# Dehydroxymethylepoxyquinomicin, a novel nuclear factor-κB inhibitor, induces apoptosis in multiple myeloma cells in an IκBα-independent manner

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## Abstract

**Nuclear factor-κB (NF-κB) is constitutively activated in multiple myeloma cells. Several proteasome inhibitors have been shown to be effective against multiple myeloma and may act by inhibiting degradation of IκBα. Here, we examined the biological effects of a new type of NF-κB inhibitor, dehydroxymethylepoxyquinomicin (DHMEQ), which is reported to directly inhibit the cytoplasm-to-nucleus translocation of NF-κB. A multiple myeloma cell line, 12PE, which is defective for IκBα protein, was utilized to determine if IκBα is concerned with the action of DHMEQ. Meanwhile, U266 was used as a multiple myeloma cell line with normal IκBα. A proteasome inhibitor, gliotoxin, which is an inhibitor of degradation of phosphorylated IκBα, failed to inhibit translocation of NF-κB in 12PE. In contrast, DHMEQ equally inhibited translocation of NF-κB to the nucleus and induced apoptosis to both multiple myeloma cell lines, suggesting that apoptosis resulting from DHMEQ is IκBα independent. DHMEQ also induced apoptosis in freshly isolated multiple myeloma cells. After DHMEQ treatment, cleavage of caspase-3 and down-regulation of cyclin D1 were observed in both cell lines. In addition, administration of DHMEQ resulted in a significant reduction in tumor volume in a plasmacytoma mice model compared with control mice. Our results show that DHMEQ could potentially be a new type of molecular target agent for multiple myeloma. [Mol Cancer Ther 2005;4(7):1114–20]**

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## Introduction

Multiple myeloma is a human B-cell neoplasm characterized by plasma cell overgrowth and malfunctions in bone marrow and is associated with various clinical manifestations, including anemia, opportunistic infections, and bone destructions. Combination chemotherapy offers initial response rates of 40% to 70% in multiple myeloma patients, but a complete cure is difficult to achieve. Although high-dose chemotherapy with stem cell transplantation has achieved a higher response rate than conventional therapy, only a few patients remain in long-term remission, highlighting the urgent need for novel therapeutic strategies (1).

Recent studies have shown that the nuclear factor-κB (NF-κB) transcription factor family may play a role in the pathogenesis of lymphoid malignancies, including multiple myeloma (2–4). Under normal conditions, NF-κB is present in the cytoplasm as an inactive heterodimer consisting of p50, p65, and IκBα subunits. On activation, IκBα is degraded in the 26S proteasome after phosphorylation and ubiquitination, allowing NF-κB nuclear translocation and binding to specific consensus sequences (κB sites), which transactivates its target genes (5). NF-κB activity promotes growth, survival, and drug resistance in multiple myeloma cells and these effects can be inhibited by blocking NF-κB (6–8). NF-κB also induces adhesion molecule and cytokine expression of multiple myeloma cells and bone marrow stromal cells (9, 10). Recently, it was reported that NF-κB inhibitors, such as PS-1145 (2), SN50 (11), and curcumin (12), are effective in multiple myeloma cells.

PS-341 (8), a proteasome inhibitor that inhibits the activity of the 26S proteasome, blocks the degradation of IκBα in the proteasome, thereby inhibiting nuclear translocation of NF-κB (13). PS-341 not only inhibits the growth of multiple myeloma cells but also induces apoptosis of multiple myeloma cells. It also inhibits binding of multiple myeloma cells to bone marrow stroma cells, thus disrupting the microenvironment (14). In a phase II clinical trial, the efficacy of PS-341 in patients with relapsed, refractory myeloma was considered promising (15).

Because NF-κB is a key molecule for the development of new therapeutic strategies for myeloma, IκBα is also likely to play an important role in myeloma cells. Recently, mutations and polymorphisms of the *IκBα* gene have been reported in lymphoma (16–18) and multiple myeloma (19), respectively. These studies suggest that such mutations prevent the IκBα protein from interacting with NF-κB, leading to transformation to lymphoma or myeloma cells.

