Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

Preclinical Development

Molecular Cancer Therapeutics

Luteinizing Hormone-Releasing Hormone (LHRH)-I Antagonist Cetrorelix Inhibits Myeloma Cell Growth *In vitro* and *In vivo*

Jianguo Wen[1], Yongdong Feng[2], Chad C. Bjorklund[3], Michael Wang[3], Robert Z. Orlowski[3,4], Zheng-Zheng Shi[5], Bing Liao[6], Jacqueline O'Hare[1], Youli Zu[1,7], Andrew V. Schally[8,9], and Chung-Che Chang[1,7]

Abstract

The objective of this study was to determine the effects of an luteinizing hormone-releasing hormone (LHRH)-I antagonist, Cetrorelix, on human multiple myeloma (MM) cells and to elucidate the mechanisms of action. We showed that *LHRH-I* and *LHRHR-I* genes were expressed in MM cell lines and primary MM cells. Treatment with Cetrorelix inhibited growth and colony-forming ability of myeloma cells, including cell lines resistant to arsenic trioxide, bortezomib, or lenalidomide. Cetrorelix induced apoptosis in myeloma cells including primary myeloma cells. In addition, Cetrorelix inhibited the growth of human myeloma cells xenografted into mice without any apparent side effects. Cetrorelix downregulated the nuclear factor-kappa B (NF-κB) pathway activity and the expression of cytokines, including interleukin 6, insulin-like growth factor 1, VEGF-A, and stromal-derived factor 1, important for myeloma cell growth and survival in myeloma cells and/or marrow stromal cells from myeloma patients. Cetrorelix decreased the phosphorylation of extracellular signal regulated kinase 1/2 and STAT3 in myeloma cells, two crucial pathways for myeloma cells growth and survival. Moreover, the expression of p21 and p53 was increased, whereas that of antiapoptotic proteins Bcl-2 and Bcl-x_L was reduced by Cetrorelix. Our findings indicate that Cetrorelix induces cytotoxicity in myeloma cells through various mechanisms and provide a rationale for investigating Cetrorelix for the treatment of MM. *Mol Cancer Ther; 10(1); 148–58. ©2010 AACR.*

Introduction

Multiple myeloma (MM) is the second most common hematologic cancer in the United States, representing 10% of all hematopoietic malignancies (1). It is incurable, although novel approaches such as the use of proteasome inhibitor bortezomib, have improved the treatment outcome (2, 3). However, the majority of patients with refractory or relapsed disease show resistance to these therapies (4, 5). Thus, the development of novel agents to treat MM remains an important task.

We observed in our gene array experiments that RPMI 8226 myeloma cells expressed the luteinizing hormone-releasing hormone (LHRH)-I and LHRHR-I, a finding that has not been reported previously (data not shown). Hypothalamic LHRH is the primary link between the brain and the pituitary in the regulation of gonadal functions and plays a key role in control of vertebrate reproduction (6). It has been shown that most vertebrate species express at least 2 forms of LHRH (7). LHRH-I and its cognate receptor, LHRHR-I, have been found in the human endometrium, placenta, breast, ovary, testis, and prostate (8–12), as well as several malignant tumors and cell lines (10).

In a number of human cancers, including endometrial, prostatic, colorectal, lung, and ovarian tumors, the proliferation of cancer cells can be inhibited by agonistic or antagonistic analogues of LHRH (13–24). Cetrorelix especially, a third-generation LHRH antagonist, has been shown to induce apoptosis, growth inhibition, and cell-cycle arrest of cancer cells (13, 16, 22). Thus, Cetrorelix decreased the levels of cyclin A and Cdk2 (cyclin-dependent kinase) but increased the level of p21 and p53 in epithelial ovarian cancer cells (22). In leiomyoma cells

Authors' Affiliations: [1]Department of Pathology, The Methodist Hospital and The Methodist Hospital Research Institute, Houston, Texas; [2]Department of General Surgery, Tongji Hospital, Tongji Medical College in Huazhong University of Science and Technology, Wuhan, China; Departments of [3]Lymphoma and Myeloma, and [4]Experimental Therapeutics, The University of Texas M. D. Anderson Cancer Center; Departments of [5]Radiology and [6]Neurology, The Methodist Hospital and Research Institute, Houston, Texas; [7]Department of Pathology, Weill Medical College of Cornell University, New York; [8]Veterans Affairs Medical Center and South Florida Vetarans Affairs Foundation for Research and Education; and [9]Department of Pathology and Medicine, Division of Hematology/Oncology, University of Miami Miller School of Medicine, Miami, Florida

J. Wen and Y. Feng contributed equally to this work.

Corresponding Author: Chung-Che Chang, Department of Pathology, The Methodist Hospital and The Methodist Hospital Research Institute, 6565 Fannin MS205, Houston, TX 77030. Phone: 7134414458; Fax: 7137902681. E-mail: jeffchang@tmhs.org

doi: 10.1158/1535-7163.MCT-10-0829

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

treated with Cetrorelix, an augmented expression of Fas, Fas ligand, Bcl-x_L, Bax, and caspase 3 and reduced expression of Bcl-2 have been reported (19).

These findings prompted us to examine cultured human MM cells and primary myeloma cells for the presence of LHRH-I and LHRHR-I and to determine the effects of the LHRH antagonist Cetrorelix on cell growth. Our work shows that Cetrorelix induces apoptosis and inhibits the growth of myeloma cells, including some cell lines resistant to arsenic trioxide (ATO), bortezomib (BZM), or lenalidomide, *in vitro* and *in vivo*. This effect is likely exerted through the suppression of NF-κB pathway and expression of several key growth/survival factors of myeloma cells, including IL-6 (interleukin 6), IGF-1 (insulin like growth factor 1), VEGF-A, SDF1-α (stromal-derived factor 1-α), expressed by myeloma cells and/or myeloma bone marrow stromal cells (BMSC) from myeloma patients; inhibition of the activation of ERK (extracellular signal regulated kinase) and STAT3 pathways; upregulation of p53 and p21 expression; and downregulation of Bcl-2 and Bcl-x_L expression. Altogether, these findings support the merit of further evaluation of LHRH-I antagonists in the treatment of MM.

Materials and Methods

Reagents and antibodies

The LHRH-I antagonist Cetrorelix [Ac-D-Nal(2)[1], D-Phe (4Cl)[2], D-Pal(3)[3], D-Cit[6], D-Ala[10]]LH-RH [Nal(2) is 3-(2-naphthyl)alanine, Pal(3) is 3-(3-pyridyl)alanine, and Cit is citrulline], was originally synthesized in our laboratory (25). Cetrorelix was dissolved in distilled water containing 5% mannitol (vehicle solution for injection). Antibodies against phospho-ERK1/2, ERK1/2, phospho-STAT3, STAT3, p21, p27, p53, Mcl-1, Bcl-2, Bcl-x_L (Cell Signaling Technology), CD138, LHRHR-I (Abcam), β-actin (Sigma-Aldrich), and Alexa 647–conjugated phospho-NF-κB p65 (RelA; Becton Dickinson) were purchased from the indicated vendors.

Myeloma cell lines and primary cell cultures

Dexamethasone-sensitive (MM.1S) and -resistant (MM.1R) human MM cell lines were kindly provided by Dr. Steven Rosen (Northwestern University, Chicago, IL), whereas RPMI 8226 and doxorubicin-resistant RPMI 8226-Dox40 cells were gifts from Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). ATO-resistant cells (RPMI 8226-ATOR, stably maintained at 0.5 μmol/L ATO), and BZM-resistant cells (RPMI 8226-BZMR, stably maintained at 5 nmol/L BZM) were generated from RPMI 8226 in our laboratory by slowly increasing concentrations of ATO or BZM for selection (26). KAS-6/1 IL-6–dependent cells were kindly provided by Dr. Diane Jelinek (Department of Immunology, Mayo Clinic/Foundation, Rochester, MN). Lenalidomide-resistant KAS-6/R10R cells were generated from KAS-6/1 cells by exposing them to slowly increasing drug concentrations and were propagated in 10 μmol/L lenalidomide. All cell lines were cultured as reported (26). No authentication was done by the authors. Bone marrow aspirates were collected from myeloma patients or healthy donors under protocols approved by the institutional review boards of The University of Texas M. D. Anderson Cancer Center and The Methodist Hospital Research Institute and informed consent was obtained in compliance with the Declaration of Helsinki. Bone marrow mononuclear cells were isolated by density gradient centrifugation with Lymphocyte Separation Medium (MP Biomedicals). $CD138^+$ plasma cells were isolated with AutoMACS, using CD138 antibodies conjugated with magnetic beads (Miltenyi Biotec). BMSC layers were established from MM patients as described previously (27). Samples were provided from The Methodist Hospital and the M. D. Anderson Cancer Center Department of Lymphoma and Myeloma Tissue Bank. Usage of these samples was approved by the institutional review board of The Methodist Hospital Research Institute.

