Growth Inhibition of Multiple Myeloma Cells by a Novel IκB Kinase Inhibitor

Takaomi Sanda,[1,2] Shinsuke Iida,[2] Hiroka Ogura,[1] Kaori Asamitsu,[1] Toshiki Murata,[3] Kevin B. Bacon,[4] Ryuzo Ueda,[2] and Takashi Okamoto[1]

Departments of [1]Molecular and Cellular Biology, and [2]Internal Medicine and Molecular Science, Nagoya City University Graduate School of Medical Sciences, Nagoya, Japan and Departments of [3]Chemistry and [4]Biology, Research Center Kyoto, Bayer Yakuhin, Ltd., Kyoto, Japan

ABSTRACT

Involvement of nuclear factor-κB (NF-κB) in cell survival and proliferation of multiple myeloma has been well established. In this study we observed that NF-κB is constitutively activated in all human myeloma cell lines, thus confirming the previous studies. In addition, we found the phosphorylation of p65 subunit of NF-κB in addition to the phosphorylation of IκBα and the activation of NF-κB DNA binding and that various target genes of NF-κB including *bcl-x_L*, *XIAP*, *c-IAP1*, *cyclin D1*, and *IL-6* are up-regulated. We then examined the effect of a novel IκB kinase inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile (ACHP). When myeloma cells were treated with ACHP, the cell growth was efficiently inhibited with IC_{50} values ranging from 18 to 35 μmol/L concomitantly with inhibition of the phosphorylation of IκBα/p65 and NF-κB DNA-binding, down-regulation of the NF-κB target genes, and induction of apoptosis. In addition, we observed the treatment of ACHP augmented the cytotoxic effects of vincristine and melphalan (L-phenylalanine mustard), conventional antimyeloma drugs. These findings indicate that IκB kinase inhibitors such as ACHP can sensitize myeloma cells to the cytotoxic effects of chemotherapeutic agents by blocking the antiapoptotic nature of myeloma cells endowed by the constitutive activation of NF-κB.

INTRODUCTION

Multiple myeloma is an intractable B-cell malignancy characterized by clonal proliferation of a terminally differentiated plasma cell in bone marrow associated with monoclonal hypergammaglobulinemia and multiple osteolytic bone lesions (1, 2). Although recent combination chemotherapy, using melphalan [L-phenylalanine mustard (PAM)], vincristine, and corticosteroids, can induce complete remission in multiple myeloma patients, the long-term remission is hardly attainable mostly due to the frequent acquisition of drug resistance and low adherence (1). Therefore, novel treatment modality has been intensively investigated.

It is noted that the interaction between myeloma cells and bone marrow stromal cells plays a crucial role through the production of cytokines or growth factors and the cognate binding of adhesion molecules (1). Among various cytokines and growth factors, interleukin-6 (IL-6) and vascular endothelial growth factor were reported to stimulate myeloma cell proliferation and its migration (3). The establishment of such bone marrow microenvironment conceivably accelerates cell proliferation. In other words, a limited number of genes including IL-6, vascular endothelial growth factor, and adhesion molecules are the principal pathophysiologic determinants of multiple myeloma. Interestingly, gene expression of these genes is under the control of a common transcription factor, nuclear factor-κB (NF-κB; refs. 1, 4). Moreover, extracellular stimuli for the growth of myeloma cells, such as CD40 ligand (CD40L) expressed on activated T cells, insulin-like growth factor I, and tumor necrosis factor α (TNFα), are known to promote the NF-κB activation pathway at various steps (5–7). A similar effect of NF-κB is also noted in other malignancies including adult T-cell leukemia (8), chronic lymphocytic leukemia (9), activated B-cell diffuse large B-cell lymphoma (9), Hodgkin's disease (9), hepatocellular carcinoma (10), and colorectal cancer (11). In fact, NF-κB inhibitors were found effective in the treatment of some cancers (12). Thus, NF-κB and its signal transduction pathway are considered as the feasible molecular target for novel cancer therapy.

NF-κB is a hetero- or homodimer consisting of Rel family proteins, p65 (RelA), RelB, c-Rel, p50/p105, and p52/p100, and normally present in the cytoplasm in association with its inhibitor, IκB (13). Stimulation by the inflammatory cytokines such as TNFα and IL-1β results in the activation of IκB kinase (IKK) complex through mitogen-activated protein kinase/extracellular signal-regulated kinase kinase kinase 1,3 or NF-κB-inducing kinase (14, 15). IKK is a large molecular weight complex consisting of three subunits, IKKα, IKKβ, and IKKγ/NEMO, in which IKKα and IKKβ serve as catalytic subunits that phosphorylate IκBα on two serine residues (Ser32/Ser36; refs. 16–18). Recent reports by us and others have shown that IKKα also phosphorylates p65 at Ser536, which is crucial for the transcriptional competence of NF-κB when bound to the promoter sequence of target genes in the nucleus (19, 20).

In this study, we examined the effect of a novel IKK inhibitor, 2-amino-6-[2-(cyclopropylmethoxy)-6-hydroxyphenyl]-4-piperidin-4-yl nicotinonitrile (ACHP), on the growth and survival of myeloma cell lines. This compound was initially

Received 9/22/04; revised 11/19/04; accepted 12/6/04.
Grant support: Ministry of Education, Culture, Sports, Science, and Technology, and the Ministry of Health, Labor, and Welfare of Japan. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked *advertisement* in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

Requests for reprints: Takashi Okamoto, Department of Molecular and Cellular Biology, Nagoya City University Graduate School of Medical Sciences, 1 Kawasumi, Mizuho-cho, Mizuho-ku, Nagoya, Aichi 467-08601, Japan. Phone: 81-52-853-8205; Fax: 81-52-859-1235; E-mail: tokamoto@med.nagoya-cu.ac.jp.

synthesized by Murata et al. (21) based on the massive screening. Among these compounds, ACHP exhibited the highest selectivity for IKKβ and IKKα (IC_{50} values for IKKβ and IKKα are 8.5 and 250 nmol/L, respectively, measured by *in vitro* kinase assays) over other kinases such as IKK3, Syk, and mitogen-activated protein kinase kinase kinase 4 ($IC_{50} > 20$ μmol/L for these kinases; ref. 22). In addition, ACHP showed good aqueous solubility and cell-permeability, thus demonstrating a very high oral bioavailability in mice and rats.

Here we show that IκBα and p65 are constitutively phosphorylated in myeloma cells, indicating the persistent activation of NF-κB, and ACHP could efficiently block NF-κB pathway in myeloma cells, thus arresting cell growth and inducing apoptotic cell death. An apparent synergism was also detected between ACHP and other conventional anticancer drugs used in the treatment of myeloma.

MATERIALS AND METHODS

Cell Lines. Human myeloma cell lines, ILKM-2, ILKM-3, KM5, U266, NCUMM-2, AMO1, and NOP1, and a B-cell lymphoma cell line, BJAB, were used in this study as described previously (23). These cell lines were cultured in RPMI 1640 supplemented with 10% fetal bovine serum, streptomycin, and penicillin at 37°C in 5% CO_2 incubator. Exogenous IL-6 is required for the growth of ILKM-2 and ILKM-3, whereas other cell lines can grow without exogenous IL-6. U266 cells produce IL-6 in an autocrine fashion. Although U266 and BJAB cells are known to have constitutively activated NF-κB (24, 25), constitutive activation of NF-κB has not been definitively reported in other cell lines.