Panepoxydone isolated from the basidiomycete *Lentinus crinitus* was found to inhibit tumor necrosis factor-α–induced activation of NF-κB (20). Epoxyquinomicin C was originally isolated from *Amycolatopsis* as a weak antibiotic

and anti-inflammatory agent, and has a 4-hydroxy-5,6-epoxycyclohexenone structure like panepoxydone, which prevents type II collagen–induced rheumatoid arthritis in mice (21). 5-Dehydroxymethylepoxyquinomicin (DHMEQ), which is a 5-dehydroxymethyl derivative of epoxyquinomicin C, was recently designed and synthesized. DHMEQ inhibits nuclear translocation of NF-κB and has a different mechanism from that of proteasome inhibitors (22). It did not inhibit nuclear translocation of large T antigen or Smad2 (21), suggesting that it inhibits the translocation of NF-κB specifically. The target molecule is now being studied by preparing immobilized DHMEQ. DHMEQ showed anti–NF-κB activity in Jurkat cells, a human T-cell leukemia line (23), and was also effective against hormone-refractory prostate cancer by inhibiting NF-κB (24). These findings suggest that DHMEQ may be a new therapeutic agent for multiple myeloma by inhibiting NF-κB activity.

Therefore, we analyzed the apoptotic effects of DHMEQ utilizing multiple myeloma cell lines and freshly isolated multiple myeloma cells. We utilized an IκBα-deficient multiple myeloma cell line to confirm the role of IκBα in DHMEQ-induced cytotoxicity. Apoptosis and cell cycle–related molecules, such as caspase-3, Bcl-2, Bcl-xL, A1/Bfl-1 (25), and cyclin D1, were simultaneously examined to explain the apoptotic pathway induced by DHMEQ. Finally, we evaluated the efficacy of DHMEQ *in vivo*.

## Materials and Methods

### Cell Lines

Myeloma cell lines, U266 (26) and KMS-12PE (27), were grown in RPMI 1640 (Sigma Chemical, St. Louis, MO) supplemented with 10% heat-inactivated FCS (Sigma), 100 μg/mL kanamycin (Meiji, Tokyo, Japan), and 100 IU/mL penicillin (Banyu Pharmaceutical, Tokyo, Japan). The myeloma cell line KMS-12PE was obtained from Dr. T. Ohtsuki (Kawasaki Medical School, Kurashiki, Japan). 12PE, which does not express IκBα protein, is a subclone of KMS-12PE. Nucleotide sequence analysis showed that there was a genomic mutation in the splicing acceptor site of intron 4 (AG to TG) of *IκBα* gene in 12PE. Sequence analysis revealed that the splicing donor site of intron 4 is connected to a new cryptic splicing acceptor site located at the 128th nucleotide of exon 5, resulting in 129 bp deletion of exon 5. Because this mutation generates a truncated mRNA, it is suggested that the absence of IκBα in 12PE cells is possibly due to instability of the truncated mRNA or IκBα protein.

### Patient Samples

After obtaining informed consent, myeloma cells were obtained from the bone marrow of myeloma patients admitted to Kumamoto University Hospital. Myeloma cells were purified using immunomagnetic beads as previously described (28).

### Agents

DHMEQ (a 5-dehydroxymethyl derivative of epoxyquinomycin C) was synthesized in the Faculty of Science and Technology, Keio University. For *in vivo* experiments, a chiral form of DHMEQ, (−)-DHMEQ, was used by dissolving in DMSO to prepare a 10 mg/mL solution. This solution was then diluted in culture medium for further experiments. A proteasome inhibitor, gliotoxin, was purchased from Calbiochem (La Jolla, CA) and dissolved in chloroform at a concentration of 10 mg/mL.

### Electrophoretic Mobility Shift Assay

Cells were treated with 10 μg/mL DHMEQ. Cells were harvested after various intervals, then washed with PBS, suspended in 400 μL of lysis buffer [10 mmol/L HEPES-KOH (pH 7.9), 5 mmol/L $MgCl_2$, 10 mmol/L KCl, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride], and incubated on ice for 1 hour. Nuclei were pelleted by centrifugation for 1 minute at 14,000 rpm, then resuspended in 40 μL of washing buffer [20 mmol/L HEPES-KOH (pH 7.9), 1.5 mmol/L $MgCl_2$, 420 mmol/L NaCl, 0.2 mmol/L EDTA, 0.5 mmol/L DTT, 0.2 mmol/L phenylmethylsulfonyl fluoride]. After 20 minutes incubation on ice, the sample was centrifuged for 2 minutes at 14,000 rpm at 4°C. The supernatant was used as a nuclear extract. The binding reaction mixture containing 5 mg of nuclear extract, 2 μg of poly-deoxyinosinic-dCMP, and $^{32}P$-labeled probe, was incubated for 20 minutes at room temperature. The DNA probe used for NF-κB binding was a double-strand oligonucleotide containing the interleukin-6 κB binding sequence (5′-TCGACATGTGGGATTTTCCCATGAC-3′). The complexes were separated from free DNA on 4% polyacrylamide gel and visualized by autoradiography.