Flow cytometric analysis for phosphorylaiton NF-κB p65 (RelA) and quantitative RT-PCR in cocultures

MM BMSCs were harvested and seeded into 12-well plates and incubated overnight. The medium was exchanged and RPMI 8226 cells were seeded at the top of confluent, nonproliferative monolayer of BMSCs and allowed to adhere for 12 hours. The cultures were then treated with 5 μmol/L Cetrorelix for 3, 6, or 12 hours. The MM cells were collected with gentle pipetting up and down without touching the monolayer and then BMSCs were collected with trypsinization. Phospho-NF-κB p65 was tested using methods previously described (26) and cytokine quantification by quantitative reverse transcription PCR (qRT-PCR) was described in the following text.

RNA extraction, reverse transcription, and PCR

Total cellular RNA was extracted and cDNA was synthesized as previously described (26). For qRT-PCR, amplification was conducted using the LightCycler, using FastStart DNA Master SYBR Green (Roche Applied Science), and 18S RNA was used as a control to evaluate the expression of the genes listed in text, using the following primers: 5′-ATCACCAGCCACAGAGATCC-3′ and 5′-CAAGGGGGCCTCTAATTTTC-3′ for human LHRH-I, 5′-CAAGGCTTGAAGCTCTGTCC-3′ and 5′-AAGGTCAGAGTGGGGAGGTT-3 for human LHRHR-I, 5′-CTCTGCTGACCCAACAAACAG-3′ and 5′-TTTTCCGGGATCTCTCCCCAT-3′ for human APRIL, 5′-GCTACCTGGAGCACCAACTC-3′ and 5′-GCCACGCAAGTAACACAGAA-3′ for human BSF3, 5-TTTATTTCAACAAGCCCACAGG-3′ and 5′-GCAATACATCTCCAGCCTCCTTA-3′ for human IGF-1, 5′-AGAGCCAACGTCAAGCATCT-3′ and 5′-CTTTAGCTTCGGGTCAATGC-3′ for human SDF1-α, 5′-CGACTACTACGCCAAGGAGG-3′ and 5′-CGGAGCTCTGATGTGTTGAA-3′ for human TGF-β, 5′-CCTTGCCTTGCTGCTCTACCTC-3′ and 5-CCACCAGGGTCTCGATTGGAT-3′ for human VEGF, 5′-TACCCCCAGGAGAAGATTCC-3′

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

and 5′-TTTTCTGCCAGTGCCTCTTT-3′ for human IL-6, and 5′-TTCGGAACTGAGGCCATGAT-3′ and 5′-TTTCGCTCTGGTCCGTCTTG-3′ for human 18SrRNA. Each sample was measure in triplicate, and the results were analyzed as previously reported (28).

Sequencing and BLAST analysis

PCR products of LHRH-I and LHRHR-I genes, purified using QIAquick Gel Extraction Kit (Qiagen), were sequenced at Baylor College of Medicine Sequencing Core Laboratory. These were then submitted through the NCBI server for BLAST analysis to confirm the expression of these genes.

Immunohistochemistry

The immunohistochemistry using antibody against CD138 and LHRHR-I were done on bone marrow biopsies from a MM patient with previously reported methods (29).

Western blotting analysis

Cells were treated as specified in the figure legends, collected, and immunoblotted with the antibodies listed earlier using previously described methods (26).

Growth inhibition assay, colony assay, and evaluation of apoptosis

Myeloma cells were treated as specified in each figure legend. The growth inhibitory effect on MM cell lines was then assessed by MTT assays (Chemicon International) as previously described (26). Colony formation was assayed by soft-agar method as shown (26). The induction of apoptosis was evaluated using the Annexin V assay (BD Pharmingen) as reported previously (26).

Flow cytometric evaluation of caspase 3 activity

Cells were treated as specified in the figure legends, collected, and caspase 3 activity was evaluated by flow cytometry using previously described methods (26).

Transient transfection of siRNA

The scrambled siRNA (siSCR; catalogue no. sc-37007) and siRNA to knock down LHRHR-I (catalogue no. sc-4002) were obtained from Santa Cruz Biotechnology, Inc. RPMI 8226 cells were transfected with siRNA, using the RNAiFect transfection system (Qiagen) as reported (26). Cells were incubated at 37°C for 40 hours before replating onto 12-well plates. Cells were allowed to attach overnight (about 8 hours), then treated with Cetrorelix for 24 hours, followed by apoptosis and Western blot.

Animal study

The pLenti7.3/V5-GFP/luciferase plasmid developed at our laboratory, along with packaging mix, was transfected into 293FT with Lipofetamin 2000 according to the manufacturer's procedure. RPMI 8226 cells were transduced with the collected virus particles and stably transduced cells were purified on the basis of GFP expression by an Aria flow cytometer (BD Biosciences). Then, cells were injected intravenously into sixteen 8- to 10-week-old female NOD/SCID Il2rg$^{-/-}$ mice (Jackson Laboratory). For imaging, each mouse was anesthetized and imaged in a Xenogen/Caliper IVIS 200 optical scanner (Wavemetrics) at approximately 10 minutes following the i.p. administration of D-luciferin (150 mg/kg). Semiquantitative region of interest analysis was conducted with the dedicated software Living Image v3.1. The mice were subjected to imaging weekly after injection. As soon as the imaging study showed tumor growth, the mice were injected i.p. daily with vehicle (5% mannitol; $n = 8$ mice) or Cetrorelix (75 μg per mouse per day; $n = 8$ mice) and imaged every week for 8 weeks to monitor the tumor size. The mice were sacrificed when signs of discomfort emerged or when the tumor volume exceeded 2.0 cm. All experiments were conducted in accord with NIH guidelines and with approval of The Methodist Hospital Research Institute Committee for the Protection of Animals in Research.

Statistical analysis

Statistical analysis was conducted with the SPSS 11.5, using t test or 1-way ANOVA.

Results

Expression of LHRH and LHRHR in MM cells lines and plasma cells from myeloma patients or healthy donors

Because there is no information on the expression of LHRH and receptor in MM cells, we first determined the expression of LHRH-I and LHRHR-I by RT-PCR in 3 myeloma cell lines and samples from myeloma patients. As presented in Figure 1A, amplified products with the predicted size of 139 bp (LHRH-I) and 128 bp (LHRHR-I) were observed in human MM cell lines RPMI 8226, MM.1S, and U266 and in CD138$^+$ plasma cells from bone marrow samples of healthy donors (NM1–5) and myeloma patients (MM1–5). Products from RPMI 8226 cDNA were sequenced and confirmed by BLAST analysis as a part of human *LHRH-I* and *LHRHR-I* genes. MM cell lines Dox40, MM.1R, ATOR, BZMR, KAS-6/1, and KAS-6/R10R all have LHRH-I and LHRHR-I expression, verified by PCR (data not shown). Western blot also revealed that the LHRHR-I is expressed in MM cells lines and primary myeloma cells (Fig. 1B). The immunohistochemical staining showed that LHRHR-I was expressed by the neoplastic CD138$^+$ plasma cells (Fig. 1C). We next studied by qRT-PCR the expression levels of LHRHR-I in CD138$^+$ plasma cells and CD138$^-$ cells from bone marrow samples of control individuals and myeloma patients. As shown in Figure 1D, the neoplastic plasma cells showed the highest level of expression of LHRHR-I, whereas there were no significant differences among normal CD138$^+$ cells and CD138$^-$ cells from controls or myeloma