Reagents. The novel IKK inhibitor, ACHP, was a kind gift from Bayer Yakuhin (Kyoto, Japan). Melphalan (PAM), vincristine, and dexamethasone were obtained from Sigma (St. Louis, MO). PAM was dissolved in dimethyl sulfoxide whereas vincristine and dexamethasone were resolved in PBS. In each experiment, equal amounts of dimethyl sulfoxide or PBS were added to control cells. We confirmed that dimethyl sulfoxide concentrations used in this study did not affect cell viability (data not shown). Human recombinant TNFα was purchased from Roche (Mannheim, Germany) and used at 5 ng/mL for NF-κB stimulation. Recombinant human IL-6 (Diaclone Reseach, Basaon, France) was added at a final concentration of 10 ng/mL.

Immunoblot Analysis. For analysis of various proteins, ~1.0×10^6 cells were maintained with or without ACHP at 37°C. These cells were washed once with cold PBS and resuspended in 50 μL of hypotonic lysis buffer [20 mmol/L HEPES-KOH (pH 7.9), 10 mmol/L KCl, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 0.2% Triton X-100, protease inhibitor]. After 20 minutes of incubation on ice, the samples were centrifuged and the supernatant was collected as cytoplasmic extract. Protein concentration was measured using detergent-compatible protein assay (Bio-Rad, Hercules, CA) and equal amounts of the proteins were electrophoresed on 10% SDS-PAGE and transferred onto the nitrocellulose membrane. The membranes were blocked with TBS-T [10 mmol/L Tris-HCl (pH 8.0), 15 mmol/L NaCl, 0.1% Tween 20] containing 5% nonfat milk for 2 hours at room temperature, and incubated with TBS-T containing 5% nonfat milk and 1:1,000 diluted antibodies against either phospho-IκB-α (Ser32) or phospho-p65 (Ser536; Cell Signaling Technology, Beverly, MA) overnight at 4°C. For antibodies against p65, p52/p100, IκB-α, and α-tubulin (Santa Cruz, Santa Cruz, CA), incubation was done at room temperature for 2 hours. After incubation, the membranes were rinsed with TBS-T and further incubated with horseradish peroxidase-conjugated secondary antibodies (Amersham Biosciences, Buckinghamshire, United Kingdom) in TBS-T with 5% nonfat milk at room temperature for 1 hour. Each protein was detected by chemiluminescence using SuperSignal (Pierce, Rockford, IL).

Electrophoretic Mobility Shift Assay. Electrophoretic mobility shift assay was done as described previously (26). Briefly, 1.0×10^6 cells were cultured with or without ACHP at 37°C, washed with PBS, and treated with hypotonic lysis buffer. After 20 minutes of incubation on ice, the cells were centrifuged to remove supernatant and resuspended in 50 μL of hypertonic lysis buffer [50 mmol/L HEPES-KOH (pH 7.9), 400 mmol/L NaCl, 1 mmol/L EDTA, 1 mmol/L Na_3VO_4, 5 mmol/L NaF, 1 mmol/L phenylmethylsulfonyl fluoride, 0.2% Triton X-100, protease inhibitor]. Thirty minutes after incubation at 4°C, the supernatant was collected as nuclear extract. Electrophoretic mobility shift assay was done using double stranded oligonucleotides containing the κB sequence taken from HIV long terminal repeat (5′-TGT CGA ATG CAA ATC ACT AGA A-3′). The probe DNA was 5′ end-labeled using T4 polynucleotide kinase and [γ-^{32}P]-ATP (Amersham Biosciences). DNA binding reactions were done at 30°C for 15 minutes with labeled DNA and 25 μg nuclear extract in 20 μL binding buffer [22 mmol/L HEPES-KOH (pH 7.9), 80 mmol/L KCl, 5% glycerol, 0.1% NP40, 1 mmol/L DTT, 2 μg poly dI-dC, 2 μg tRNA, and protease inhibitor]. The samples were loaded on 5% non-denaturing polyacrylamide gel with 0.5× Tris-Borate-EDTA buffer at 4°C, followed by autoradiography.

Transient Luciferase Assay. Approximately 2.0×10^6/well U266 cells were transfected in 12-well plates in triplicates using DEMRIE-C reagent (Invitrogen, Carlsbad, CA) according to the recommendation of the manufacturer. For each transfection, 2.5 μg of reporter plasmid, 4κBwt-Luc, or 4κBmut-Luc, and 1.5 μg of the internal control plasmid, pRL-TK, expressing Renilla luciferase, were used. The construction of these plasmids was described previously (26). Twenty-four hours after transfection, the cells were treated with ACHP. After 4 hours of incubation, the cells were harvested and the luciferase activity was measured by luminometer as described (27). The cells were treated with TNFα and harvested after 30 minutes of treatment. The luciferase activity was normalized with Renilla luciferase activity used as an internal control. The efficiency of transfection was about 0.9% as estimated from the experiment with a plasmid expressing green fluorescent protein (data not shown).

Reverse Transcription-PCR. To detect mRNA expression of various genes, 1.0×10^6 cells in 1 mL were maintained at 37°C in CO_2 incubator, washed once with PBS, homogenized with QIAshredder (Qiagen, Alameda, CA), and total RNA was purified using RNeasy (Qiagen) according to the protocol of the manufacturer. After incubation with DNase I (Invitrogen), 1 μg of total RNA was reverse transcribed using SuperScript First-Strand synthesis System (Invitrogen). One-seventh of each sample was subjected to PCR amplification for 33 cycles, and the products

were analyzed by agarose gel electrophoresis. The oligonucleotideprimers were as follows: *bcl-2*, sense 5′-TCG CTA CCG TCG TGA CTT C-3′ and antisense, 5′-AAA CAG AGG TCG CAT GCT G-3′; *bcl-x_L*, sense 5′-GTT GTA CCT GCT TGC TGT CGC CGG-3′ and antisense 5′-AGC TTG TAG GAG AGA AAG TCG ACC-3′; *cyclin D1*, sense 5′-CCC TCG GTG TCC TAC TTC AAA-3′ and antisense 5′-CAC CTC CTC CTC CTC CTC TTC-3′; *XIAP*, sense 5′-CTT GCA TAC TGT CTT TCT GAG C-3′ and antisense, 5′-ACA CCA TAT ACC CGA GGA AC-3′; *c-IAP1*, sense 5′-CCT GTG GTT AAA TCT GCC TTG-3′ and antisense 5′-CAA TTC GGC ACC ATA ACT CTG-3′; *β-actin*, sense CCA GGC ACC AGG GCG TGA TG-3′ and antisense 5′ -CGG CCA GCC AGG TCC AGA CG-3′.

Interleukin-6 Production Assay. To measure IL-6 production, 2.0×10^5 of U266 cells in 500 μL medium were cultured at 37°C for 48 hours, centrifuged to collect supernatant, and the IL-6 concentrations were determined using ELISA kit according to the instruction of the manufacturer (Amersham Biosciences).

Growth Inhibition Assay. Growth inhibitory effects of compounds were analyzed using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (27). Approximately 1.0×10^4 to 1.5×10^4 cells (in 100 μL/well) were cultured in 96-well plates in triplicates in the presence or absence of each reagent or in combination at 37°C. After incubation, 10 μL (5 mg/mL) of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide solution (Sigma) were added to each well, the cells were incubated for 4 hours at 37°C, and 100 μL of lysis buffer (0.04 mol/L HCl, isopropanol) were added. Absorbances at 570 and 630 nm were measured with the aid of multiplate reader using plain medium as blank. Cell viability (%) was calculated as follows: $(A_{630} - A_{570}$ of the samples / $A_{630} - A_{570}$ of the control) × 100 (%).