### Western Blot Analysis

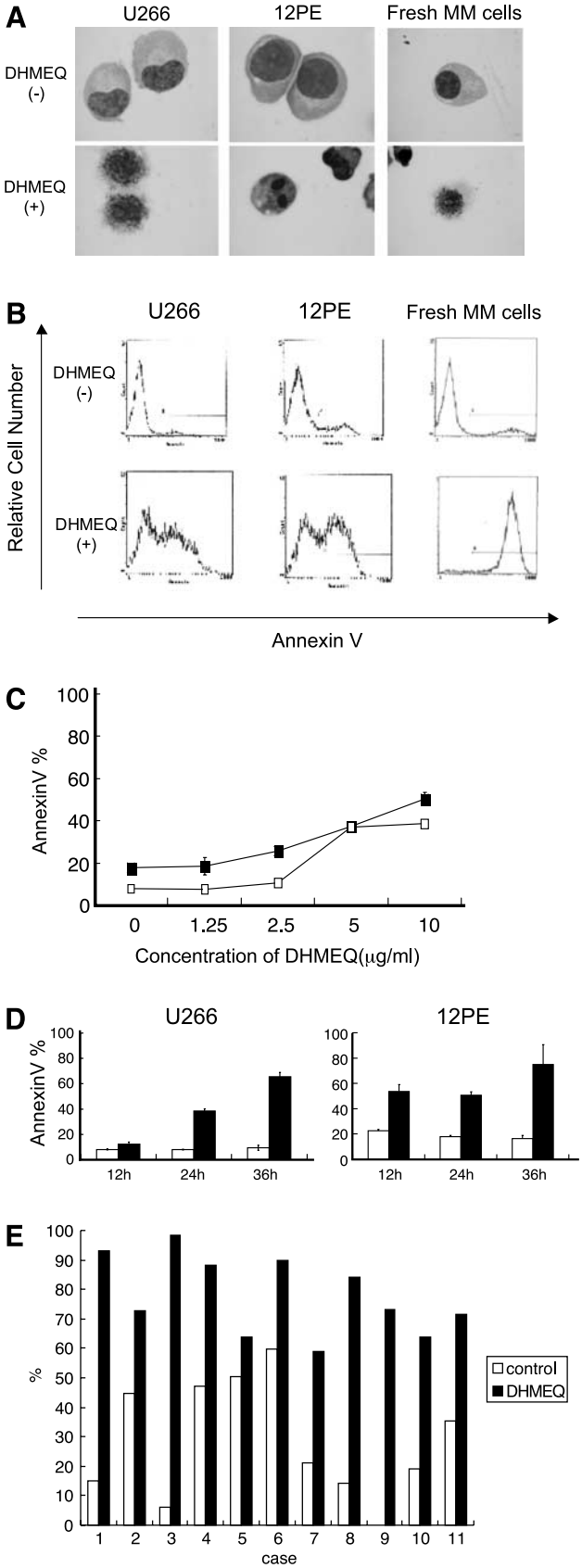
Cells were lysed in radioimmunoprecipitation assay buffer (0.5% sodium deoxycholate, 1% NP40, 0.1% SDS, protease inhibitor cocktail; Roche Diagnostics GmbH, Mannheim, Germany). Cell lysates were subjected to electrophoresis on a SDS polyacrylamide gel and transferred onto nitrocellulose membranes. The membranes were blocked with TBS containing 0.05% Tween and 5% nonfat milk at 4°C overnight. Subsequently, they were incubated for 1 hour with anti–caspase-3, anti-Bcl-2, anti–Bcl-Xs/L (Santa Cruz Biotechnology, Santa Cruz, CA), anti–A1/Bfl-1 (Santa Cruz), antiactin (Sigma), anti-IκBα (Cell Signal, Beverly, MA), or anti–phosphorylated IκBα (Cell Signal) antibodies. The membranes were then incubated for 1 hour with peroxidase-labeled anti-rabbit or anti-mouse secondary antibody, and developed using an enhanced chemiluminescence system (Amersham Life Science, Inc., Little Chalfont, United Kingdom).

### Detection of Apoptosis

Apoptosis was quantified by using the Annexin V/propidium iodide staining kit (Medical and Biological Laboratories, Nagoya, Japan; ref. 29). Briefly, after treatment with various concentrations of DHMEQ or gliotoxin, cells were harvested, washed, and then incubated with Annexin V-FITC and propidium iodide for 15 minutes in the dark. Fluorescence was analyzed by flow cytometry (EPICS V, Coulter, Miami, FL).