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

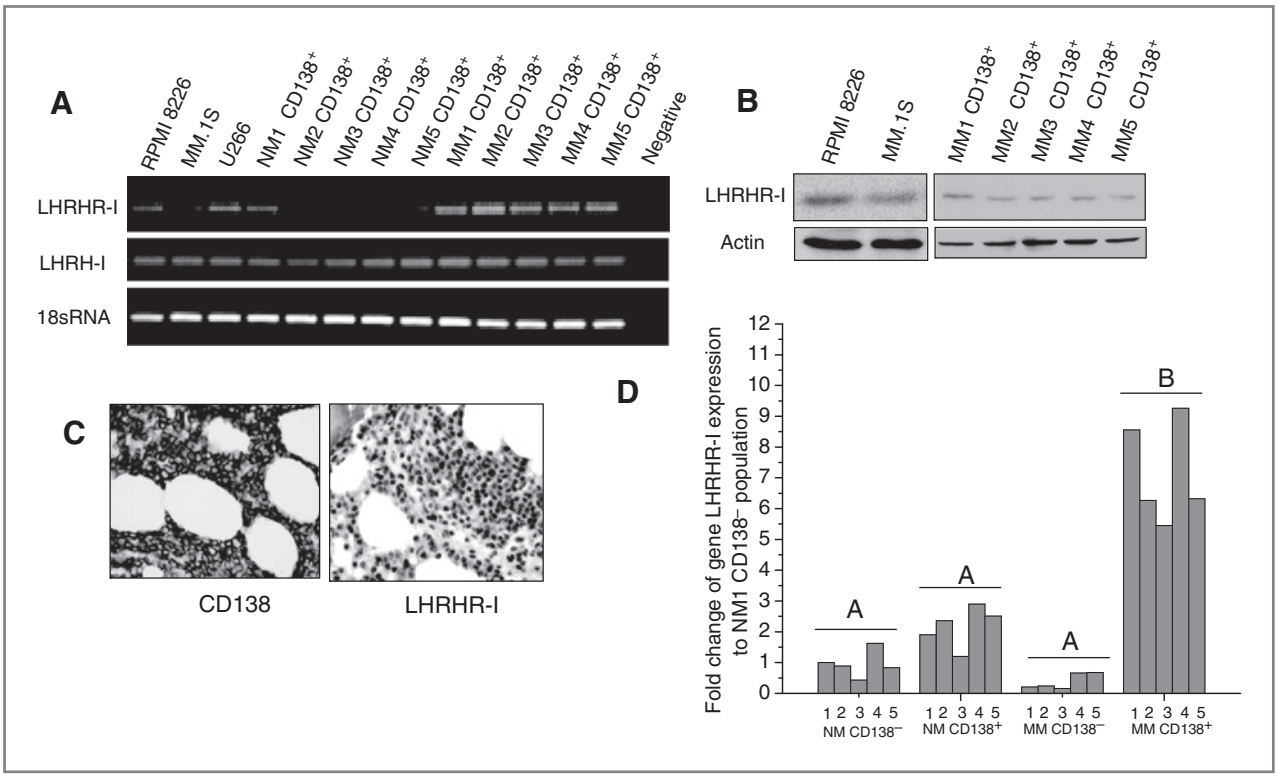

Figure 1. The presence of LHRH receptor mRNA and LHRH receptor proteins in MM cell lines and primary samples from myeloma patients. A, RNA was extracted from RPMI 8226, MM.1S, U266, primary normal CD138$^+$ cells (NM1–5), and MM CD138$^+$ cells (MM1–5) and then *LHRH-I* and *LHRHR-I* gene expression was assayed with RT-PCR. PCR products were electrophoresed on a 2% agarose gel. Results are from 1 experiment representative of 3. B, total cell lysates from RPMI 8226, MM.1S, and primary MM CD138$^+$ cells (MM1–5) were immunoblotted with antibodies against the LHRHR-I. C, immunohistochemical staining shows the expression of LHRHR-I in the neoplastic CD138$^+$ cells. D, mRNA levels of LHRHR-I in primary normal CD138$^{+/-}$ and MM CD138$^{+/-}$ cells were assayed by qRT-PCR. The results of all samples are shown as individual bars of relative mRNA levels normalized to primary normal 1 (NM1) CD138$^{/-}$ cells. Groups with the same letter indicate no significant differences ($P > 0.05$).

patients. This raises the possibility of targeting LHRHR-I for the treatment of myeloma patients.

Cetrorelix decreases MM cell growth and colony-forming ability

To investigate the effect of LHRH-I antagonist on myeloma cell growth, we used the Cetrorelix to treat 8 MM cells lines, including the RPMI 8226–derived ATO- or BZM-resistant cells, and KAS-6/1–derived, lenalidomide-resistant myeloma cells generated in our laboratories. Cetrorelix treatment reduced cell growth after 48 hours in all cell lines. At the concentration of 1 to 2 μmol/L (a concentration comparable with the previous studies; ref. 16), a 20% to 50% decrease of cell growth as compared with the vehicle was seen and a maximal response was reached at 4 μmol/L (Fig. 2A). Importantly, although RPMI 8226-ATOR cells, RPMI 8226-BZMR, and KAS-6/R10R cells show resistance to ATO, BZM, and lenalidomide, respectively (Fig. 2A inset), they remained sensitive to Cetrorelix treatment. The growth inhibition was further confirmed by colony formation assays (Fig. 2B), showing that Cetrorelix (1 μmol/L) reduced colony-forming ability of the two MM cell lines tested.

Cetrorelix induces apoptosis in MM cells

The changes of cell growth and viability indicated earlier suggested the induction of cell death by Cetrorelix in MM cells. Therefore, we investigated the Cetrorelix-induced apoptosis with flow cytometry. As shown in Figure 3A, as compared with controls, treatment with Cetrorelix for 24 hours significantly increased apoptosis in MM cells in a dose-dependent manner. We also investigated the effect of Cetrorelix on caspase-mediated apoptosis. Results showed that Cetrorelix significantly induced the activities of caspase 3 in RPMI 8226, MM.1S, and U266 cells (Fig. 3A). Furthermore, Cetrorelix showed a similar cytotoxic effect in all primary MM CD138$^+$ cells (MM1–5). Of note, there were no significant toxicities to CD138$^-$ cells (Fig. 3B), suggesting that the toxicity of Cetrorelix is specific to myeloma cells. We next explored whether this Cetrorelix-induced apoptosis is mediated through LHRHR-I. Compared with siSCR-transfected cells, the apoptosis was largely abrogated by the