Cell Cycle Analysis. Cytofluorometric analysis was done with $\sim 1.0 \times 10^6$ cells as previously described (28). After incubation with or without ACHP, the cells were washed with cold PBS and fixed with 70% ethanol at −30°C overnight. The cell pellets were resuspended in 500 μL PBS containing 2 mg/mL RNase A (Roche) and kept at 37°C for 30 minutes. Then, the cell pellets were resuspended in 500 μL PBS containing 20 mg/mL propiodium iodide (PI) followed by incubation at room temperature for 30 minutes. The DNA content of each cell preparation was analyzed by flow cytometry (FACScan, BD Bioscience, San Jose, CA) using CellQuest analysis program.

Apoptosis Assay. Briefly, cells undergoing apoptosis were detected as previously reported (29). After treating the cells (2.0×10^5 cells) at 37°C with or without ACHP for 8 hours, the cells were washed with cold PBS and resuspended in staining buffer containing PI and FITC-conjugated Annexin V (MEBCYTO apoptosis kit, MBL, Nagoya, Japan). After 20 minutes of incubation in the dark at room temperature, the cells were analyzed by flow cytometry.

RESULTS

Constitutive Phosphorylation of IκBα and p65 in Myeloma Cell Lines. In order to see the status of NF-κB signaling in myeloma cells, we examined the phosphorylation of IκBα and p65 subunit in a number of myeloma cell lines and BJAB B-cell line. As shown in Fig. 1, the phosphorylation of IκBα at Ser32 was detected in most of the cell lines examined, especially in U266, ILKM-2, NCUMM-2, and BJAB. The upper bands, corresponding to heavily phosphorylated IκBα, were detected in ILKM-2 and NCUMM-2. In addition, p65 is phosphorylated at Ser536 in all the cell lines examined. This constitutive phosphorylation of IκBα was observed even when cells were cultured in serum-free medium (data not shown). These findings suggest that NF-κB is constitutively activated in myeloma cells.

Constitutive Activation of Nuclear Factor-κB DNA Binding and Inhibition by 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile. We then examined if NF-κB DNA-binding is constitutively activated in these myeloma cells by electrophoretic mobility shift assay. Representative results are shown in Fig. 2 with U266 and NCUMM-2 cells, in which the NF-κB DNA-binding is constitutively activated. We also examined the effect of ACHP on the NF-κB DNA binding in these cells and found that ACHP, specific inhibitor of IKKα and IKKβ, could inhibit the DNA binding activity of NF-κB (Fig. 2*B*). The inhibitory effect was observed at ACHP concentrations greater than 10 μmol/L, and was evident after only 4 hours of treatment with ACHP (Fig. 2*C*).

Inhibition of IκBα and p65 Phosphorylation by 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile. We then examined the effect of ACHP on the phosphorylation of IκBα and p65. As shown in Fig. 3*A*, ACHP efficiently inhibited the phosphorylation of IκBα and p65 at 1 μmol/L, and phosphorylated forms of these proteins disappeared at higher concentrations. This inhibitory action was observed as early as 20 minutes after treatment (Fig. 3*B*). No effect of ACHP on the processing of p100/p52, another subunit of NF-κB, was observed. Similar effects of ACHP were observed with other myeloma cell lines (data not shown).

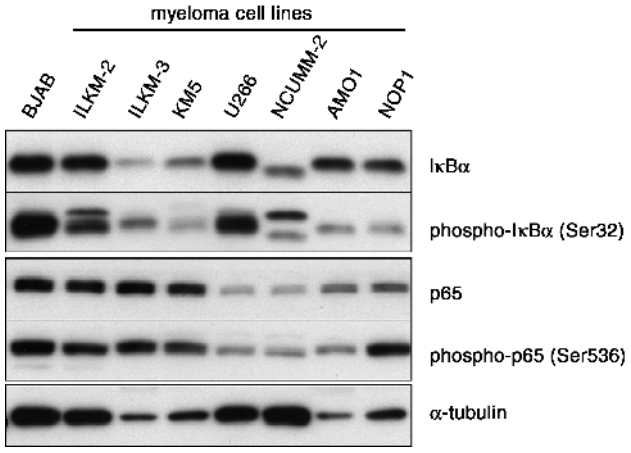

Fig. 1 Constitutive phosphorylation of IκBα and p65 in myeloma cell lines. The cytoplasmic extracts obtained from seven myeloma cell lines (U266, ILKM-2, NCUMM-2, ILKM-3, KM5, AMO1, and NOP1) and a B-cell line (BJAB), maintained in culture without any stimulation, were examined by Western blotting analyses with specific antibodies against IκBα, phospho-IκBα (at Ser32), p65, and phospho-p65 (at Ser536). Anti-α-tubulin antibody was used as an internal control. The mobility shift of the phosphorylated IκBα is noted in ILKM-2 and NCUMM-2 cells, suggesting phosphorylation at multiple sites.

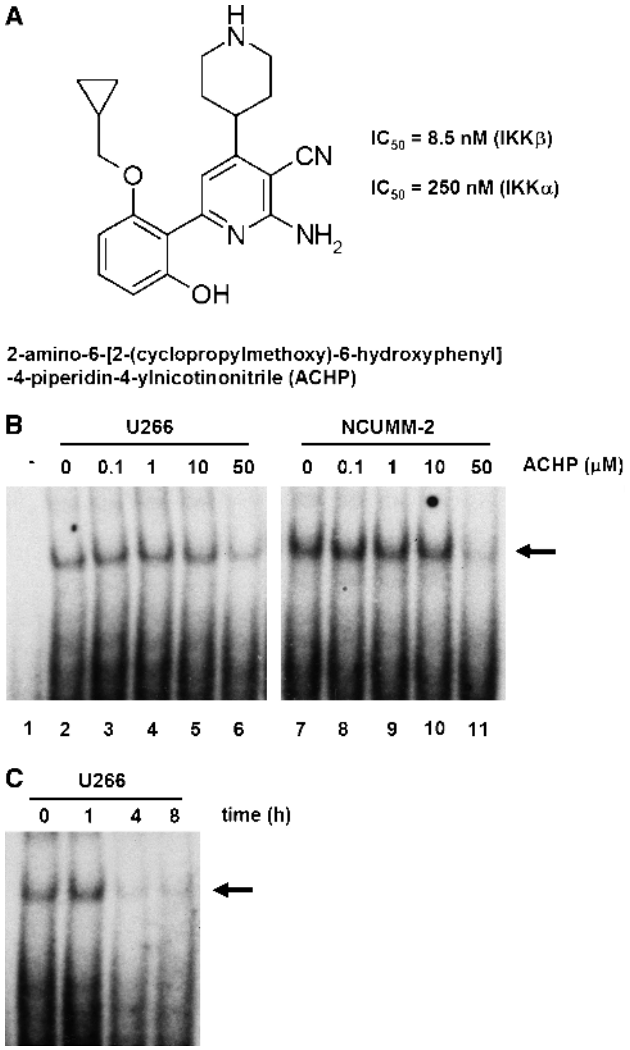

Fig. 2 Constitutive activation of NF-κB DNA binding in myeloma cells and inhibitory effect of ACHP. *A,* structure of ACHP. *B,* dose-dependent inhibition of NF-κB DNA binding by ACHP. The myeloma cell lines, U266 and NCUMM-2, were cultured in the absence of any stimulation and treated with ACHP (0-50 μmol/L) for 4 hours. Nuclear extracts were prepared and subjected to electrophoretic mobility shift assay. Arrow, location of the DNA-NF-κB complex, which was confirmed by the competition assay using unlabelled κB DNA probe and the supershift assay using antibodies for NF-κB subunits (data not shown). Lane 1, probe DNA only; lanes 2-6 and 7-11, fixed amounts (~25 μg) of nuclear extracts from U266 and NCUMM-2 cells, respectively, were incubated with the DNA probe. The indicated concentrations of ACHP were added in cell cultures for 4 hours before preparation of the nuclear extract. *C,* time-dependent inhibition of NF-κB DNA binding by ACHP. U266 cells were treated with ACHP (50 μmol/L) for 0 to 8 hours before preparation of the nuclear extract.