### Mouse Model

All procedures involving animals and their care described in this study were approved by the Animal Care Committee of Kumamoto University in accordance with




institutional and Japanese government guidelines for animal experiments. CB17/Icr female mice (6 weeks old) were obtained from Clea Japan (Tokyo, Japan). 12PE cells were implanted s.c. in the flank of each mouse. When animals developed palpable tumors, they were randomly assigned into two groups. DHMEQ was administered i.p. at a dosage of 12 mg/kg once daily for 14 consecutive days. Control animals received only a vehicle medium injection. Each experimental group consisted of five mice. Tumor size was measured twice a week over 7 weeks. Tumor volume was calculated according to the following formula: $(\text{smallest diameter})^2 \times (\text{largest diameter}) \times 0.52$.

## Results

### DHMEQ-Induced Apoptosis in Multiple Myeloma Cells

We first evaluated the effects of DHMEQ on two multiple myeloma cell lines (U266 and 12PE) and freshly purified multiple myeloma samples to determine whether DHMEQ can induce apoptosis. Morphologically, DHMEQ significantly induced apoptosis as shown by cell shrinkage and nuclear fragmentation in both multiple myeloma cell lines and the freshly purified multiple myeloma samples from 11 cases (Fig. 1A). Induction of apoptosis by DHMEQ was further confirmed by fluorescence-activated cell sorting analysis (Annexin V/propidium iodide staining). Treatment with DHMEQ at a concentration of 10 μg/mL for 24 hours induced apoptosis in U266, 12PE, and fresh multiple myeloma cells at 38.5%, 50.5%, and 93% of cells, respectively (Fig. 1B). Exposure of cells to various concentrations of DHMEQ revealed dose-dependent apoptosis of both U266 and 12PE (Fig. 1C). When cells were treated with 10 μg/mL of DHMEQ, U266 cells showed a time-dependent increase of cell death, whereas 12PE cells showed a more

**Figure 1.** **A**, morphologic examination of multiple myeloma cells after treatment with DHMEQ. U266, 12PE, and freshly purified myeloma cells (case 1 in **E**) were treated with DHMEQ (10 μg/mL) for 24 h (magnification ×400, May Giemsa staining). The majority of cells treated with DHMEQ underwent apoptosis, which is evident by cellular shrinkage and nuclear fragmentation. **B,** detection of apoptosis by flow cytometry. U266, 12PE, and purified fresh myeloma cells were treated with DHMEQ at a concentration of 10 μg/mL for 24 h. Cells treated with DMSO served as the control. Apoptosis was measured by the amount of Annexin V. DHMEQ induced apoptosis of all cells tested in this study. **C,** dose-dependent induction of apoptosis by DHMEQ. Cells were treated with various concentrations of DHMEQ for 24 h. □, U266; ■, 12PE. *Points,* mean of at least three individual experiments; *bars,* SD. Sensitivity of cells to DHMEQ is not significantly different. **D,** time course analysis of DHMEQ-induced apoptosis. Cells were treated with 10 μg/mL of DHMEQ and incubated for various periods. *Columns,* mean of at least three individual experiments; *bars,* SD. □, control; ■, DHMEQ. U266 showed time-dependent induction of apoptosis compared with the rapid induction of apoptosis in 12PE. **E,** DHMEQ induced apoptosis in fresh myeloma cells. Apoptosis was assessed as expression of Annexin V (%). □, control; ■, DHMEQ (1 μg/mL for 24 h). All cases were previously treated except cases 9 and 11. All pretreated cases were refractory to preceding treatments at the time of analysis. Preceding treatments were as follows: MP (cases 1, 3, 5, 6, 7, and 8), VAD (cases 1, 4, and 6), high-dose melphalan (cases 2 and 10), thalidomide (cases 1, 2, and 10), high-dose dexamethasone (cases 5, 8, and 10). Induction of apoptosis by DHMEQ was found in all cases.

