagle's medium (DMEM, Nissui, Tokyo, Japan) supplemented with 10% heat-inactivated fetal bovine serum (JRH Biosciences, Lenexa, KS), 200 μ g/mL kanamycin, 100 units/mL penicillin G, 600 μ g/mL L-glutamine, and 2.25 g/L NaHCO₃.

2. Transfection with Plasmids. HeLa cells (3×10^5) were grown in 60 mm dishes. Cells were transfected with the DNA by using Lipofectamine LTX (Invitrogen, Grand Island, NY), as described by the manufacturer. A total of 24 h after transfection, the cells were treated with the chemical prior to EMSA and Western blotting.

3. Nuclear Protein Extraction. Nuclear extracts were prepared according to the method of Andrews and Fallers.³⁸ Cells (3×10^5) were grown in 60 mm dishes and incubated with the desired chemicals. They were then harvested and washed with phosphate-buffered saline (PBS), suspended in 400 μ L of buffer A (10 mM HEPES [pH 7.8], 10 mM KCl, 2 mM MgCl₂, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF), and incubated on ice for 15 min. Nuclei

were pelleted by centrifugation for 5 min at 14000 rpm, resuspended in 40 μ L of buffer C (50 mM HEPES [pH 7.8], 50 mM KCl, 300 mM NaCl, 0.1 mM EDTA, 1 mM DTT, 0.1 mM PMSF, 25% glycerol [v/v]), incubated on ice for 20 min, and centrifuged for 5 min at 14000 rpm at 4 °C. The supernatant was used as the nuclear extract.

4. Western Blotting. Total cell extracts and the nuclear extracts were boiled in Laemmli loading buffer and subjected to SDS-polyacrylamide gel electrophoresis. Proteins were transferred at 200 mA for 1 h onto Hybond-P membranes (GE Healthcare). Nonspecific binding sites were blocked for 30 min by immersing the membrane in a blocking solution, Tris-buffered saline with Tween 20 (TBST): 10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 0.1% Tween 20 (v/v), and 5% (w/v) nonfat dry milk. After a short wash in TBST, the membrane was incubated in a 1:3000 dilution of a primary antibody in TBST for 1 h at RT followed by 30 min of washing with TBST. The bound antibody was then detected with horseradish peroxidase-conjugated secondary antibody (diluted at 1:3000 in TBST) by incubation with it for 1 h at RT. After having been washed for 30 min in TBST, the immunocomplexes were detected by using ECL reagent, Immobilon Western (Millipore, Billerica, MA). Exposure to RX-U films (Fuji Film, Kanagawa, Japan) was carried out for 10 s to 2 min.

Acknowledgment. This work was financially supported in part by grants from the programs Grants-in-Aid for Scientific Research on Priority Areas of the Ministry of Education, Culture, Sports, Science and Technology (MEXT). This work was also supported by High-Tech Research Center Project for Private Universities: matching fund subsidy from MEXT, 2006–2011, and the Program for Promotion of Fundamental Studies in Health Sciences of the National Institute of Biomedical Innovation (NIBIO).

