Luteinizing hormone-releasing hormone (LHRH)-I antagonist Cetrorelix inhibits myeloma cell growth \textit{in vitro} and \textit{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 OHare$^1$, Youli Zu$^{1,7}$, Andrew V. Schally$^{8,9}$, Chung-Che Chang$^{1,7}$

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

*: Both authors contributed equally to this work.

\textbf{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 University of Miami Miller School of Medicine Departments of Pathology and Medicine, Division of Hematology/Oncology (AVS) and The Methodist Hospital Research Institute Scholar Grant (CC).

\textbf{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.

\textbf{Corresponding Author}: Chung-che Chang, Department of Pathology, The Methodist Hospital and Research Institute, 6565 Fannin, MS205, Houston, TX 77030. Phone: 713-441-4458; Fax: 713-441-1565. E-mail: jeffchang@tmhs.org

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Copyright © 2010 American Association for Cancer Research

Downloaded from mct.aacrjournals.org on June 19, 2017. © 2010 American Association for Cancer Research.
Running title: Cetrorelix inhibits myeloma cell growth in vitro and in vivo

Key words: LHRH; antagonist; Cetrorelix; multiple myeloma

Abstract:

The objective of this study was to determine the effects of an LHRH-I antagonist, Cetrorelix, on human multiple myeloma (MM) cells and to elucidate the mechanisms of action. We demonstrated 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, or 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 down-regulated the Nuclear factor-kappa B (NF-κB) pathway activity and the expression of cytokines important for myeloma cell growth and survival, including IL-6, IGF-1, VEGF-A and SDF1, in myeloma cells and/or marrow stromal cells from myeloma patients. Cetrorelix decreased the phosphorylation of ERK1/2 and STAT3 in myeloma cells, two crucial pathways for myeloma cells growth and survival. Moreover, the expression of p21 and p53 was increased, while the expression of anti-apoptotic proteins Bcl-2 and Bcl-XL 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 treatment of multiple myeloma.
Introduction

Multiple myeloma (MM) is the second most common hematological cancer in the United States, representing 10% of all hematopoietic malignancies(1). MM is incurable, although novel approaches such as the 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 which 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 two forms of LHRH (7). LHRH-I and its cognate receptor, LHRHR-I, have been also 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, but increased the level of p21 and p53 in
epithelial ovarian cancer cells (22). In leiomyoma cells treated with Cetrorelix, an augmented expression of Fas, Fas ligand, Bcl-xL, Bax, and caspase-3 and reduced Bcl-2 expression 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 demonstrates that Cetrorelix induces apoptosis and inhibits the growth of myeloma cells, including some cell lines resistant to arsenic trioxide, bortezomib 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, IGF-1, VEGF-A, SDF1-α, expressed by myeloma cells and/or myeloma bone marrow stromal cells (BMSCs) from myeloma patients; inhibition of the activation of ERK and STAT3 pathways; up-regulation of p53 and p21 expression; and down-regulation of Bcl-2 and Bcl-xL expression. Altogether, these findings support the merit of further evaluation of LHRH-I antagonists in the treatment of multiple myeloma.
Materials and methods

Reagents and antibodies

The LHRH-I antagonist Cetrorelix [Ac-D-Nal(2), d-Phe(4Cl)2, d-Pal(3), d-Cit6, d-Ala10]LHRH [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-xL (Cell Signaling Technology, Danvers, MA), CD138, LHRHR-I (Abcam, Cambridge, MA), beta-actin (Sigma-Aldrich, St. Louis, MO), and Alexa 647 conjugated phospho-NF-κB p65 (RelA) (Becton Dickinson, San Jose, CA) 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), while RPMI 8226 and doxorubicin-resistant RPMI 8226-Dox40 cells were gifts from Dr. William Dalton (Moffitt Cancer Center, Tampa, FL). Arsenic trioxide (ATO) resistant cells (RPMI 8226-ATOR, stably maintained at 0.5 μM of ATO), and bortezomib (BZM) resistant cells (RPMI 8226-BZMR, stably maintained at 5 nM 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 μM lenalidomide. All cell lines were cultured as reported (26). There is 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 Board 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, Solon, OH). CD138+ plasma cells were isolated with AutoMACS using CD138 antibodies conjugated with magnetic beads (Miltenyi Biotec, Auburn, CA). Bone marrow stromal cells (BMSCs) 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 cytometry analysis for phosphorylation NF-κB p65 (RelA) and quantitative RT-PCR (qRT-PCR) in cocultures**

MM BMSCs were harvested and seeded into 12 well plates and incubated overnight. The medium was exchanged and RPMI8226 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 μM Cetrorelix for 3, 6, or 12 hours. The MM cells were collected with gentle pipetting up and down without touching monolayer and then BMSCs were collected with
trypsinization. Phospho-NF-κB p65 was tested using methods previously described (26) and cytokine quantification by qRT-PCR was described below.