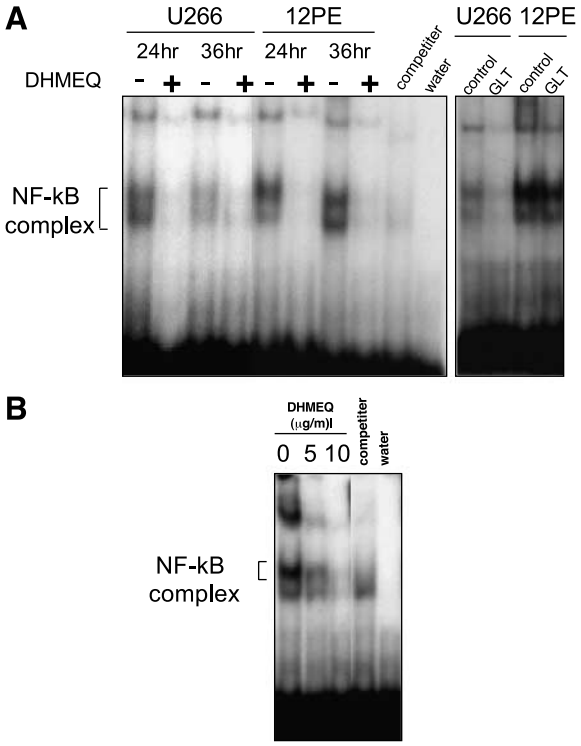



**Figure 2.** Electrophoretic mobility shift assay analysis of NF-κB. **A,** U266 and 12PE were treated with 10 μg/mL of DHMEQ (*left*) or 10 μg/mL of gliotoxin (*right*) for the indicated periods. DNA binding of NF-κB was found in both untreated U266 and 12PE cells (indicated as a NF-κB complex). DHMEQ decreased DNA binding of NF-κB at 24 and 36 h posttreatment in both cell lines (*left*), whereas gliotoxin inhibited NF-κB only in U266 (*right*). **B,** DHMEQ inhibited translocation of NF-κB to nucleus in fresh myeloma cells in a dose-dependent manner. Fresh myeloma cells from case 1 in Fig. 1E were treated with DHMEQ (1 μg/mL) for 24 h. DHMEQ inhibited translocation of NF-κB to nucleus at a dose-dependent manner.

prompt response as early as 12 hours from the start of treatment (Fig. 1D). We finally evaluated fresh isolated myeloma cells from 11 cases. DHMEQ also induced apoptosis to myeloma cells from all patients although some of them are very refractory to conventional chemotherapy (Fig. 1E).

### DHMEQ-Induced Inhibition of NF-κB Activity

We next analyzed the NF-κB inhibitory effect of DHMEQ by electrophoretic mobility shift assay. DNA-binding activity of NF-κB was found in both U266 and 12PE cell lines before DHMEQ treatment (Fig. 2A). DHMEQ treatment equally reduced DNA binding activity of NF-κB in both U266 and 12PE at 24 and 36 hours after treatment (Fig. 2A, *left*). In contrast, a proteasome inhibitor, gliotoxin, failed to inhibit DNA-binding activity of NF-κB in 12PE at 8 hours after treatment, whereas inhibition was seen in U266 (Fig. 2A, *right*). The sensitivity of 12PE to gliotoxin ($IC_{50}$ > 8 μmol/L) was significantly less than that of U266 ($IC_{50}$ 0.5-1.0 μmol/L).

We also evaluated NF-κB inhibitory effect of DHMEQ in freshly isolated myeloma cells. Translocation of NF-κB to nucleus was inhibited by DHMEQ treatment also in fresh myeloma cells (Fig. 2B).

### DHMEQ Caused Apoptosis Independent of IκBα

Because IκBα is thought to be critical in the NF-κB pathway, we next investigated IκBα and phosphorylated-IκBα protein levels before and after treatment with DHMEQ or gliotoxin (Fig. 3). Gliotoxin is a well-characterized proteasome inhibitor that blocks degradation of phosphorylated IkB, leading to accumulation of phosphorylated and unphosphorylated IkB protein. In U266, DHMEQ did not change the protein level of IκBα or phosphorylated IκBα, whereas phosphorylated IκBα accumulated after treatment with gliotoxin. Meanwhile, there was no expression of IκBα in 12PE cells. Because DHMEQ can induce apoptosis in 12PE, these results indicate that DHMEQ-induced apoptosis is IκBα independent.

### DHMEQ Down-Regulates Cyclin D1 and Activates Caspase-3

Because Bcl-2, Bcl-xL, and cyclin D1 have been shown to be regulated by NF-κB (30, 31), we examined the effects of DHMEQ on the expression of these proteins (Fig. 4). DHMEQ decreased Bcl-2 and Bcl-xL expression level in U266 but not in 12PE, suggesting that regulation of translation of Bcl-2 and Bcl-xL is different in U266 compared with 12PE although both cell lines similarly undergo apoptosis after treatment with DHMEQ. Bfl-1, a novel Bcl-2 family protein, was expressed in both cell lines and its expression was not changed by treatment with DHMEQ.