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

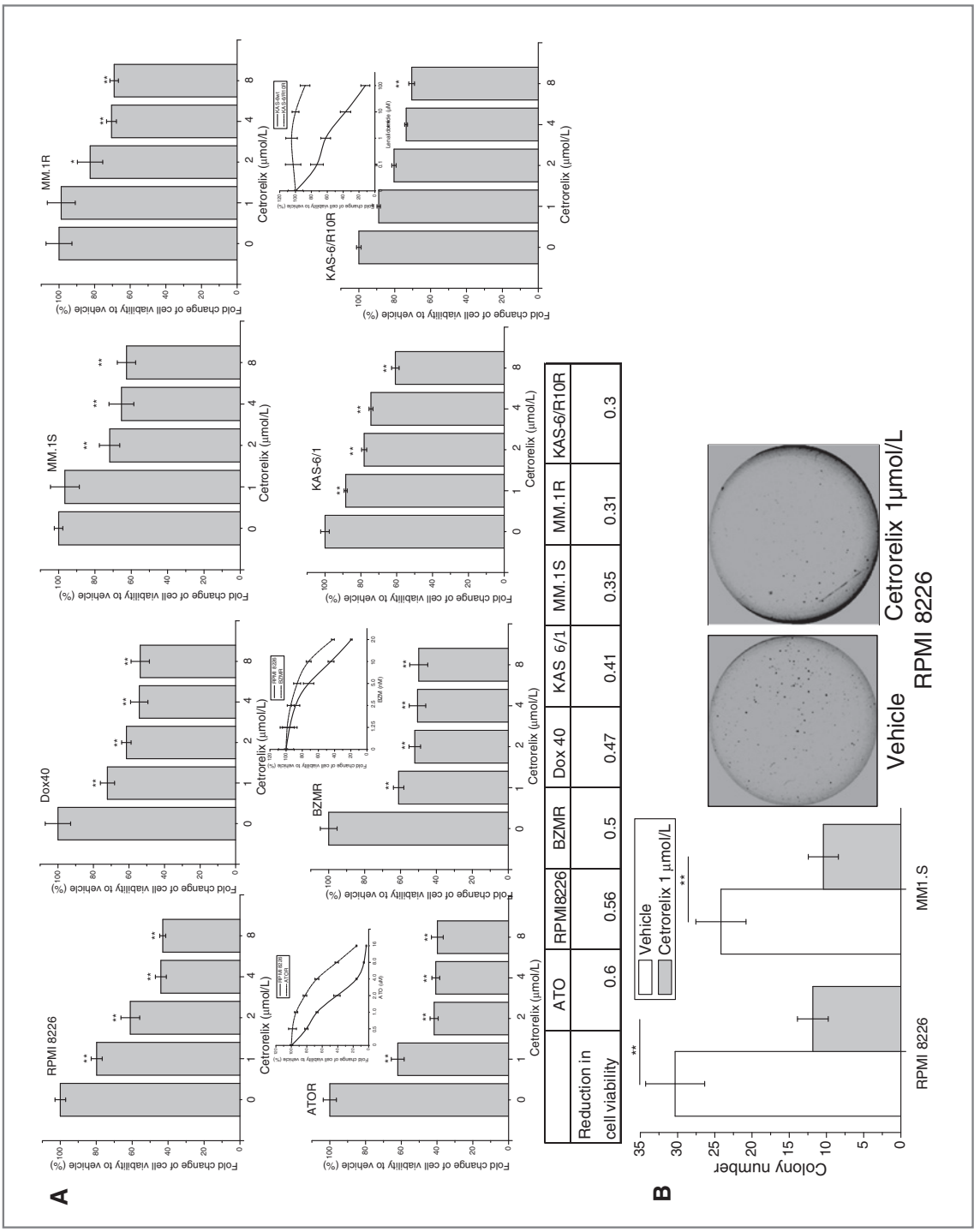

Figure 2. Effects of Cetrorelix on the growth and colony-forming ability of MM cells. A, RPMI 8226, RPMI 8226-Dox40, MM.1S, MM.1R, ATOR, BZMR, KAS-6/1, and KAS-6/R10R cells were cultured in 96-well plate and treated with Cetrorelix (1, 2, 4, and 8 μmol/L). The MTT analysis was done after 48 hours of culture. Inset, RPMI 8226 and ATOR were cultured and treated with ATO (0, 1, 2, 4, 8, and 16 μmol/L); RPMI 8226 and BZMR were cultured and treated with BZM (0, 1.25, 2.5, 5, 10, and 20 nmol/L) to show the resistance to ATO and BZM, respectively; KAS-6 and KAS-6/R10R were cultured and treated with lenalidomide (0, 0.1, 1, 10, and 100 μmol/L) to show the resistance. The MTT analysis was done after 48 hours of culture. Data are mean ± SD of 3 independent experiments. *, $P < 0.05$; **, $P < 0.01$ versus vehicle. The reduction in cell viability (= 1 − relative viability normalized to vehicle) caused by Cetrorelix (4 μmol/L) in different MM cell lines are shown in the table. B, RPMI 8226 and MM.1S cells were cultured in agar with 1 μmol/L Cetrorelix in a 6-well plate. After 2 weeks, the dishes were stained with methylene blue and colonies were photographed and counted. Data are mean ± SD of 3 independent experiments. **, $P < 0.01$ versus vehicle. Representative images of colonies are presented.

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

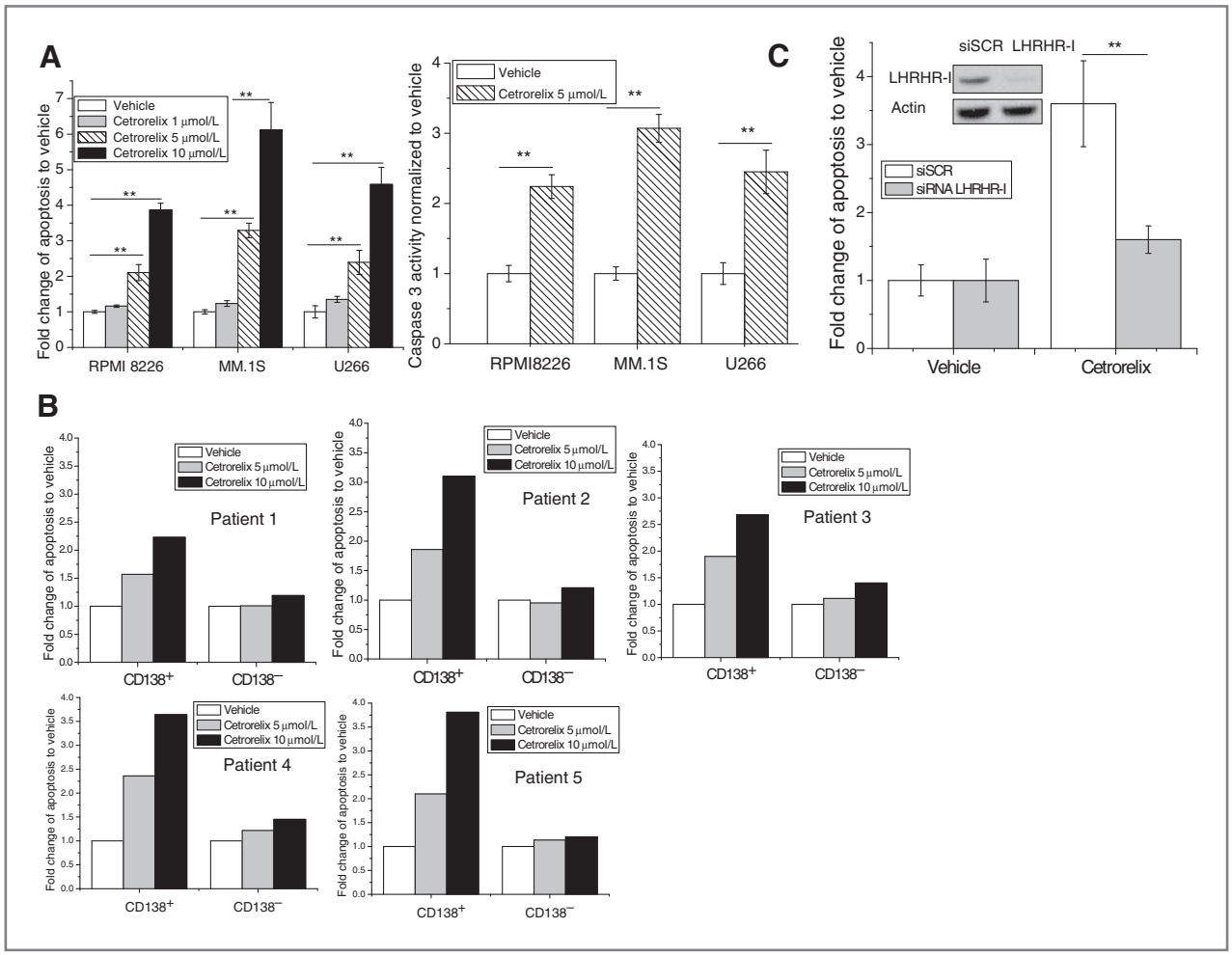

Figure 3. Effects of Cetrorelix on the apoptosis of MM cells. A, left, RPMI 8226, MM.1S, and U226 cells were cultured in 6-well plates and treated with Cetrorelix (1, 5, and 10 μmol/L) for 24 hours and the analysis for apoptosis was done. Right, RPMI 8226, MM.1S, and U226 cells were cultured in 6-well plates and treated with Cetrorelix (5 μmol/L) for 24 hours and the analysis for caspase 3 was done. Data are mean ± SD of 3 independent experiments. **, P <0.01 versus vehicle. B, $CD138^+$ and $CD138^-$ cells from primary MM samples (MM1–5) were cultured in 6-well plate and treated with Cetrorelix (5 and 10 μmol/L) for 24 hours and the analysis for apoptosis was done. C, RPMI 8226 cells were transfected with siSCR or siRNA to knockdown LHRHR-I. Forty hours after transfection, cells were replated and cultured overnight, allowed to attach, and then treated with Cetrorelix for another 24 hours. Cells were collected for apoptosis as well as Western blot to determine the knockdown efficiency. Data are mean ± SD of 3 independent experiments, normalized to vehicle treatment. **, $P < 0.01$ versus siSCR control.