**RNA extraction, reverse transcription and PCR**

Total cellular RNA was extracted and cDNA was synthesized as previously described (26). For qRT-PCR, amplification was performed in LightCycler using FastStart DNA Master SYBR Green (Roche Applied Science, Indianapolis, IN), and 18S RNA was used as a control to evaluate the expression of the genes listed in text, using the following primers: 5'-ATCACCAGCCACAGATCC-3' and 5'-CAAGGGGGCCTCTAATTTC-3' for human LHRH-I, 5'-CAAGGCTTGAAGCTCTGTCC-3' and 5'-AAGGTCAGAGTGCGGAGGT-3' for human LHRHR-I, 5'-CTCTGCTGACCCAACAAACAG-3' and 5'-TTTCCGGATCTCTCCCAT-3' for human APRIL, 5'-GCTACCTGGAGCACCAACTC-3' and 5'-GCCACGCAAGTAACACAGAA-3' for human BSF3, 5'-TTATTGTCACAAGCACCAGG-3' and 5'-GCAATACATCTCCAGCCTCCTTA-3' for human IGF-1, 5'-AGAGCCACGTCAGCATCT-3' and 5'-CTTTCAGCTCTCGGAGGTCAATGC-3' for human SDF1-α, 5'-CGACTACTACGCGCAAGAGG-3' and 5'-CGGAGCTCTGTGTTGAA-3' for human TGF-β, 5'-CCTTGCTGTGCTGCCTACCTC-3' and 5'-CCACCAGGGGTCTCGATGGT-3' for human VEGF, 5'-TACCCCAAGGAGAGATTCC-3' and 5'-TTTCTGACGTGGCTCTCTT-3' for human IL-6, 5'-TTGGAACGTAGGCCATG-3' and 5'-TTTCGCTCTGCTCCGTTT-3' for human 18SrRNA. Each sample was measured 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, Valencia, CA), 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 methods previously reported (29).

Western blotting analysis

Cells were treated as specified in the figure legends, collected, and immunoblotted with the antibodies listed above using methods previously described (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, Temecula, CA) 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, San Diego, CA) as reported previously (26).

Flow cytometric evaluation caspase3 activity
Cells were treated as specified in the figure legends, collected, and caspase3 activity was evaluated by flow cytometry using methods previously described (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. (Santa Cruz, CA). 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 analysis 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 Lipofetamin2000 according to the manufacturer’s procedure. RPMI 8226 cells were transduced with the collected virus particles and stably transduced cells were purified based on GFP expression by an Aria flow cytometer (BD Biosciences, San Jose, CA). Then, cells were injected intravenously into 16 eight-to 10-week-old female NOD/SCID Il2rg/- mice (Jackson Laboratory, Bar Harbor, ME). For imaging, each mouse was anesthetized and imaged in a Xenogen/Caliper IVIS 200 optical scanner (Wavemetrics, Seattle, WA) at approximately 10 minutes following the i.p. administration of D-luciferin (150 mg/kg). Semi-quantitative region of interest (ROI) analysis was performed with the dedicated software, Living Image v3.1. The mice were subjected to imaging weekly after injection. As soon as the imaging study showing tumor growth, the mice were injected...
intraperitoneally daily with vehicle (5% mannitol, n=8 mice) or Cetrorelix (75 μg/mouse/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 National Institutes of Health guidelines, and with approval of the The Methodist Hospital Research Institute Committee for the Protection of Animals in Research.