DHMEQ treatment inhibited expression of cyclin D1 at all periods examined in both U266 and 12PE. An alternative transcript of cyclin D1, termed cyclin D1b (32), was found in 12PE. Similar to the response seen with cyclin D1, treatment with DHMEQ markedly reduced cyclin D1b expression in 12PE. We also

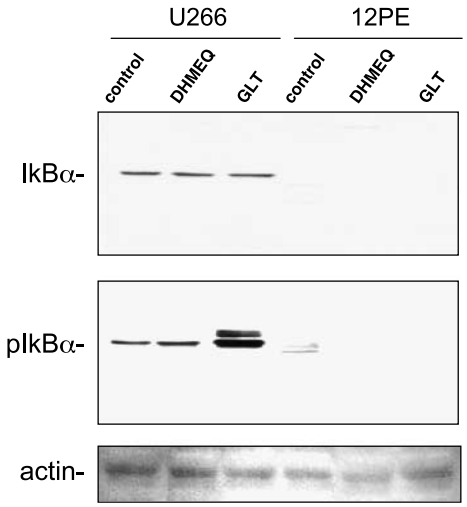



**Figure 3.** Western blot analysis of IκBα. Accumulation of phosphorylated-IκBα was detected in U266 cells after treatment with gliotoxin, whereas there was no expression of IκBα or phosphorylated IκBα either before or after treatment with gliotoxin in 12PE cells. DHMEQ did not change the expression of IκBα in both cell lines.

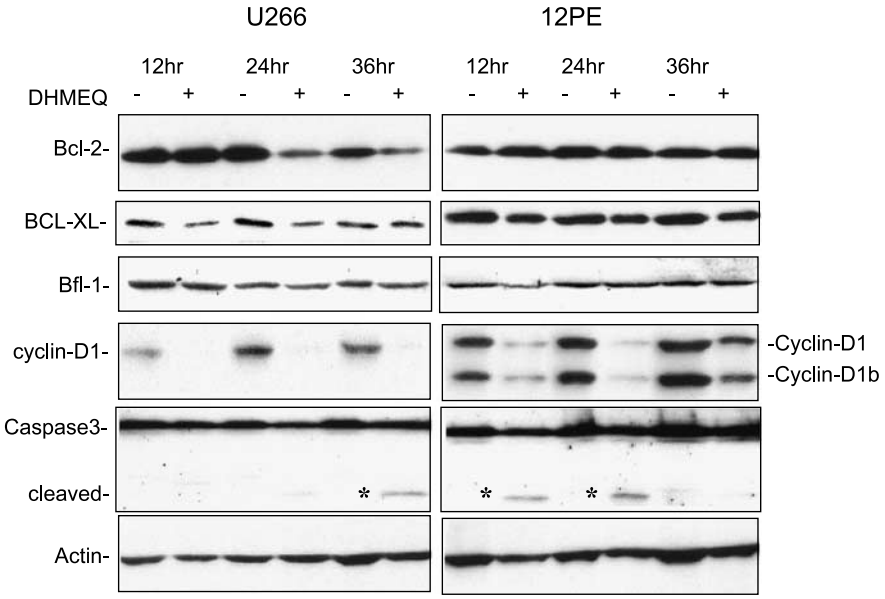



**Figure 4.** Western blot analysis of cyclin D1, Bcl-2, Bcl-xL, Bfl-1, and caspase-3. U266 and 12PE were treated with 10 μg/mL of DHMEQ for 12, 24, and 36 h. Down-regulation of Bcl-2 and Bcl-xL was found only in U266 and not in 12PE. DHMEQ down-regulated cyclin D1 in both cell lines. The aberrant form of cyclin D1 (D1b) was also down-regulated in 12PE. DHMEQ treatment resulted in the cleaved form of caspase-3 in both cell lines (*), indicating that DHMEQ-induced apoptosis was at least partly mediated through activation of caspase-3.

examined the expression of the *mdr-1* gene because NF-κB induces *mdr-1* gene expression (33). However, neither expression nor induction of *mdr-1* was detected by reverse transcription-PCR in either cell line (data not shown).

Another hallmark of apoptosis is activation of caspases. Therefore, we next examined whether blocking the NF-κB pathway with DHMEQ activated the caspase pathway. Western blot analysis showed that DHMEQ activated caspase-3 in both cell lines (Fig. 4), indicating that DHMEQ-induced apoptosis is at least partly mediated by the caspase-3 pathway. Significant activation of caspase-3 was found at 24 hours in 12PE but not in U266; however, activation was found at 36 hours in U266. This is compatible with the fact that 12PE undergoes apoptosis sooner than U266 after treatment with DHMEQ (Fig. 1D).

### DHMEQ Inhibits Tumor Growth *In vivo*

The efficacy of DHMEQ treatment in the plasmacytoma *in vivo* model was examined. We s.c. transplanted 12PE cells into severe combined immunodeficient mice. After the mice developed palpable tumors, they received DHMEQ at a dosage of 12 mg/kg daily for 14 days. Tumor growth in treated mice was significantly inhibited (2.91 ± 1.39 mL) compared with those of control mice (7.91 ± 1.54 mL) 21 days after the start of the treatment ($P < 0.01$; Fig. 5). No apparent toxic effect was noted in the mice during treatment. These data suggested that DHMEQ may be an applicable agent for multiple myeloma treatment *in vivo*.