LHRHR-I knockdown with siRNA transfection. This result suggested that the LHRHR-I is necessary for Cetrorelix to function in myeloma cells. The remaining apoptosis effect may come from the residual LHRHR-I or another unknown target receptor (Fig. 3C).

Cetrorelix inhibits myeloma cell growth *in vivo*

To show the *in vivo* activity of Cetrorelix, we next treated NOD/SCID $Il2rg^{-/-}$ mice bearing human myeloma RPMI 8226 tumors with Cetrorelix. At a dose of 75 μg Cetrorelix per animal per day, a dose lower than the previous studies (30, 31), bioluminescence imaging began to show significantly decreased tumor volume with Cetrorelix treatment at 4 weeks (Fig. 4A and B) and the inhibition of tumor growth continued for the 8-week duration of the experiment. The Kaplan–Meier survival curve revealed that there was a significant improvement in overall survival of mice treated with Cetrorelix compared with vehicle-treated mice (Fig. 4C; $P < 0.05$).

Cetrorelix regulates the expression of cytokines and NF-κB pathway

To understand the mechanisms involved in the induction of cytotoxicity by Cetrorelix in myeloma cells, we conducted several studies. First, because several cytokines expressed by myeloma cells and/or the microenvironment have been shown to be important for myeloma cell growth and survival through autocrine and/or

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

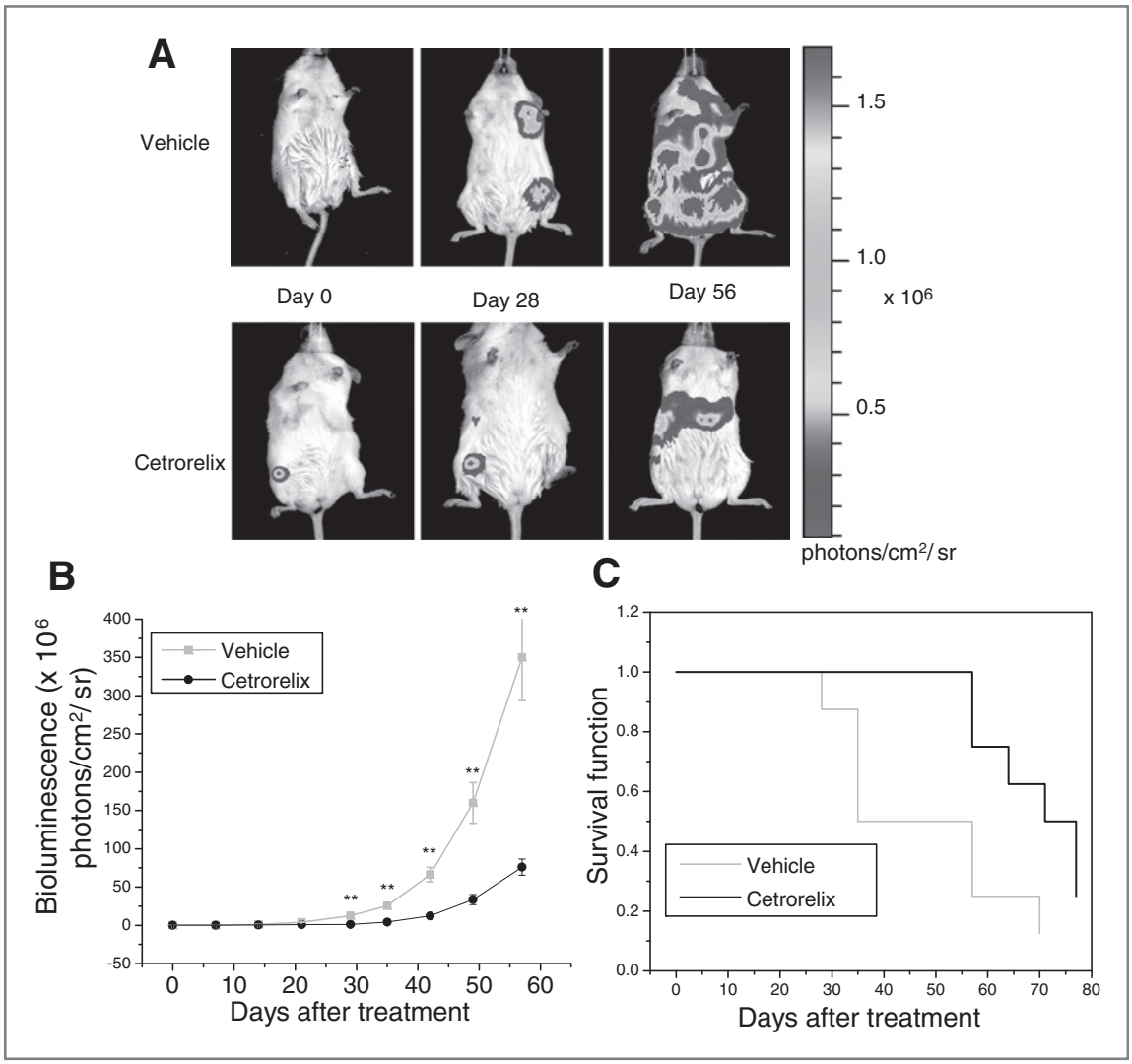

Figure 4. Cetrorelix is active in an MM xenograft model. A, NOD/SCID Il2rg$^{-/-}$ mice bearing RPMI 8226 tumors were injected i.p. daily with vehicle ($n = 8$) or Cetrorelix (75 μg/mouse/day; $n = 8$) for 8 weeks. Tumor volume was monitored with bioluminescence imaging and a representative example from each group is shown. Color gradation scale ranges from purple (low signal; low tumor burden) to red (high signal; high tumor burden). Units are 10^6 photons/s/cm^2/sr. B, mice receiving Cetrorelix or vehicle were imaged weekly. The images were analyzed using Living Image software, and a region of interest tool was used to measure the fluorescence efficiency. Data are mean of 8 independent experiments. **, $P < 0.01$ versus vehicle. C, the Kaplan–Meier survival curve for mouse groups that received Cetrorelix ($n = 8$) or vehicle ($n = 8$).

paracrine signaling, we evaluated the effects of Cetrorelix on these cytokines and growth factors. Thus, we used a qRT-PCR to assess the effect of Cetrorelix on mRNA expression of IL-6, IGF-1, VEGF-A, A proliferation-inducing ligand (APRIL), B-cell–stimulating factor-3 (BSF3), SDF1-α, and TGF-β in RPMI 8226 cells and BMSCs established from myeloma patients when they were cocultured. As shown in Figure 5A, Cetrorelix significantly decreased the mRNA expression for IL-6, IGF-1, VEFG-A, APRIL, BSF3, SDF1-α, and TGF-β in RPMI 8226 cells. In addition, Cetrorelix also significantly decreased the expression of mRNA for IL-6, VEGF-A, and BSF3 in BMSCs. Because LHRH pathway has been shown to induce activation of NF-κB (32), and many of these cytokines expression are regulated by NF-κB pathway (33, 34), we further examined the effect of Cetrorelix on NF-κB activity in the MM coculture system. The phosphorylation of p65 (RelA), an indicator for NF-κB activation, was significantly decreased after 6 hours of Cetrorelix treatment in both MM cells and BMSCs (left and right panels, respectively, Fig. 5C). This suggests that the decreased NF-κB activity and the decreased expression of these cytokines. which are important for myeloma cell growth and survival. may play a role in Cetrorelix-induced cytotoxicity and growth inhibition.