Effect of 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile on the Tumor Necrosis Factor α–mediated Nuclear Factor-κB Transactivation. We then examined the inhibitory effect of ACHP on NF-κB transactivation activity. By transfection of NF-κB-dependent luciferase reporter plasmid to U266 cells, ~4-fold increase in the extent of gene expression was observed (Fig. 4*A*). When cells were pretreated with ACHP 4 hours before the stimulation with TNFα, a dose-dependent inhibition of gene expression was observed. The inhibitory action was evident at 0.1 μmol/L ACHP. No such effect of ACHP was observed in control transcription using a reporter plasmid devoid of NF-κB binding sites.

Inhibition of Gene Expressions of *cyclin D1*, *bcl-x_L*, *XIAP*, and *c-IAP1* by 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile. In myeloma cells, constitutive NF-B activation and transcriptional induction of target antiapoptotic genes such as *bcl-x_L*, *XIAP*, and *c-IAPs* are ascribable to the resistance to apoptotic stimuli by anticancer agents (30, 31). In addition, NF-B also contributes to cell proliferation of myeloma cells by up-regulating growth-promoting genes such as *cyclin D1* (30). We thus examined the effect of ACHP on gene expression of these genes. As shown in Fig. 4*B*, whereas gene expression levels of β-*actin* (control) were not changed by the treatment of U266 with ACHP, inhibition of gene expressions of *bcl-x_L*, *XIAP*, *c-IAP1*, and *cyclin D1*

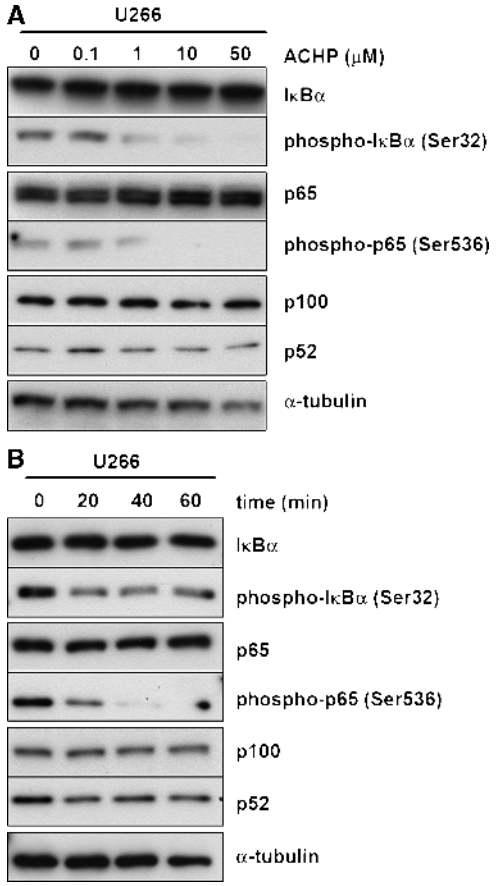

Fig. 3 Inhibition of IκBα and p65 phosphorylation by ACHP. *A,* dose-dependent inhibition by ACHP. U266 cells were treated with ACHP (0-50 μmol/L) for 20 minutes. Cytoplasmic extracts were prepared and subjected to immunoblots with the indicated antibodies. The IC_{50} values for phospho-IκBα and phospho-p65 were 1.0 and 7.6 μmol/L, respectively. *B,* time-dependent inhibition of IκBα and p65 phosphorylation by ACHP. U266 cells were treated with ACHP (10 μmol/L) for 0 to 60 minutes and the nuclear extract was obtained for the immunoblot analyses.

was observed. The expression of *bcl-2* was not remarkably affected by ACHP. The inhibitory action of ACHP was observed after 4 hours (Fig. 4*C*). Similar results were observed with NCUMM-2 and ILKM-2 cells (data not shown). Moreover, the effect of ACHP on the production of a growth promoting cytokine, IL-6, was examined with IL-6-secreting myeloma cell line, U266. As shown in Fig. 4*D*, a significant reduction of IL-6 was evident at concentrations of ACHP greater than 0.1 mol/L. Increasing concentration of ACHP resulted in further reduction of IL-6 production associated with repression of cell growth.

Suppression of Cell Cycle Progression and Induction of Apoptosis by 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile. When U266 and NCUMM-2 cells were treated with 10 μmol/L ACHP for 24 hours, cell cycle progression was affected. As shown in Fig. 5*A*, whereas 24% of U266 cells were at S phase, only 17.0% of U266 cells were at S phase after treatment with ACHP. Similar results were obtained with NCUMM-2 cells. ACHP also induced apoptosis in myeloma cell lines. In Fig. 5*B*, the number of cells undergoing apoptosis (Annexin V (+) and PI (−)) was measured. Although the sensitivity of apoptosis to ACHP varied among different cell lines, ACHP could efficiently induce cell death. For example, in U266 cells, which were relatively resistant to the ACHP-induced apoptosis, 50 μmol/L ACHP treatment increased the fraction of apoptotic cells from 4.2% (no treatment) to 16.0%. In NCUMM-2 cells, even a lower concentration of ACHP (10 μmol/L) could efficiently induce apoptosis (15.8%) and a higher concentration of ACHP (50 μmol/L) induced apoptosis in 43.7% of the cells. These findings illustrate the effect of ACHP in down-regulating antiapoptotic genes (Fig. 4).

Growth Inhibitory Effects of 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile. We then assessed the net effects of ACHP on the growth of myeloma cell lines (U266, NCUMM-2, and ILKM-2) and B-cell line (BJAB). As shown in Fig. 6*A*, ACHP inhibited cell growth in a dose-dependent manner with mean IC_{50} of 26.8 μmol/L in three myeloma cell lines. There was a sharp decline in cell growth property between 10 and 50 μmol/L of ACHP in most of the cells, which corresponded with the effective ACHP concentration that inhibited expression of antiapoptotic genes (Fig. 4). Figure 6*B* shows the time course of cell growth property of U266 cells with various concentrations of ACHP. Higher concentrations of ACHP were necessary to inhibit cell growth. Lower ACHP concentrations (10 μmol/L and below) eventually allowed myeloma cells to grow for a longer time. Similar observations were obtained with other myeloma cells examined (data not shown).