## Discussion

In the present study, we investigated the effects of the NF-κB inhibitor, DHMEQ, on multiple myeloma cell lines and patient myeloma cells. The major findings of the present study were as follows: (*a*) DHMEQ induced apoptosis of multiple myeloma cell lines and multiple myeloma patient cells, (*b*) DHMEQ suppressed constitutive NF-κB activation in an IκBα-independent manner, (*c*) down-regulation of cyclin D1 and activation of caspase-3 were involved in apoptosis by DHMEQ, and (*d*) DHMEQ effectively reduced plasma cell tumor growth *in vivo*.

A proteasome inhibitor, gliotoxin, did not induce apoptosis in the 12PE cell line, whereas apoptosis was

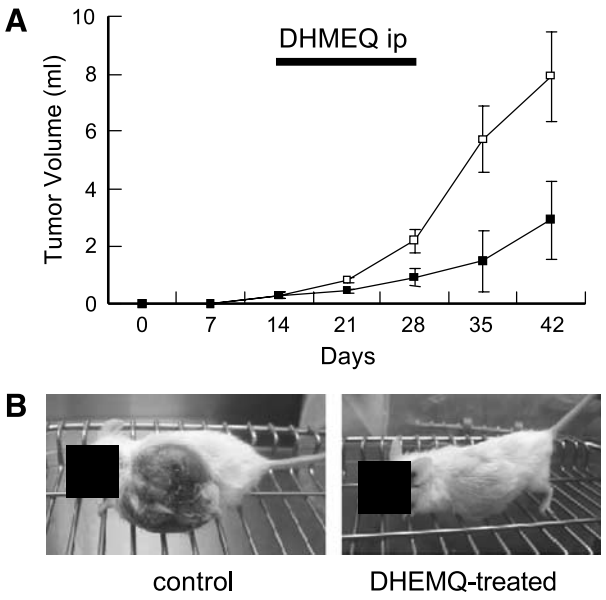



**Figure 5.** **A**, antitumor effects of DHMEQ *in vivo*. 12PE cells were implanted s.c. in the flank of severe combined immunodeficient mice. After the growth of a palpable tumor, each mouse received i.p. injections of DHMEQ once daily for 14 d (*horizontal thick line*). *Points,* serial changes in mean tumor volume (in mL); *bars,* SD. DHMEQ significantly inhibited growth of tumor. **B,** photographs of representative mice. *Left,* untreated mouse with expansive tumor and skin necrosis. *Right,* DHMEQ-treated mouse has a smaller tumor.

significantly induced in the U266 cell line 8 hours after application. Resistance of 12PE to gliotoxin is recognized as an absence of NF-κB blocking in 12PE cells by electrophoretic mobility shift assay. Moreover, gliotoxin induced an accumulation of phosphorylated IκBα in U266, indicating gliotoxin actually inhibited degradation of IκBα at the proteasome as previously reported (13). Because IκBα was not detected in 12PE, these results suggest that IκBα should be critical for gliotoxin-induced apoptosis.

Although 12PE was defective in IκBα, the sensitivity of 12PE to DHMEQ was equal to that of U266, suggesting that DHMEQ induces apoptosis independent of IκBα. Electrophoretic mobility shift assay analysis actually showed that DHMEQ inhibited translocation of NF-κB in both cell lines. This finding suggests that DHMEQ inhibit NF-κB by direct interaction but not by an IκBα-mediated mechanism as previously reported in other proteasome inhibitors (22).

It is clear that the absence of IκBα is not critical for the growth of 12PE cells. However, regulation of NF-κB in 12PE is unknown. It has been suggested that a defect in IκBα leads to constitutive translocation of NF-κB. However, whether this abnormal regulation of NF-κB causes unexpected physiologic phenomena is unknown. The 12PE cell line seems to have very efficient proliferation with a doubling time of ~24 hours. The possibility that mechanisms regulating NF-κB other than IκBα may function in 12PE cannot be ruled out. Further detailed analysis is needed to clarify the mechanisms regulating NF-κB without IκBα. Emmerich et al. (17) also reported a mutation of IkB in a Reed Sternberg cell-derived cell line, L428. However, detailed mechanisms for regulating NF-κB in the cell line were not examined.