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

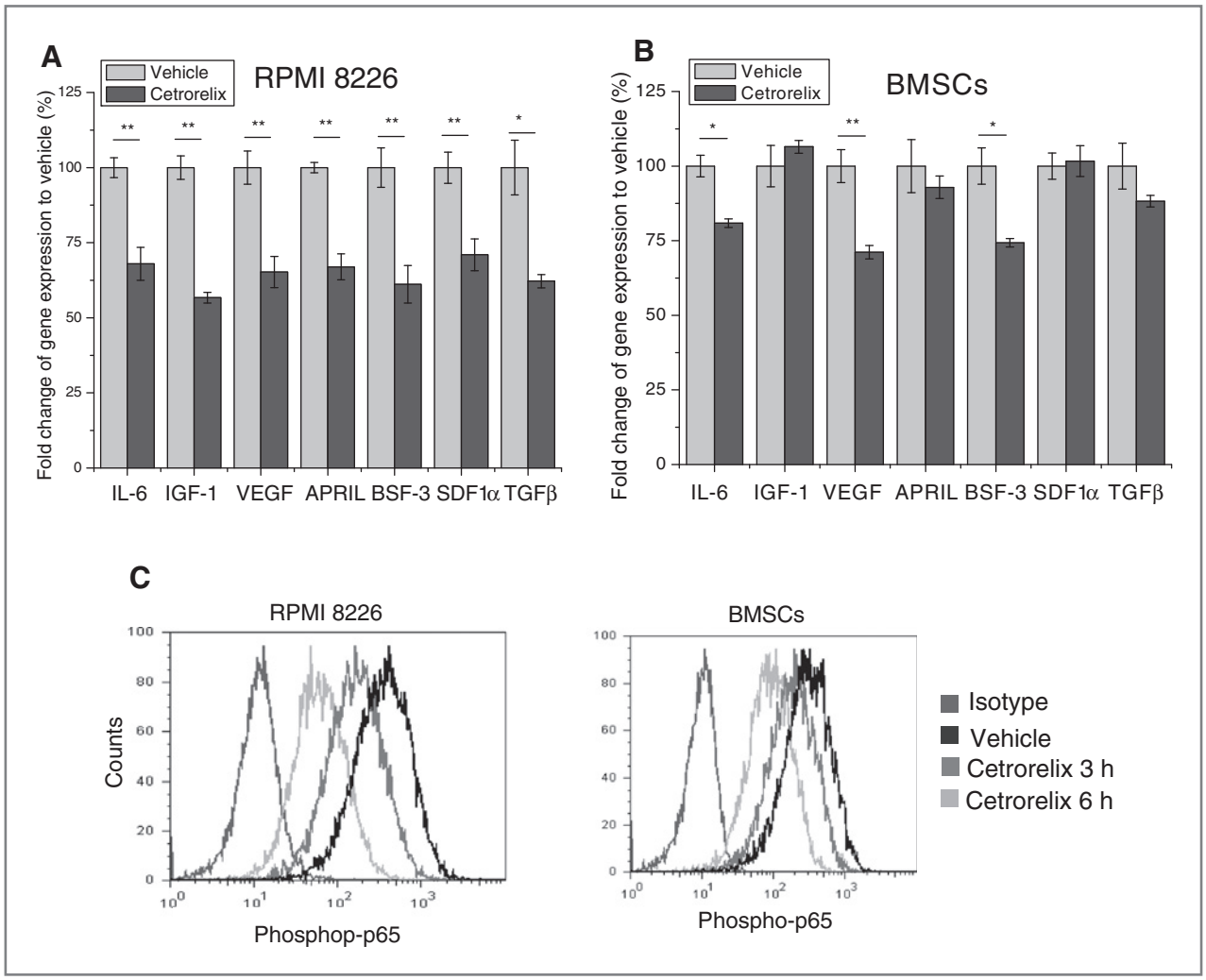

Figure 5. Cetrorelix regulates the expression of cytokines and NF-κB pathway. RPMI 8226 cells cocultured with BMSCs were starved overnight and cultured without cytokine. A, cells were then treated with Cetrorelix (5 μmol/L) for 12 hours. RNA was extracted from RPMI 8226 and BMSCs separately and qRT-PCR was done. Data are mean ± SD of 3 independent experiments after normalizing the data to the vehicle. *, P <0.05; **, P <0.01 versus vehicle. B, cells were treated with Cetrorelix (5 μmol/L) for 3 or 6 hours and then collected, fixed, permeabilized, and labeled with phycoerythrin-conjugated antibody against phosphorylated form of p65 followed by flow cytometric analysis. The *x*-axis shows the fluorescence intensity of PE, and the *y*-axis represents the cell counts. Data are from 3 independent experiments.

Cetrorelix regulates the phosphorylation of STAT3 and ERK: expression of cell-cycle–related and apoptosis-related proteins

We next examined whether Cetrorelix inhibited signaling pathways important for myeloma growth and survival, including STAT3, ERK, JNK (c-*jun* NH, kinase), and AKT pathways. As shown in Figure 5B, Cetrorelix markedly inhibited phosphorylation of STAT3 and ERK. We then studied the influence of Cetrorelix on cell-cycle–related and apoptosis-related protein expression in myeloma cells (Fig. 6). Results showed that Cetrorelix increased the expression of genes inhibiting cell cycling, *p21* and *p53*, but decreased the expression of antiapoptotic genes, including *Bcl-2* and *Bcl-*x_L. These results suggest that Cetrorelix generates antimyeloma effects through various pathways important for the survival and growth of myeloma cells. Of note, JNK and AKT did not show significant changes with Cetrorelix treatment (data not shown).

Discussion

Various investigators showed that Cetrorelix inhibits *in vivo* and *in vitro* growth of human ovarian, endometrial, mammary, and prostatic cancers (13, 15–18, 21–24). Here, we report that Cetrorelix induced cell growth inhibition and apoptosis in multiple myeloma cells. To the best of our knowledge, our study is the first to show the antitumor properties of Cetrorelix in a hematologic malignancy.

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

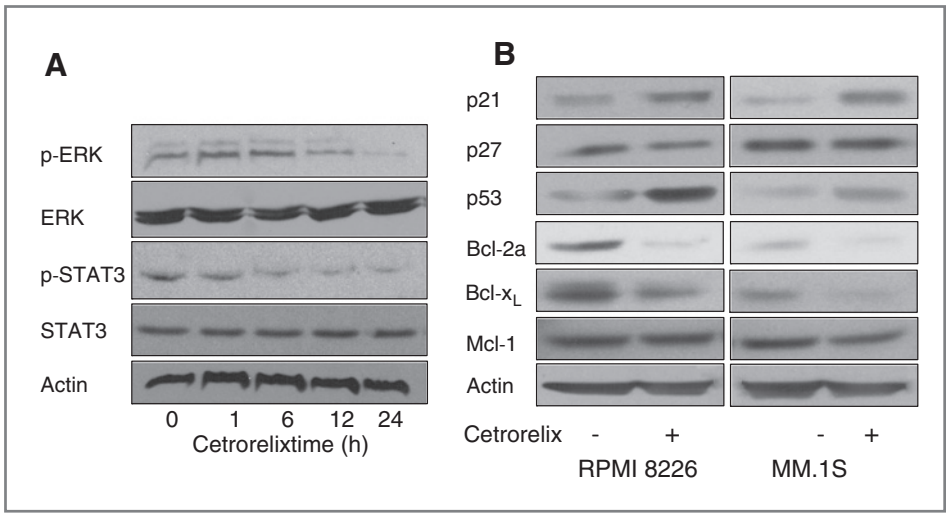

Figure 6. Cetrorelix regulates the phosphorylation of ERK and STAT3; expression of cell-cycle–related and apoptosis-related protein. A, RPMI 8226 cells were starved overnight and cultured without cytokine. Cells were then exposed to Cetrorelix (5 μmol/L) for 0, 1, 6, and 12 hours. Lysates from unstimulated or stimulated myeloma cells were immunoblotted with either anti-phospho–specific ERK antibody and then reprobed with anti-ERK antibody or with anti-phospho–specific STAT3 antibody and reprobed with anti-STAT3 antibody. The membrane was reprobed again with anti-actin antibody as a loading control. Data are from 3 independent experiments. B, RPMI 8226 and MM.1S cells were starved overnight and cultured without cytokines. Cells were then exposed to Cetrorelix (5 μmol/L) for 12 hours. Lysates from unstimulated or stimulated myeloma cells were immunoblotted with anti-p21, anti-p27, anti-p53, anti-Bcl-2, or anti-Bcl-x_L antibody. The membrane was reprobed again with anti-actin antibody as a loading control. Data are from 3 independent experiments.