Statistical analysis

Statistical analysis was performed with the SPSS 11.5 (Chicago, IL) using t test or one way analysis of variance (ANOVA).
Results

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

Since 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, 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 the LHRHR-I is expressed in MM cells lines and primary myeloma cells (Figure 1B). The immunohistochemistry staining showed that LHRHR-I was expressed by the neoplastic CD138+ plasma cells (Figure 1C). We next studied the expression levels of LHRHR-I in CD138+ plasma cells and CD138- cells from bone marrow samples of control individuals and myeloma patients by qRT-PCR. As shown in Figure 1D, the neoplastic plasma cells showed the highest level of expression of LHRHR-I, while there were no significant differences among normal CD138+ cells and CD138- cells from controls or myeloma patients. This raises the possibility of targeting LHRHR-I for 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 cell 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 1-2 μM (a concentration comparable to the previous studies(16)), a 20-50% decrease of cell growth as compared to the vehicle was seen, and a maximal response was reached at 4 μM (Figure 2A). Importantly, although RPMI 8226-ATOR cells, RPMI 8226-BZMR, and KAS-6/R10R cells show resistance to ATO, BZM, and lenalidomide, respectively (Figure 2A inset), they remained sensitive to Cetrorelix treatment. The growth inhibition was further confirmed by colony formation assays (Figure 2B), showing that Cetrorelix (1 μM) 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 above 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, treatment with Cetrorelix for 24 hours significantly increased apoptosis in MM cells in a dose-dependent manner, as compared with controls. We also investigated the effect of Cetrorelix on caspase-mediated apoptosis. Results showed that Cetrorelix significantly induced the activities of caspase -3 in RPMI8226, MM1S and U266 cells (Figure 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 (Figure 3B) suggesting that the toxicity of Cetrorelix is specific to myeloma cells. We next explored if this Cetrorelix-induced apoptosis is mediated
through LHRHR-I. Compared with scrambled siRNA transfected cells, the apoptosis was largely abrogated by the LHRHR-I knock down 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 (Figure 3C).

**Cetrorelix inhibits myeloma cell growth in vivo.** To demonstrate 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 75µg Cetrorelix/animal/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 (Figure 4A and 4B), 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 (Figure 4C; P<0.05).

**Cetrorelix regulates the expression of cytokines and NF-κB pathway.** To understand the mechanisms involved in induction of cytotoxicity by Cetrorelix in myeloma cells, we performed several studies. First, since 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 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 interleukin 6 (IL-6), insulin-like growth factor 1 (IGF-1), vascular endothelial growth factor (VEGF-A), A proliferation-inducing ligand (APRIL), B cell-stimulating factor-3 (BSF3), stromal-derived factor 1-α (SDF1-α), and transforming growth...
factor-beta (TGF-β) in RPMI 8226 cells and BMSC established from myeloma patients when they were co-cultured. As shown in Figure 5A, Cetrorelix significantly decreased the mRNA expression for IL-6, IGF-1, VEGF-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 BMSC. Since 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 Cetrorelix treatment in both MM cells and BMSCs (left and right panel respectively, Figure 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.

**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, and AKT. 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. Results showed that Cetrorelix increased the expression of genes inhibiting cell cycling, p21 and p53, but decreased the expression of anti-apoptotic genes, including Bcl-2 and Bcl-xL. These results suggest that Cetrorelix generates anti-myeloma 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 anti-tumor properties of Cetrorelix in a hematologic malignancy.

Our results indicate that the anti-myeloma effects of Cetrorelix are likely to be mediated at several levels. Firstly, 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 bone marrow stromal cells. 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 TNFα 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 demonstrated that Cetrorelix decreased the mRNA expression of IL-6, VEGF, and BSF3 in both myeloma cells and BMSCs. Cetrorelix also down-regulated the mRNA expression of IGF-1, APRIL, SDF1-α, and TGF-β in myeloma cells. Previous studies on the anti-cancer 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/ERK signaling pathway, whereas IL-6-induced JAK/STAT3 signaling promotes MM cell survival by modulating Bcl-xL 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 anti-apoptotic proteins, Bcl-2, and Bcl-xL, 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 up-regulated 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 myeloma cells from patients compared to normal plasma cells from controls (Figure 1). These results agree with those of gene expression profiling study database from Multiple Myeloma Genomics Portal (MMGP, 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, 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 suggesting 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 resistant and BZM resistant cells are sensitive to Cetrorelix treatment. The proteasome inhibitor bortezomib 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 bortezomib or ATO treatment (4-5). Future studies, including clinical trials, are warranted to evaluate the possible benefits of using Cetrorelix in treatment of myeloma.