Effects of 2-Amino-6-[2-(Cyclopropylmethoxy)-6-Hydroxyphenyl]-4-Piperidin-4-yl Nicotinonitrile with Other Antimyeloma Agents. Finally, we examined the feasibility of ACHP as an adjuvant chemotherapeutic agent in myeloma treatment. In the experiments described in Fig. 6*C*, U266 cells were cultured in the presence of PAM, vincristine, and dexamethasone together with ACHP and the effects on cell growth were assessed. Although 10 μmol/L of ACHP alone did not efficiently inhibit U266 cell growth (Fig. 6*A*), combination with either PAM, vincristine, or dexamethasone showed more

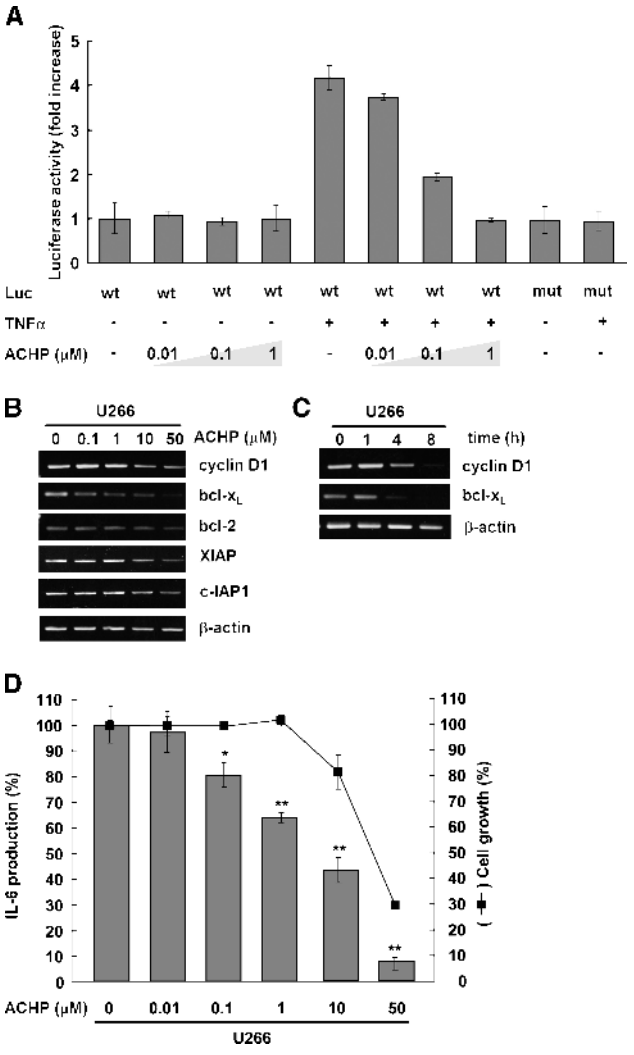

Fig. 4 Inhibition of the NF-κB dependent transactivation and gene expression by ACHP. Luciferase reporter plasmid containing wild-type NF-κB binding sequence (*wt*) or its mutant (*mut*) was transfected into U266 cells together with an internal control plasmid (pRL-TK). Forty hours after transfection, the cells were treated with ACHP (0-1 μmol/L) for 4 hours, stimulated by TNFα (5 μg/mL) for 30 minutes, and harvested for luciferase assay. The luciferase activity is indicated as fold increase compared with the untreated control (=1.0). Transfection efficiency was ~0.9% as evaluated by green fluorescent protein assay (data not shown). Experiments were done in triplicates; columns, mean; bars, SD. *B,* dose-dependent inhibition by ACHP. After U266 cells were treated with ACHP (0-50 μmol/L) for 4 hours, total RNA samples were prepared for reverse transcription-PCR, and the mRNA levels of *cyclin D1*, *bcl-xL*, *bcl-2*, *XIAP*, *c-IAP1*, and *β-actin* were examined with specific primers. *C,* time-dependent inhibition by ACHP. U266 cells were treated with ACHP (50 μmol/L) for 0 to 8 hours, total RNA samples were prepared, and the mRNA level of each gene was assessed by reverse transcription-PCR. *D,* down-regulation of IL-6 production by ACHP. U266 cells were incubated in the presence of ACHP (0-50 μmol/L) for 48 hours and the IL-6 levels in the supernatant were measured by ELISA method (*solid bar*). The effect of ACHP on the net cell growth was examined by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay (*square*). The results are expressed as percentage compared with the untreated control. Experiments were done in triplicates; columns, mean; bars, SD. *, $P = 0.05$; **, $P < 0.01$.

than additive effects in blocking cell growth in a dose-dependent manner (Fig. 6*C*). When cell cultures were maintained for up to 5 days, a greater effect was observed with vincristine and ACHP (Fig. 6*D*). Although similar effects were observed with PAM and ACHP for the first 3 days, the synergistic effect was diminished after 5 days, presumably due to the short half-life of PAM.

DISCUSSION

In spite of a significant advancement in conventional chemotherapy and wider applicability of high-dose treatment with transplantation of hematopoietic stem cells, multiple mye loma still remains incurable (3). Thus, the pursuit for novel therapeutic modalities has been attempted by many laboratories. One such approach is the chemotherapeutic intervention of the NF-κB activation cascade (25, 32–34). In this study, we examined the effect of ACHP, a newly developed IKK inhibitor, on the growth of myeloma cells. We confirmed the previous observations of Bharti et al. (25, 35) that NF-κB is constitutively activated in myeloma cells and found that ACHP could effectively inhibit the myeloma cell growth. We also found that the cell growth inhibitory effect of conventional antimyeloma compounds, vincristine and PAM, was significantly augmented when combined with ACHP. These findings support the idea that NF-κB could be a feasible molecular target for the treatment of multiple myeloma.

Regarding the mechanism by which NF-κB is activated in myeloma cells, we found the constitutive phosphorylation of p65 subunit of NF-κB at Ser536 as well as that of IκBα at Ser32. Although the constitutive phosphorylation of IκBα at Ser32 has been reported in myeloma cells (25, 35), the constitutive phosphorylation of p65 has not been explored. There are at least three phosphorylation sites, Ser276, Ser529, and Ser536, within p65. Among these phosphorylation sites of p65, Ser536 phosphorylation plays crucial roles in the NF-κB-mediated transactivation (36). For example, the point mutation of Ser536