Our results indicate that the antimyeloma effects of Cetrorelix are likely to be mediated at several levels. First, Cetrorelix is capable of regulating the NF-κB activity and expression of several key cytokines important for survival and growth of myeloma cells and in BMSCs. It has been shown that adhesion of multiple myeloma cells to BMSCs triggers the NF-κB–dependent transcription and secretion of cytokines such as IL-6, VEGF, IGF-1, SDF1-α, BAFF, APRIL, HGF, and TNF-α (tumor necrosis factor α) in BMSCs (33, 34). These cytokines then enhance the growth, survival, drug resistance, and migration of myeloma cells (33, 34). In addition, myeloma cells localized in the bone marrow milieu can secrete cytokines such as TNF-α, TGF-β, and VEGF, which further augment secretion of IL-6 from BMSCs, forming a positive feedback loop between myeloma cells and BMSCs to support their survival and growth (34–37). IL-6 has been shown to be one of the most important factors for myeloma cell growth and survival. In the current study, we showed that Cetrorelix decreased the mRNA expression of IL-6, VEGF, and BSF3 in both myeloma cells and BMSCs. Cetrorelix also downregulated the mRNA expression of IGF-1, APRIL, SDF1-α, and TGF-β in myeloma cells. Previous studies on the anticancer activities of Cetrorelix have not evaluated the effect of Cetrorelix in regulating expression of these cytokines, except for IGF-1.

It has been reported that IL-6 induces proliferation of MM cells through the activation of the Ras/Raf/MEK (MAP/ERK kinase)/ERK signaling pathway whereas IL-6-induced JAK/STAT3 signaling promotes MM cell survival by modulating Bcl-x_L and/or Mcl-1 protein levels (38–42). Of note, it has been shown that engagement of the LHRHR by LHRH initiates a complex series of signaling events that include the activation of ERK1/2 (43). Therefore, the abrogation of ERK and STAT3 activation may contribute to the growth inhibition and apoptosis induced by Cetrorelix in myeloma cells. This phenomenon is further supported by our finding that Cetrorelix decreased the expression of STAT3-regulated antiapoptoic proteins, Bcl-2 and Bcl-x_L, consistent with the results by Kwon et al. (19). However, effects of Cetrorelix on STAT3 activation have not been previously evaluated.

Our findings suggest that the cell-cycle regulatory genes *p53* and *p21* play significant roles in Cetrorelix-induced growth inhibition. *p53* is known to induce cell-cycle arrest and apoptosis. *p21* is an inhibitor of cyclin-dependent kinases and plays a critical role in controlling the cell cycle. It is known that p21 can be upregulated by both p53-dependent and p53-independent pathways (22). In our study, the expression of p53 protein was increased in association with the increase of p21 protein, suggesting that Cetrorelix-induced upregulation of p21 may be mediated by p53. Although RPMI 8226 is regarded as a mutant *p53*-expressing cell line, RPMI 8226 cells still produce small amounts of wild-type p53 with correct conformation (44, 45).

Our results revealed that the LHRH-I and LHRHR-I were expressed in myeloma cells lines and the expression levels of LHRHR-I were upregulated in the neoplastic

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

myeloma cells from patients compared with normal plasma cells from controls (Fig. 1). These results agree with those of gene expression profiling study database from Multiple Myeloma Genomics Portal (http://www.broadinstitute.org/mmgp/home). In that database, LHRH-I and LHRHR-I are expressed in neoplastic plasma cells isolated from all patients of MGUS (monoclonal gammopathy of undetermined significance), smoldering myeloma, and MM of different prognostic subtypes. Although we do not observe significantly different expressions of these genes among these groups of plasma cell dyscrasias, the wide expression of LHRHR-I in myeloma patients suggests that targeting these molecules may represent a treatment option for most of myeloma patients.

In conclusion, our results show for the first time that Cetrorelix significantly suppressed growth of multiple myeloma cells *in vitro* and *in vivo* through various mechanisms. In addition, we found that RPMI 8226–derived, ATO- and BZM-resistant cells are sensitive to Cetrorelix treatment. The proteasome inhibitor BZM and ATO are recent additions to the MM treatment armamentarium, and both drugs show significant beneficial effects in myeloma treatment (46, 47). However, more than 50% of patients with refractory or relapsed diseases show resistance to BZM or ATO treatment (4, 5). Future studies, including clinical trials, are warranted to evaluate the possible benefits of using Cetrorelix in the treatment of myeloma patients.

Disclosure of Potential Conflicts of Interest

Dr. Andrew V. Schally is a co-inventor on the patent for LHRH antagonist Cetrorelix, which is assigned to Tulane University School of Medicine.

Acknowledgments

We thank the Multiple Myeloma Tissue Bank at the Department of Lymphoma and Myeloma of The M. D. Anderson Cancer Center for providing the myeloma samples for this study.

Grant Support

This work was supported by the Medical Research Service of the Veterans Affairs Department and South Florida Veterans Affairs Foundation for Research and Education and the University of Miami Miller School of Medicine Departments of Pathology and Medicine, Division of Hematology/Oncology (A.V. Schally) and The Methodist Hospital Research Institute Scholar Grant (C.-C. Chang).

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.

Received September 2, 2010; revised October 15, 2010; accepted November 1, 2010; published OnlineFirst November 9, 2010

References

1. Jemal A, Siegel R, Ward E, Murray T, Xu J, Thun MJ. Cancer statistics, 2007. CA Cancer J Clin 2007;57:43–66.
2. Cavo M. Proteasome inhibitor bortezomib for the treatment of multiple myeloma. Leukemia 2006;20:1341–52.
3. Nencioni A, Grunebach F, Patrone F, Ballestrero A, Brossart P. Proteasome inhibitors: antitumor effects and beyond. Leukemia 2007;21:30–6.
4. Richardson PG, Barlogie B, Berenson J, et al. A phase 2 study of bortezomib in relapsed, refractory myeloma. N Engl J Med 2003;348:2609–17.
5. Munshi NC, Tricot G, Desikan R, et al. Clinical activity of arsenic trioxide for the treatment of multiple myeloma. Leukemia 2002;16:1835–7.
6. Stojilkovic SS, Catt KJ. Expression and signal transduction pathways of gonadotropin-releasing hormone receptors. Recent Prog Horm Res 1995;50:161–205.
7. Sherwood NM, Lovejoy DA, Coe IR. Origin of mammalian gonadotropin-releasing hormones. Endocr Rev 1993;14:241–54.
8. Clayton RN, Catt KJ. Gonadotropin-releasing hormone receptors: characterization, physiological regulation, and relationship to reproductive function. Endocr Rev 1981;2:186–209.
9. Adelman JP, Mason AJ, Hayflick JS, Seeburg PH. Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. Proc Natl Acad Sci U S A 1986;83:179–83.
10. Limonta P, Moretti RM, Marelli MM, Motta M. The biology of gonadotropin hormone-releasing hormone: role in the control of tumor growth and progression in humans. Front Neuroendocrinol 2003;24:279–95.
11. Oikawa M, Dargan C, Ny T, Hsueh AJ. Expression of gonadotropin-releasing hormone and prothymosin-alpha messenger ribonucleic acid in the ovary. Endocrinology 1990;127:2350–6.
12. Palmon A, Ben Aroya N, Tel-Or S, Burstein Y, Fridkin M, Koch Y. The gene for the neuropeptide gonadotropin-releasing hormone is expressed in the mammary gland of lactating rats. Proc Natl Acad Sci USA 1994;91:4994–6.
13. Chen W, Yoshida S, Ohara N, Matsuo H, Morizane M, Maruo T. Gonadotropin-releasing hormone antagonist Cetrorelix down-regulates proliferating cell nuclear antigen and epidermal growth factor expression and up-regulates apoptosis in association with enhanced poly(adenosine 5'-diphosphate-ribose) polymerase expression in cultured human leiomyoma cells. J Clin Endocrinol Metab 2005;90:884–92.
14. Emons G, Ortmann O, Becker M, et al. High affinity binding and direct antiproliferative effects of LHRH analogues in human ovarian cancer cell lines. Cancer Res 1993;53:5439–46.
15. Emons G, Schally AV. The use of luteinizing hormone releasing hormone agonists and antagonists in gynaecological cancers. Hum Reprod 1994;9:1364–79.
16. Grundker C, Schlotawa L, Viereck V, et al. Antiproliferative effects of the GnRH antagonist Cetrorelix and of GnRH-II on human endometrial and ovarian cancer cells are not mediated through the GnRH type I receptor. Eur J Endocrinol 2004;151:141–9.
17. Halmos G, Schally AV, Kahan Z. Down-regulation and change in subcellular distribution of receptors for luteinizing hormone-releasing hormone in OV-1063 human epithelial ovarian cancers during therapy with LH-RH antagonist Cetrorelix. Int J Oncol 2000;17:367–73.
18. Kleinman D, Douvdevani A, Schally AV, Levy J, Sharoni Y. Direct growth inhibition of human endometrial cancer cells by the gonadotropin-releasing hormone antagonist SB-75: role of apoptosis. Am J Obstet Gynecol 1994 Jan;170:96–102.
19. Kwon JY, Park KH, Park YN, Cho NH. Effect of Cetrorelix acetate on apoptosis and apoptosis regulatory factors in cultured uterine leiomyoma cells. Fertil Steril 2005;84:1526–8.