References

- Schmitz, M. L.; Mattioli, I.; Buss, H.; Kracht, M. NF- κ B: A multifaceted transcription factor regulated at several levels. *Chem-BioChem* **2004**, *5*, 1348–58.
- Plaksin, D.; Baeuerle, P. A.; Eisenbach, L. KBF1 (p50 NF- κ B homodimer) acts as a repressor of H-2Kb gene expression in metastatic tumor cells. *J. Exp. Med.* **1993**, *177*, 1651–62.
- Baeuerle, P. A.; Baltimore, D. NF- κ B: Ten years after. *Cell* **1996**, *87*, 13–20.
- Ghosh, S.; Karin, M. Missing pieces in the NF- κ B puzzle. *Cell* **2002**, *109*, 81–96.
- Chen, Z.; Hagler, J.; Palombella, V. J.; Melandri, F.; Scherer, D.; Ballard, D.; Maniatis, T. Signal-induced site-specific phosphorylation targets I κ B α to the ubiquitin-proteasome pathway. *Genes Dev.* **1995**, *9*, 1586–97.
- Fagerlund, R.; Kinnunen, L.; Köhler, M.; Julkunen, I.; Melén, K. NF- κ B is transported into the nucleus by importin α 3 and importin α 4. *J. Biol. Chem.* **2005**, *280*, 15942–51.
- Senftleben, U.; Cao, Y.; Xiao, G.; Greten, F. R.; Krähn, G.; Bonizzi, G.; Chen, Y.; Hu, Y.; Fong, A.; Sun, S. C.; Karin, M. Activation by IKK α of a second, evolutionary conserved, NF- κ B signaling pathway. *Science* **2001**, *293*, 1495–9.
- Matsumoto, N.; Ariga, A.; To-e, S.; Nakamura, H.; Agata, N.; Hirano, S.; Inoue, J.; Umezawa, K. Synthesis of NF- κ B activation inhibitors derived from epoxyquinomicin C. *Bioorg. Med. Chem. Lett.* **2000**, *10*, 865–9.
- Suzuki, Y.; Sugiyama, C.; Ohno, O.; Umezawa, K. Preparation and biological activities of optically active dehydroxymethyl epoxyquinomicin, a novel NF- κ B inhibitor. *Tetrahedron* **2004**, *60*, 7061–6.
- Ariga, A.; Namekawa, J.; Matsumoto, N.; Inoue, J.; Umezawa, K. Inhibition of tumor necrosis factor- α -induced nuclear translocation and activation of NF- κ B by dehydroxymethyl epoxyquinomicin. *J. Biol. Chem.* **2002**, *277*, 24625–30.
- Wakamatsu, K.; Nanki, T.; Miyasaka, N.; Umezawa, K.; Kubota, T. Effect of a small molecule inhibitor of nuclear factor- κ B nuclear translocation in a murine model of arthritis and cultured human synovial cells. *Arthritis Res. Ther.* **2005**, *7*, R1348–59.
- Miyajima, A.; Kosaka, T.; Seta, K.; Asano, T.; Umezawa, K.; Hayakawa, M. Novel nuclear factor κ B activation inhibitor prevents inflammatory injury in unilateral ureteral obstruction. *J. Urol.* **2003**, *169*, 1559–63.
- Kuroda, K.; Horiguchi, Y.; Nakashima, J.; Kikuchi, E.; Kanao, K.; Miyajima, A.; Ohigashi, T.; Umezawa, K.; Murai, M. Prevention of cancer cachexia by a novel nuclear factor κ B inhibitor in prostate cancer. *Clin. Cancer Res.* **2005**, *11*, 5590–4.
- Kubota, T.; Hoshino, M.; Aoki, K.; Ohya, K.; Komano, Y.; Nanki, T.; Miyasaka, N.; Umezawa, K. NF- κ B inhibitor dehydroxymethyl epoxyquinomicin suppresses osteoclastogenesis and expression of NFATc1 in mouse arthritis without affecting expression of RANKL, osteoprotegerin or macrophage colony-stimulating factor. *Arthritis Res. Ther.* **2007**, *9*, R97.
- Kikuchi, E.; Horiguchi, Y.; Nakashima, J.; Kuroda, K.; Oya, M.; Ohigashi, T.; Takahashi, N.; Shima, Y.; Umezawa, K.; Murai, M. Suppression of hormone-refractory prostate cancer by a novel nuclear factor κ B inhibitor in nude mice. *Cancer Res.* **2003**, *63*, 107–10.
- Starenki, D. V.; Namba, H.; Saenko, V. A.; Ohtsuru, A.; Maeda, S.; Umezawa, K.; Yamashita, S. Induction of thyroid cancer cell apoptosis by a novel nuclear factor κ B inhibitor, dehydroxymethyl epoxyquinomicin. *Clin. Cancer Res.* **2004**, *10*, 6821–9.
- Matsumoto, G.; Namekawa, J.; Muta, M.; Nakamura, T.; Bando, H.; Tohyama, K.; Toi, M.; Umezawa, K. Targeting of nuclear factor κ B pathways by dehydroxymethyl epoxyquinomicin, a novel inhibitor of breast carcinomas: Antitumor and antiangiogenic potential in vivo. *Clin. Cancer Res.* **2005**, *11*, 1287–93.
- Matsumoto, G.; Muta, M.; Umezawa, K.; Suzuki, T.; Misumi, K.; Tsuruta, K.; Okamoto, A.; Toi, M. Enhancement of the caspase-independent apoptotic sensitivity of pancreatic cancer cells by DH-MEQ, an NF- κ B inhibitor. *Int. J. Oncol.* **2005**, *27*, 1247–55.
- Tatetsu, H.; Okuno, Y.; Nakamura, M.; Matsuno, F.; Sonoki, T.; Taniguchi, I.; Uneda, S.; Umezawa, K.; Mitsuya, H.; Hata, H. Dehydroxymethyl epoxyquinomicin, a novel nuclear factor- κ B inhibitor, induces apoptosis in multiple myeloma cells in an I κ B α -independent manner. *Mol. Cancer Ther.* **2005**, *4*, 1114–20.
- Watanabe, M.; Ohsugi, T.; Shoda, M.; Ishida, T.; Aizawa, S.; Maruyama-Nagai, M.; Utsunomiya, A.; Koga, S.; Yamada, Y.; Kamihira, S.; Okayama, A.; Kikuchi, H.; Uozumi, K.; Yamaguchi, K.; Higashihara, M.; Umezawa, K.; Watanabe, T.; Horie, R. Dual targeting of transformed and untransformed HTLV-1-infected T cells by DHMEQ, a potent and selective inhibitor of NF- κ B, as a strategy for chemoprevention and therapy of adult T-cell leukemia. *Blood* **2005**, *106*, 2462–71.
- Umezawa, K. Inhibition of tumor growth by NF- κ B inhibitors. *Cancer Sci.* **2006**, *97*, 990–5.
- García-Piñeres, A. J.; Lindenmeyer, M. T.; Merfort, I. Role of cysteine residues of p65/NF- κ B on the inhibition by the sesquiterpene lactone parthenolide and N-ethyl maleimide, and on its transactivating potential. *Life Sci.* **2004**, *75*, 841–56.
- Ryseck, R. P.; Weih, F.; Carrasco, D.; Bravo, R. RelB, a member of the Rel/NF- κ B family of transcription factors. *Braz. J. Med. Biol. Res.* **1996**, *29*, 895–903.
- Park, S. W.; Huq, M. D.; Hu, X.; Wei, L. N. Tyrosine nitration on p65: A novel mechanism to rapidly inactivate nuclear factor- κ B. *Mol. Cell. Proteomics* **2005**, *4*, 300–9.
- Qanungo, S.; Starke, D. W.; Pai, H. V.; Mielay, J. J.; Nieminen, A. L. Glutathione supplementation potentiates hypoxic apoptosis by S-glutathionylation of p65-NF κ B. *J. Biol. Chem.* **2007**, *282*, 18427–36.
- Straus, D. S.; Pascual, G.; Li, M.; Welch, J. S.; Ricote, M.; Hsiang, C. H.; Sengchanthalangsy, L. L.; Ghosh, G.; Glass, C. K. 15-Deoxy- δ -12,14-prostaglandin J2 inhibits multiple steps in the NF- κ B signaling pathway. *Proc. Natl. Acad. Sci. U.S.A.* **2000**, *97*, 4844–9.
- García-Piñeres, A. J.; Castro, V.; Mora, G.; Schmidt, T. J.; Strunck, E.; Pahl, H. L.; Merfort, I. Cysteine 38 in p65/NF- κ B plays a crucial role in DNA binding inhibition by sesquiterpene lactones. *J. Biol. Chem.* **2001**, *276*, 39713–20.
- Han, Y.; Englert, J. A.; Delude, R. L.; Fink, M. P. Ethacrynic acid inhibits multiple steps in the NF- κ B signaling pathway. *Shock* **2005**, *23*, 45–53.
- Liang, M. C.; Bardhan, S.; Pace, E. A.; Rosman, D.; Beutler, J. A.; Gilmore, T. D. Inhibition of transcription factor NF- κ B signaling proteins IKK β and p65 through specific cysteine residues by epoxyquinone A monomer: Correlation with its anticancer cell growth activity. *Biochem. Pharmacol.* **2006**, *71*, 634–45.
- Chen, F. E.; Huang, D. B.; Chen, Y. Q.; Ghosh, G. Crystal structure of p50/p65 heterodimer of transcription factor NF- κ B bound to DNA. *Nature* **1998**, *391*, 410–3.
- Moorthy, A. K.; Huang, D. B.; Wang, V. Y.; Vu, D.; Ghosh, G. X-ray structure of a NF- κ B p50/RelB/DNA complex reveals assembly of multiple dimers on tandem κ B sites. *J. Mol. Biol.* **2007**, *373*, 723–34.
- Cramer, P.; Larson, C. J.; Verdine, G. L.; Müller, C. W. Structure of the human NF- κ B p52 homodimer-DNA complex at 2.1 Å resolution. *EMBO J.* **1997**, *16*, 7078–90.
- Watanabe, M.; Dewan, M. Z.; Taira, M.; Shoda, M.; Honda, M.; Sata, T.; Higashihara, M.; Kadin, M. E.; Watanabe, T.; Yamamoto, N.; Umezawa, K.; Horie, R. I κ B α independent induction of NF- κ B and