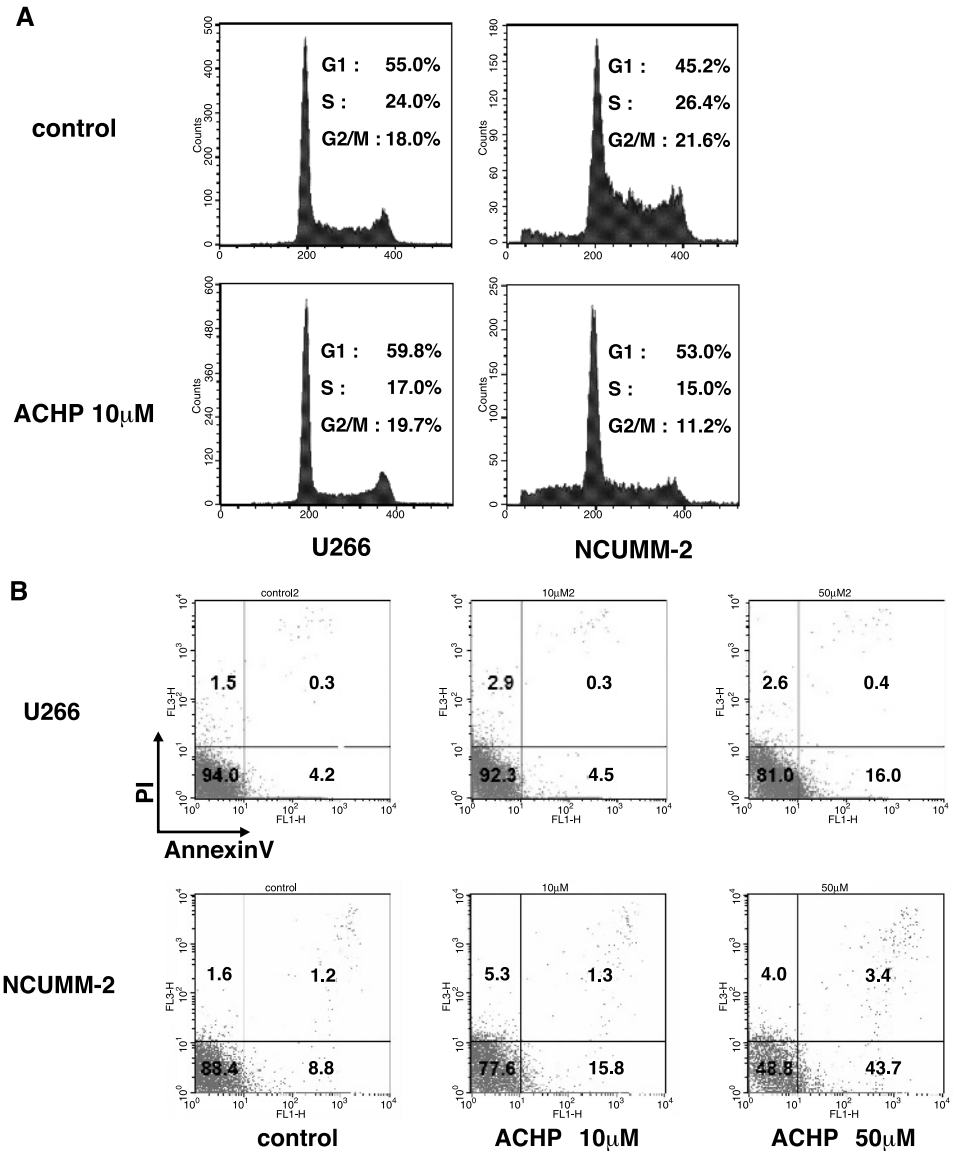

Fig. 5 Effects of ACHP on cell cycle and induction of apoptosis. *A,* inhibition of cell cycle progression by ACHP. U266 and NCUMM-2 cells were treated with ACHP (10 μmol/L) for 24 hours. Cells were stained with PI and subjected to flow cytometric analysis. The fractions of each cell cycle phase (%) are shown as percentage using the CellQuest analysis program. *B,* induction of apoptotic cells by ACHP. U266 and NCUMM-2 cells were treated with ACHP (0, 10, and 50 μmol/L) for 8 hours, stained with FITC-conjugated Annexin V and PI, and analyzed by flow cytometry. Normal cells do not stain with Annexin V or PI, whereas apoptotic cells stain with Annexin V but not PI. The percentage of each fraction is indicated.

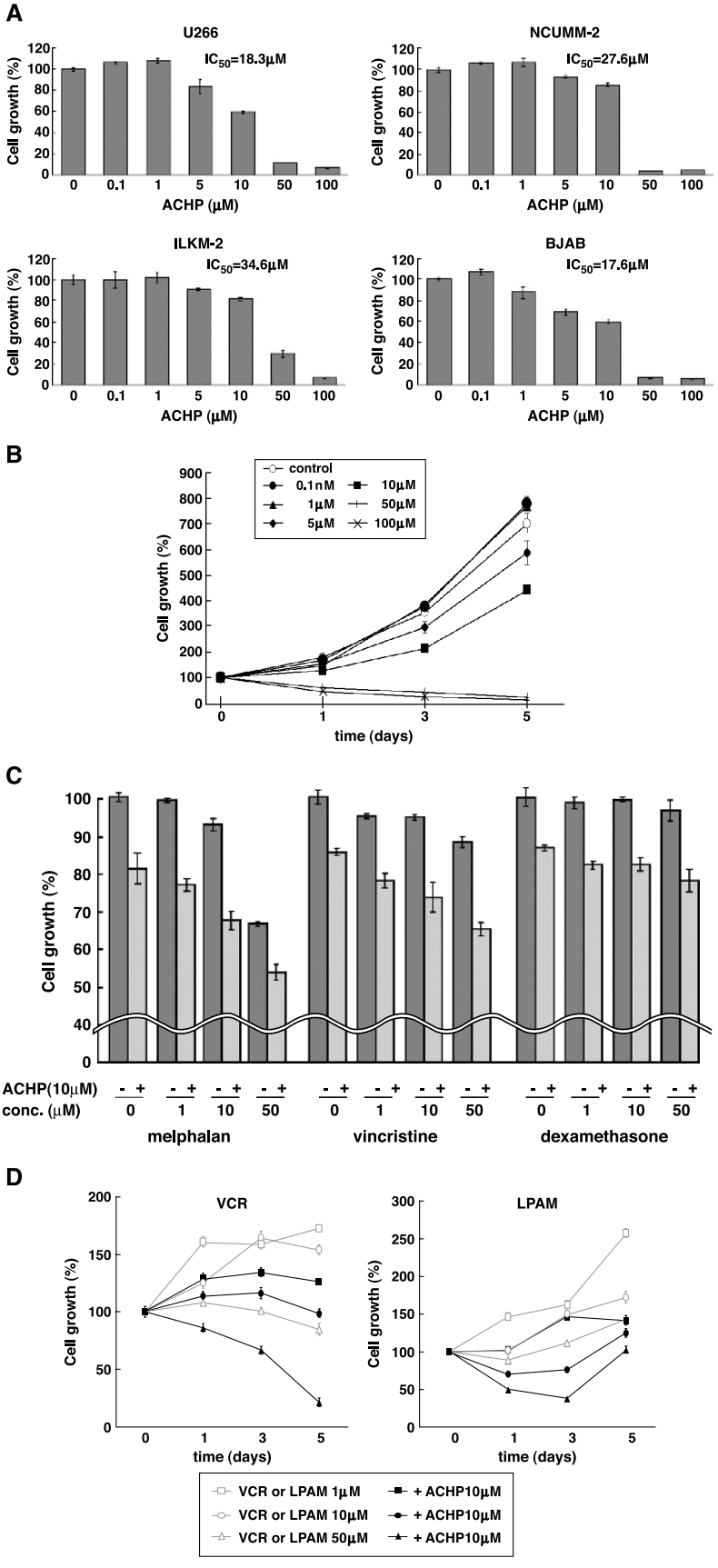

Fig. 6 Growth inhibitory effects of ACHP. Cell growth was monitored by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide assay. The results are indicated as percentage compared with the untreated control or day 0. Experiments were done in triplicates; columns, mean; bars, SD. *A,* cell growth analysis of myeloma cell lines and the inhibitory effects of ACHP. Each myeloma cell and BJAB was treated with the indicated concentrations of ACHP for 3 days. *B,* effects of ACHP on the temporal profiles of U266 cell growth. U266 cells were treated with indicated concentrations of ACHP (0-100 μmol/L) up to 5 days. *C,* synergistic effects of ACHP with other antimyeloma agents. The U266 myeloma cells were cultured with indicated concentrations of antimyeloma agents in the absence or presence (10 μmol/L) of ACHP for 1 day. *D,* the temporal profile of U266 cells. The combined effects of ACHP and vincristine or PAM. U266 cells were cultured with vincristine or PAM (1-50 μmol/L) and combined with ACHP (10 μmol/L) for up to 5 days. Cell growth was assessed at day 1, day 3, and day 5. The growth of untreated control U266 cells at day 1, day 3, and day 5 was 175%, 448%, and 972%, respectively.