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

20. Noci I, Coronnello M, Borri P, et al. Inhibitory effect of luteinising hormone-releasing hormone analogues on human endometrial cancer *in vitro*. Cancer Lett 2000;150:71–8.
21. Schally AV. Luteinizing hormone-releasing hormone analogs: their impact on the control of tumorigenesis. Peptides 1999;20:1247–62.
22. Tang X, Yano T, Osuga Y, et al. Cellular mechanisms of growth inhibition of human epithelial ovarian cancer cell line by LH-releasing hormone antagonist Cetrorelix. J Clin Endocrinol Metab 2002;87: 3721–7.
23. Yano T, Pinski J, Radulovic S, Schally AV. Inhibition of human epithelial ovarian cancer cell growth *in vitro* by agonistic and antagonistic analogues of luteinizing hormone-releasing hormone. Proc Natl Acad Sci USA 1994 Mar 1;91:1701–5.
24. Engel JB, Schally AV. Drug Insight: clinical use of agonists and antagonists of luteinizing-hormone-releasing hormone. Nat Clin Pract Endocrinol Metab 2007;3:157–67.
25. Bajusz S, Csernus VJ, Janaky T, Bokser L, Fekete M, Schally AV. New antagonists of LHRH. II. Inhibition and potentiation of LHRH by closely related analogues. Int J Pept Protein Res 1988;32:425–35.
26. Wen J, Cheng HY, Feng Y, et al. P38 MAPK inhibition enhancing ATO-induced cytotoxicity against multiple myeloma cells. Br J Haematol 2008;140:169–80.
27. Stromberg T, Ekman S, Girnita L, et al. IGF-1 receptor tyrosine kinase inhibition by the cyclolignan PPP induces G_2/M-phase accumulation and apoptosis in multiple myeloma cells. Blood 2006 15;107:669–78.
28. Pfaffl MW. A new mathematical model for relative quantification in real-time RT-PCR. Nucleic Acids Res 2001;29:e45.
29. Shahjahan M, Dunphy CH, Ewton A, et al. p38 mitogen-activated protein kinase has different degrees of activation in myeloproliferative disorders and myelodysplastic syndromes. Am J Clin Pathol 2008;130:635–41.
30. Yano T, Pinski J, Halmos G, Szepeshazi K, Groot K, Schally AV. Inhibition of growth of OV-1063 human epithelial ovarian cancer xenografts in nude mice by treatment with luteinizing hormone-releasing hormone antagonist SB-75. Proc Natl Acad Sci USA 1994;91:7090–4.
31. Jungwirth A, Pinski J, Galvan G, et al. Inhibition of growth of androgen-independent DU-145 prostate cancer *in vivo* by luteinising hormone-releasing hormone antagonist Cetrorelix and bombesin antagonists RC-3940-II and RC-3950-II. Eur J Cancer 1997;33:1141–8.
32. Grundker C, Schulz K, Gunthert AR, Emons G. Luteinizing hormone-releasing hormone induces nuclear factor kappaB-activation and inhibits apoptosis in ovarian cancer cells. J Clin Endocrinol Metab 2000;85:3815–20.
33. Chauhan D, Uchiyama H, Akbarali Y, et al. Multiple myeloma cell adhesion-induced interleukin-6 expression in bone marrow stromal cells involves activation of NF-kappa B. Blood 1996;87:1104–12.
34. Hideshima T, Chauhan D, Schlossman R, Richardson P, Anderson KC. The role of tumor necrosis factor alpha in the pathophysiology of human multiple myeloma: therapeutic applications. Oncogene 2001; 20:4519–27.
35. Urashima M, Ogata A, Chauhan D, , et al. Transforming growth factor-beta1: differential effects on multiple myeloma versus normal B cells. Blood 1996;87:1928–38.
36. 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.
37. Gupta D, Treon SP, Shima Y, et al. Adherence of multiple myeloma cells to bone marrow stromal cells upregulates vascular endothelial growth factor secretion: therapeutic applications. Leukemia 2001;15: 1950–61.
38. Hideshima T, Chauhan D, Hayashi T, et al. Proteasome inhibitor PS-341 abrogates IL-6 triggered signaling cascades via caspase-dependent downregulation of gp130 in multiple myeloma. Oncogene 2003;22:8386–93.
39. Hideshima T, Nakamura N, Chauhan D, Anderson KC. Biologic sequelae of interleukin-6 induced PI3-K/Akt signaling in multiple myeloma. Oncogene 2001;20:5991–6000.
40. Mitsiades CS, Mitsiades N, Poulaki V, et al. Activation of NF-kappaB and upregulation of intracellular anti-apoptotic proteins via the IGF-1/Akt signaling in human multiple myeloma cells: therapeutic implications. Oncogene 2002;21:5673–83.
41. Podar K, Tai YT, Cole CE, et al. Essential role of caveolae in interleukin-6- and insulin-like growth factor I-triggered Akt-1-mediated survival of multiple myeloma cells. J Biol Chem 2003;278:5794–801.
42. Puthier D, Bataille R, Amiot M. IL-6 up-regulates mcl-1 in human myeloma cells through JAK / STAT rather than ras / MAP kinase pathway. Eur J Immunol 1999;29:3945–50.
43. Caunt CJ, Finch AR, Sedgley KR, McArdle CA. GnRH receptor signalling to ERK: kinetics and compartmentalization. Trends Endocrinol Metab 2006;17:308–13.
44. Tai YT, Podar K, Gupta D, et al. CD40 activation induces p53-dependent vascular endothelial growth factor secretion in human multiple myeloma cells. Blood 2002;99:1419–27.
45. Teoh G, Tai YT, Urashima M, et al. CD40 activation mediates p53-dependent cell cycle regulation in human multiple myeloma cell lines. Blood 2000;95:1039–46.
46. Richardson PG, Sonneveld P, Schuster MW, et al. Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. N Engl J Med 2005;352:2487–98.
47. Hussein MA, Saleh M, Ravandi F, Mason J, Rifkin RM, Ellison R. Phase 2 study of arsenic trioxide in patients with relapsed or refractory multiple myeloma. Br J Haematol 2004;125:470–6.

Published OnlineFirst November 9, 2010; DOI: 10.1158/1535-7163.MCT-10-0829

AACR American Association for Cancer Research

Molecular Cancer Therapeutics

Luteinizing Hormone-Releasing Hormone (LHRH)-I Antagonist Cetrorelix Inhibits Myeloma Cell Growth *In vitro* and *In vivo*

Jianguo Wen, Yongdong Feng, Chad C. Bjorklund, et al.

Mol Cancer Ther 2011;10:148-158. Published OnlineFirst November 9, 2010.