To elucidate the downstream pathway of DHMEQ-induced apoptosis, we next evaluated caspase-3, Bcl-2, Bcl-xL, Bfl-1, and cyclin D1 protein levels after treatment with DHMEQ. Caspase-3 was cleaved after DHMEQ exposure as reported with other NF-κB inhibitors (11, 12), suggesting that DHMEQ causes apoptosis of multiple myeloma cells through the caspase-3 pathway. In contrast, down-regulation of Bcl-2 was found only in U266 and not in 12PE despite more significant cleavage of capase-3 in 12PE, suggesting that Bcl-2 is not involved with caspase-3 cleavage by DHMEQ in 12PE although the *Bcl-2* and *Bcl-xL* genes are reported to be regulated by NF-κB (30, 31). Because inhibition of NF-κB was observed in 12PE, absence of reduction of Bcl-2 and Bcl-xL in 12PE cannot be simply explained. It may be that expression of Bcl-2 and Bcl-xL is regulated by different pathways in U266 and 12PE.

It was reported that Bfl-1, a member of the Bcl-2 family of proteins, regulates apoptosis (34). Expression of Bfl-1 is regulated by NF-κB (35, 36), indicating that expression of Bfl-1 in myeloma cells after activation of NF-κB in myeloma cells is frequently found. However, Tarte et al. (25) reported that Bfl-1 is repressed in myeloma, suggesting that regulation of Bfl-1 could be independent of NF-κB in myeloma cells. Our finding that Bfl-1 was expressed but not influenced by the treatment with DHMEQ supports this hypothesis.

We also found that U266 and 12PE overexpressed cyclin D1, which was down-regulated by DHMEQ. Because NF-κB regulates transcription of cyclin D1, it is reasonable to find down-regulation of cyclin D1 by treatment with DHMEQ. Because cyclin D1 is needed for the $G_1$-S transition during the cell cycle, down-regulation of cyclin D1 by DHMEQ may lead to growth arrest in myeloma cells. Indeed, Yata et. al. (37) reported that treatment of myeloma cells with an antisense oligonucleotide of cyclin D1 inhibited the growth rate of cyclin D1–overexpressing myeloma cells. We found a variant form of cyclin D1 with a different molecular weight in 12PE and its expression was also inhibited by DHMEQ. As assessed by its molecular weight, this variant form of cyclin D1 is thought to be cyclin D1b (32), which is derived from an alternate transcript of cyclin D1. A recent study reported that cyclin D1b protein is constitutively localized in the nucleus (32) and a potential inducer of cellular transformation. DHMEQ efficiently inhibited both the normal and variant forms of cyclin D1. Moreover, given the fact that 12PE has t(11;14) and U266 has an insertion in the IgH enhancer region on chromosome 11q13 (38), overexpression of cyclin D1 should result. It is also noteworthy that DHMEQ efficiently shut off the expression of cyclin D1, which is powerfully driven by chromosome translocation or insertion.

Taken together, DHMEQ may induce apoptosis of multiple myeloma cells at least through activation of the caspase-3 pathway and down-regulation of cyclin D1. Further analysis is needed to elucidate mechanisms of apoptosis of multiple myeloma cells, including the relationship to activation of the caspase-3 pathway and down-regulation of cyclin D1.

Finally, we evaluated the efficacy of DHMEQ in an *in vivo* plasma cell tumor model. DHMEQ treatment using doses at a concentration far lower than the toxic dosage induced marked reduction in tumor volume compared with vehicle treatment. Treatment of severe combined immunodeficient mice with DHMEQ did not result in any apparent side effects or death throughout the evaluation period. Because the toxic dose of DHMEQ is ~30 times that utilized here,[3] DHMEQ is believed to be a very safe compound. These findings suggest that treatment at higher doses and for longer durations should be examined in future studies. Further analysis of the metabolism of DHMEQ is needed to select the most appropriate therapeutic regimen.

Analysis of fresh myeloma cells from 11 cases revealed that DHMEQ induced apoptosis even in cases refractory to conventional chemotherapy, including MP, VAD, high-dose melphalan, or thalidomide. Indeed, melphalan was not effective in this 12PE mouse model (data not shown), suggesting that DHMEQ should be a very useful agent for chemoresistant myeloma cells.

In conclusion, we have shown in the present study that DHMEQ could be used as a new molecular-targeted chemotherapeutic agent for multiple myeloma. Analysis

[3] K. Umezawa, unpublished result from *in vivo* experiments.

of fresh myeloma cells indicates that DHMEQ has a potential to overcome refractoriness to various chemotherapeutic drugs. Our *in vivo* model showed that DHMEQ was safe and effective. Further analysis of the exact mechanisms of the inhibitory effects of DHMEQ on nuclear translocation of NF-κB is currently under way.

**Acknowledgments**

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