eventually resulted in the lack of response to the lymphotoxin β receptor signaling (36) and the failure of nuclear translocation of NF-κB (37). Owing to Ser536 being located within the carboxyl-terminal transactivation domain of p65, it is implicated in the transcriptional activity of NF-κB once bound to the target DNA within the regulatory region of target genes by recruiting basal transcription factors and transcriptional coactivators (20, 36, 38). In contrast, although the Ser529 phosphorylation was found associated with the signal-induced NF-κB activation through casein kinase II (39), it is unlikely that Ser529 plays a regulatory role in the NF-κB activation cascade and its phosphorylation is considered to occur as a coincidence (40). Regarding the Ser276 phosphorylation of p65, both protein kinase A and mitogen- and stress-activated protein kinase 1 have been implicated and are considered to be involved in the NF-κB activation by regulating the selective interaction with the p300/CREB-binding protein coactivator over histone deacetylase 1 (41, 42). However, other studies have shown that protein kinase A plays a negative role in the action of NF-κB (43, 44). Thus, Ser536 phosphorylation plays a major role in the signal-mediated regulation of transcriptional competence of NF-κB.

More importantly, the signal transduction pathways mediated by CD40L and B-cell activating factor have been shown to play important roles in the proliferation of myeloma cells. For example, B-cell activating factor is overexpressed in myeloma cells and involved in the protection from dexamethasone-induced apoptosis (45). In addition, CD40L is known to induce the proliferation and migration of myeloma cells by inducing NF-κB through the activation of mitogen-activated protein kinases and phosphatidylinositol-3 kinase (5). In another report, it is shown that CD40/CD40L signaling activates NF-κB by inducing the Ser536 phosphorylation of p65 (46). Although both IKKα and IKKβ have been implicated in the Ser536 phosphorylation (36, 37, 40), the signaling cascades involving CD40L, B-cell activating factor, and lymphotoxin β require IKKα but not IKKβ (36, 47, 48). Thus, IKKα could be a more feasible target for the treatment of multiple myeloma.

Furthermore, recent recognition of the noncanonical NF-κB activation cascade, which is used by the signaling mediated by B-cell activating factor, CD40L, and lymphotoxin β, and primarily involving IKKα but not necessarily associated with phosphorylation of IκBα proteins followed by their degradation, has highlighted the role of IKKα (49). Interestingly, the treatment of cells with a nuclear export inhibitor leptomycin B resulted in the nuclear accumulation of NF-κB, IκBα, NF-κB-inducing kinase, and IKKα, but not IKKβ, indicating that these proteins are shuttling between the cytoplasm and the nucleus even in the absence of any stimulation (50). Therefore, the sole inhibition of IKKβ or proteasome may not be sufficient to suppress the NF-κB activation associated with multiple myeloma.

In this study, ACHP exhibited the distinctive effective concentrations in inhibiting various features of myeloma cells. Although inhibition of the TNFα-mediated gene expression could occur at low ACHP concentration (<1 μmol/L), higher concentrations (>10 μmol/L) were required to inhibit the constitutive phosphorylation of p65, expression of NF-κB-mediated genes, such as *cyclin D1*, *bcl-x$_L$*, *XIAP*, *c-IAP1*, and *IL-6*, and myeloma cell growth. These findings indicate that the growth inhibitory effect of ACHP may be through the inhibition of IKKα as well as IKKβ.

In conclusion, our findings indicate the therapeutic efficacy ofACHP in inducing myeloma cell death presumably by blocking the constitutive activation of NF-κB and the induction of antiapoptotic genes, thus sensitizing myeloma cells to cell death mediated by conventional antimyeloma agents. Although further efforts in drug development are necessary (i.e., the search for IKKα-specific compounds), our findings obtained with ACHP should give useful insights into a novel antimyeloma chemotherapy. Use of such compounds would conceivably reduce the dose of antimyeloma agents, prevent the side effects, enhance the adherence to chemotherapy, and augment the efficacy of the current myeloma chemotherapy.

ACKNOWLEDGMENTS

We thank Angelita Sarile for the language editing of this manuscript.

REFERENCES

1. Hideshima T, Anderson KC. Molecular mechanisms of novel therapeutic approaches for multiple myeloma. Nat Rev Cancer 2002;2:927–37.

2. Iida S, Ueda R. Multistep tumorigenesis of multiple myeloma: its molecular delineation. Int J Hematol 2003;77:207–12.

3. Dankbar B, Padro T, Leo R, et al. Vascular endothelial growth factor and interleukin-6 in paracrine tumor-stromal cell interactions in multiple myeloma. Blood 2000;95:2630–6.

4. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:6853–66.

5. Tai YT, Podar K, Mitsiades N, et al. CD40 induces human multiple myeloma cell migration via phosphatidylinositol 3-kinase/AKT/NF-κB signaling. Blood 2003;101:2762–9.

6. Mitsiades CS, Mitsiades N, Poulaki V, et al. Activation of NF-κB and up-regulation of intracellular antiapoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene 2002;21:5673–83.

7. Hideshima T, Chauhan D, Schlossman R, Richardson P, Anderson KC. The role of tumor necrosis factor α in the pathophysiology of human multiple myeloma: therapeutic applications. Oncogene 2001;20:4519–27.

8. Mori N, Yamada Y, Ikeda S, et al. Bay 11-7082 inhibits transcription factor NF-κB and induces apoptosis of HTLV-I-infected T-cell lines and primary adult T-cell leukemia cells. Blood 2002;100:1828–34.

9. Davis RE, Staudt LM. Molecular diagnosis of lymphoid malignancies by gene expression profiling. Curr Opin Hematol 2002;9:333–8.

10. Pikarsky E, Porat RM, Stein I, et al. NF-κB functions as a tumour promoter in inflammation-associated cancer. Nature 2004;431:461–6.

11. Greten FR, Eckmann L, Greten TF, et al. IKKβ links inflammation and tumorigenesis in a mouse model of colitis-associated cancer. Cell 2004;118:285–96.

12. Adams J. The proteasome: a suitable antineoplastic target. Nat Rev Cancer 2004;4:349–60.

13. Okamoto T, Sakurada S, Yang JP, Merin JP. Regulation of NF-κB and disease control: identification of a novel serine kinase and thioredoxin as effectors for signal transduction pathway for NF-κB activation. Curr Top Cell Regul 1997;35:149–61.

14. Nakano H, Shindo M, Sakon S, et al. Differential regulation of IκB kinase α and β by two upstream kinases, NF-κB-inducing kinase and mitogen-activated protein kinase/ERK kinase kinase-1. Proc Natl Acad Sci U S A 1998;95:3537–42.

15. Yang J, Lin Y, Guo Z, et al. The essential role of MEKK3 in TNF-induced NF-κB activation. Nat Immunol 2001;2:620–4.

16. DiDonato JA, Hayakawa M, Rothwarf DM, Zandi E, Karin M. A cytokine-responsive IκB kinase that activates the transcription factor NF-κB. Nature 1997;388:548–54.