**Acknowledgements**
We would like to also acknowledge 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 the current study.

References

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...
Figure legend:

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 genes expression were assayed with RT-PCR. PCR products were electrophoresed on a 2% agarose gel. Results are from one 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) Immunohistochemistry 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 were 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).

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 μM). 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 μM); RPMI 8226 and BZMR were cultured and treated with bortezomib (0, 1.25, 2.5, 5, 10, and 20 nM) to demonstrate the resistance to ATO and BZM respectively; KAS-6 and KAS-6/R10R were cultured and treated with lenalidomide (0, 0.1, 1, 10, 100 μM) to demonstrate the resistance. The MTT analysis was done after 48 hours of culture.
Data are mean ± SD of three 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 μM) in different MM cell lines were shown in the table. (B) RPMI 8226 and MM.1S cells were cultured in agar with 1 μM Cetrorelix in 6-well plate. After 2 weeks, the dishes were stained with methylene blue and colonies were photographed and counted. Data are mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ versus vehicle. Representative images of colonies were presented.

**Figure 3. Effects of Cetrorelix on the apoptosis of MM cells.** (A) Left panel: RPMI 8226, MM.1S, and U226 cells were cultured in 6-well plates and treated with Cetrorelix (1, 5, and 10 μM) for 24 hours and the analysis for apoptosis was done. Right panel: RPMI 8226, MM.1S, and U226 cells were cultured in 6-well plates and treated with Cetrorelix (5 μM) for 24 hours and the analysis for caspase 3 was done. Data are mean ± SD of three independent experiments. *, $P < 0.05$; **, $P < 0.01$ 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 μM) for 24 hours and the analysis for apoptosis was done. (C) RPMI 8226 cells were transfected with scrambled siRNA (siSCR) or siRNA to knock down LHRHR-I. Forty hours after transfection, cells were replated and cultured overnight, allowed to attach, then treated with Cetrorelix for another 24 hours. Cells were collected for apoptosis analysis as well as Western blot to determine the knock down efficiency. Data are mean ± SD of three independent experiments, normalized to vehicle treatment. *, $P < 0.05$; **, $P < 0.01$ versus scrambled siRNA control.
Figure 4. Cetrorelix is active in an MM xenograft model. (A) NOD/SCID Il2rg-/- mice bearing RPMI 8226 tumors were injected intraperitoneally 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/second/cm\(^2\)/steradian. (B) Mice receiving Cetrorelix or vehicle were imaged weekly, the images were analyzed using Living Image software, and an ROI tool was used to measure the fluorescence efficiency. Data are mean of eight independent experiments. *, \(P<0.05\); **, \(P<0.01\) versus vehicle. (C) The Kaplan-Meier survival curve for mouse groups that received Cetrorelix (n=8) or vehicle (n=8).

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 μM) for 12 hours. RNA was extracted from RPMI 8226 and BMSCs separately and qRT-PCR was performed. Data are mean ± SD of three independent experiments after normalizing the data to the vehicle. *, \(P<0.05\); **, \(P<0.01\) versus vehicle. (B) Cells were treated with Cetrorelix (5 μM) for 3 or 6 hours, then collected, fixed, permeabilized and labeled with phycoerythrin (PE)-conjugated antibody against phosphorylated form of p65 followed by flow cytometry analysis. The x-axis shows the fluorescence intensity of PE and the y-axis represents the cell counts. Data are from three independent experiments.
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 μM) 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 probed with anti-ERK antibody, or with anti-phospho-specific STAT3 antibody and probed with anti-STAT3 antibody. The membrane was probed again with anti-Actin antibody as a loading control. Data are from three independent experiments. (B) RPMI 8226 and MM.1S cells were starved overnight and cultured without cytokines. Cells were then exposed to Cetrorelix (5 μM) for 12 hours. Lysates from unstimulated or stimulated myeloma cells were immunoblotted with either anti-p21, or with anti-p27, or with anti-p53, or with anti-Bcl-2, or with anti-Bcl-xL antibody. The membrane was probed again with anti-actin antibody as a loading control. Data are from three independent experiments.
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
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 Published OnlineFirst November 9, 2010.

Updated version
Access the most recent version of this article at:
doi:10.1158/1535-7163.MCT-10-0829

Author Manuscript
Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts
Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions
To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions
To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.