- its inhibition by DHMEQ in Hodgkin/Reed-Sternberg cells. *Lab. Invest.* **2007**, 87, 372–82.
- (34) Harhaj, E. W.; Sun, S. C. Regulation of RelA subcellular localization by a putative nuclear export signal and p50. *Mol. Cell. Biol.* **1999**, 19, 7088–95.
- (35) Lee, S. H.; Hannink, M. The N-terminal nuclear export sequence of I κ B α is required for RanGTP-dependent binding to CRM1. *J. Biol. Chem.* **2001**, 276, 23599–606.
- (36) Fukuda, M.; Asano, S.; Nakamura, T.; Adachi, M.; Yoshida, M.; Yanagida, M.; Nishida, E. CRM1 is responsible for intracellular transport mediated by the nuclear export signal. *Nature* **1997**, 390, 308–11.
- (37) Stemmer, W. P.; Morris, S. K. Enzymatic inverse PCR: A restriction site independent, single-fragment method for high-efficiency, site-directed mutagenesis. *Biotechniques* **1992**, 13, 214–20.
- (38) Dignam, J. D.; Lebovitz, R. M.; Roeder, R. G. Accurate transcription initiation by RNA polymerase II in a soluble extract from isolated mammalian nuclei. *Nucleic Acids Res.* **1983**, 11, 1475–89.

JM8006245