17. Mercurio F, Zhu H, Murray BW, et al. IKK-1 and IKK-2: cytokine-activated IκB kinases essential for NF-κB activation. Science 1997;278:860–6.

18. Zandi E, Rothwarf DM, Delhase M, Hayakawa M, Karin M. The IκB kinase complex (IKK) contains two kinase subunits, IKKα and IKKβ, necessary for IκB phosphorylation and NF-κB activation. Cell 1997;91:243–52.

19. Sakurai H, Suzuki S, Kawasaki N, et al. Tumor necrosis factor-α–induced IKK phosphorylation of NF-κB p65 on serine 536 is mediated through the TRAF2, TRAF5, and TAK1 signaling pathway. J Biol Chem 2003;278:36916–23.

20. Jiang X, Takahashi N, Ando K, et al. NF-κB p65 transactivation domain is involved in the NF-κB-inducing kinase pathway. Biochem Biophys Res Commun 2003;301:583–90.

21. Murata T, Shimada M, Sakakibara S, et al. Discovery of novel and selective IKK-β serine-threonine protein kinase inhibitors: Part 1. Bioorg Med Chem Lett 2003;13:913–8.

22. Murata T, Shimada M, Sakakibara S, et al. Synthesis and structure-activity relationships of novel IKK-β inhibitors: Part 3. Orally active anti-inflammatory agents. Bioorg Med Chem Lett 2004;14:4019–22.

23. Kato M, Iida S, Komatsu H, Ueda R. Lack of ku80 alteration in multiple myeloma. Jpn J Cancer Res 2002;93:359–62.

24. Algarte M, Lecine P, Costello R, et al. *In vivo* regulation of interleukin-2 receptor α gene transcription by the coordinated binding of constitutive and inducible factors in human primary T cells. EMBO J 1995;14:5060–72.

25. Bharti AC, Donato N, Singh S, Aggarwal BB. Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-κB and IκBα kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. Blood 2003;101:1053–62.

26. Yang JP, Hori M, Sanda T, Okamoto T. Identification of a novel inhibitor of nuclear factor-κB, RelA-associated inhibitor. J Biol Chem 1999;274:15662–70.

27. Sato T, Asamitsu K, Yang JP, et al. Inhibition of human immunodeficiency virus type 1 replication by a bioavailable serine/threonine kinase inhibitor, fasudil hydrochloride. AIDS Res Hum Retroviruses 1998;14:293–8.

28. Ando T, Kawabe T, Ohara H, et al. Involvement of the interaction between p21 and proliferating cell nuclear antigen for the maintenance of G_2/M arrest after DNA damage. J Biol Chem 2001;276:42971–7.

29. Kajino S, Suganuma M, Teranishi F, et al. Evidence that *de novo* protein synthesis is dispensable for antiapoptotic effects of NF-κB. Oncogene 2000;19:2233–9.

30. Specht K, Haralambieva E, Bink K, et al. Different mechanisms of cyclin D1 overexpression in multiple myeloma revealed by fluorescence *in situ* hybridization and quantitative analysis of mRNA levels. Blood 2004;104:1120-6.

31. Hideshima T, Bergsagel PL, Kuehl WM, Anderson KC. Advances in Biology of Multiple Myeloma: Clinical Applications. Blood 2004;104:603–18.

32. Hideshima T, Chauhan D, Richardson P, et al. NF-κB as a therapeutic target in multiple myeloma. J Biol Chem 2002;277:16639–47.

33. Dai Y, Pei XY, Rahmani M, et al. Interruption of the NF-κB pathway by Bay 11-7082 promotes UCN-01-mediated mitochondrial dysfunction and apoptosis in human multiple myeloma cells. Blood 2004;103:2761–70.

34. Mitsiades N, Mitsiades CS, Poulaki V, et al. Biologic sequelae of nuclear factor-κB blockade in multiple myeloma: therapeutic applications. Blood 2002;99:4079–86.

35. Bharti AC, Shishodia S, Reuben JM, et al. Nuclear factor-κB and STAT3 are constitutively active in CD138+ cells derived from multiple myeloma patients, and suppression of these transcription factors leads to apoptosis. Blood 2004;103:3175–84.

36. Jiang X, Takahashi N, Matsui N, Tetsuka T, Okamoto T. The NF-κB activation in lymphotoxin β receptor signaling depends on the phosphorylation of p65 at serine 536. J Biol Chem 2003;278:919–26.

37. Mattioli I, Sebald A, Bucher C, et al. Transient and selective NF-κB p65 serine 536 phosphorylation induced by T cell costimulation is mediated by IκB kinase β and controls the kinetics of p65 Nuclear Import. J Immunol 2004;172:6336–44.

38. Asamitsu K, Tetsuka T, Kanazawa S, Okamoto T. RING finger protein AO7 supports NF-κB-mediated transcription by interacting with the transactivation domain of the p65 subunit. J Biol Chem 2003;278:26879–87.

39. Wang D, Westerheide SD, Hanson JL, Baldwin AS Jr. Tumor necrosis factor α–induced phosphorylation of RelA/p65 on Ser529 is controlled by casein kinase II. J Biol Chem 2000;275:32592–7.

40. Sakurai H, Chiba H, Miyoshi H, Sugita T, Toriumi W. IκB kinases phosphorylate NF-κB p65 subunit on serine 536 in the transactivation domain. J Biol Chem 1999;274:30353–6.

41. Zhong H, May MJ, Jimi E, Ghosh S. The phosphorylation status of nuclear NF-κB determines its association with CBP/p300 or HDAC-1. Mol Cell 2002;9:625–36.

42. Vermeulen L, De Wilde G, Van Damme P, Vanden Berghe W, Haegeman G. Transcriptional activation of the NF-κB p65 subunit by mitogen- and stress-activated protein kinase-1 (MSK1). EMBO J 2003;22:1313–24.

43. Takahashi N, Tetsuka T, Uranishi H, Okamoto T. Inhibition of the NF-κB transcriptional activity by protein kinase A. Eur J Biochem 2002;269:4559–65.

44. Neumann M, Grieshammer T, Chuvpilo S, et al. RelA/p65 is a molecular target for the immunosuppressive action of protein kinase A. EMBO J 1995;14:1991–2004.

45. Moreaux J, Legouffe E, Jourdan E, et al. BAFF and APRIL protect myeloma cells from apoptosis induced by interleukin 6 deprivation and dexamethasone. Blood 2004;103:3148–57.

46. Schwabe RF, Schnabl B, Kweon YO, Brenner DA. CD40 activates NF-κB and c-Jun N-terminal kinase and enhances chemokine secretion on activated human hepatic stellate cells. J Immunol 2001;166:6812–9.

47. Coope HJ, Atkinson PG, Huhse B, et al. CD40 regulates the processing of NF-κB2 p100 to p52. EMBO J 2002;21:5375–85.

48. Claudio E, Brown K, Park S, Wang H, Siebenlist U. BAFF-induced NEMO-independent processing of NF-κB2 in maturing B cells. Nat Immunol 2002;3:958–65.

49. Pomerantz JL, Baltimore D. Two pathways to NF-κB. Mol Cell 2002;10:693–5.

50. Birbach A, Gold P, Binder BR, et al. Signaling molecules of the NF-κB pathway shuttle constitutively between cytoplasm and nucleus. J Biol Chem 2002;277:10842–51.