Molecular Pharmacology Fast Forward. Published on February 23, 2006 as doi:10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

MOL 20479

Raloxifene-induced myeloma cell apoptosis: a study of NF-κB inhibition and gene expression signature

Sabine Olivier, Pierre Close, Emilie Castermans, Laurence de Leval, Sebastien Tabruyn, Alain Chariot, Michel Malaise, Marie-Paule Merville, Vincent Bours[1] and Nathalie Franchimont[1] .

Departments of Rheumatology (S.O., M.M., N.F), Clinical Chemistry and Human Genetics (P.C., A.C., MP.M., V.B.), Hematology (E.C.), Pathology (L.d.L.) and Genetic Engineering (S.T.), Center for Biomedical Integrative Genoproteomics, University of Liège, CHU Sart-Tilman 4000 Liège, Belgium.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

Running title: Raloxifene-induced myeloma cell apoptosis

Corresponding author:

Vincent Bours

Laboratory of Medical Chemistry and Human Genetics

CHU B35, Sart Tilman, 4000, Liège

BELGIUM

Tel : +32 43 66 81 44

Fax : +32 43 66 81 46

Email : vbours@ulg.ac.be

Abstract word count: 221

Introduction word count: 525

Discussion word count: 840

Number of text pages: 30

Number of Tables: 1

Number of figures: 5

Number of references: 41

List of abbreviations:

ChIP : Chromatin immunoprecipitation

NF-κB: Nuclear factor-kappaB

SERMs : Selective estrogen receptor modulators

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

ABSTRACT

As multiple myeloma remains associated with a poor prognosis, novel drugs targeting specific signalling pathways are needed. The efficacy of selective estrogen receptor modulators for the treatment of multiple myeloma is not well documented. In the present report, we studied the antitumor activity of raloxifene, a selective estrogen receptor modulator, on multiple myeloma cell lines. Raloxifene effects were assessed by MTS reduction assay, cell cycle analysis and western blotting. Mobility shift assay, immunoprecipitation, ChIP assay and gene expression profiling were performed to characterize the mechanisms of raloxifene-induced activity. Indeed, raloxifene, as well as tamoxifen, decreased JJN-3 and U266 myeloma cell viability and induced caspase-dependent apoptosis. Raloxifene and tamoxifen also increased the cytotoxic response to vincristin and arsenic trioxide. Moreover, raloxifene inhibited constitutive NF-κB activity in myeloma cells by removing p65 from its binding sites through ERα interaction with p65. Importantly, micro-array analysis showed that raloxifene treatment decreased the expression of known NF-κB-regulated genes involved in myeloma cell survival and myeloma-induced bone lesions (e.g. *c-myc, mip-1α, hgf, pac1*,…) and induced the expression of a subset of genes regulating cellular cycle (e.g. *p21, gadd34, cyclin G2*,…). In conclusion, raloxifene induces myeloma cell cycle arrest and apoptosis partly through NF-κB-dependent mechanisms. These findings also provide a transcriptional profile of raloxifene treatment on multiple myeloma cells offering the framework for future studies of SERMs therapy in multiple myeloma.

.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

INTRODUCTION

Multiple myeloma is a bone marrow disease characterized by uncontrolled plasma cell proliferation and by various clinical manifestations such as hyperproteinemia, renal insufficiency, anemia and skeletal destruction. Osteolysis is a major clinical complication of multiple myeloma and is associated with bone pain and pathological fractures (Roodman, 2004). Multiple myeloma treatment remains unsatisfactory and new drugs targeting key signalling pathways required for myeloma growth or survival are needed.

Raloxifene is a Selective Estrogen Receptor Modulator (SERM) registered for the treatment of osteoporosis (Delmas et al., 2002). SERMs have both estrogen agonistic and antagonistic properties depending on the tissue, the cell type and even the target gene. Raloxifene thus has *in vitro* antiestrogen activities on breast tumor cells, and raloxifene treatment is associated with a decreased incidence of invasive breast cancer (Martino et al., 2004). However, raloxifene properties on the proliferation and survival of other cancer cells, including multiple myeloma cells, have not been explored extensively.

Many studies have reported the crucial role of the transcription factor NF-κB in the growth and survival of various tumor cells (Panwalkar et al., 2004). The typical heterodimeric p50/p65 NF-κB complex is sequestered in the cytoplasm through its association with IκB family inhibitors. Activation by various stimuli leads to IκB protein phosphorylation and degradation, allowing NF-κB translocation into the nucleus where it specifically binds DNA and stimulates target gene transcription (Greten and Karin, 2004). In contrast to normal cells, NF-κB is constitutively activated in various cancer cells including myeloma cells. This constitutive activity favours cell proliferation, inhibits apoptosis and enhances resistance to cytotoxic agents (Bharti et al., 2003; Hideshima et al., 2002). Moreover, elevated levels of NF-κB activity were found in relapsing and refractory multiple myeloma (Feinman et al., 1999). Several established anti-myeloma therapies, e.g.

dexamethasone, histone deacetylase inhibitors and thalidomide, inhibit NF-κB activity (Mitsiades et al., 2004; Mitsiades et al., 2002b). Recently, it has been demonstrated that bortezomib (PS-341), a proteasome inhibitor, is highly efficient for the treatment of refractory multiple myeloma (Richardson et al., 2005) and it is considered that PS-341 efficacy against multiple myeloma is mainly due to the inhibition of NF-κB activity (Mitsiades et al., 2002a).

The rationale for the investigation of raloxifene activity against multiple myeloma cells was based on the following scientific arguments: a) some myeloma cells express estrogen receptors (ER) α and β (Otsuki et al., 2000); b) tamoxifen has been shown to induce apoptosis in multiple myeloma cells and is currently tested in clinical trials (Fassas et al., 2001; Gauduchon et al., 2005; Treon et al., 1998); c) ERs can inhibit NF-κB activity in the presence of their ligands (Galien and Garcia, 1997; Harnish et al., 2000; Valentine et al., 2000); d) raloxifene decreases osteoclastic bone resorption through modulation of cytokines such as IL-6 and the RANKL/OPG system (Cheung et al., 2003); e) raloxifene is a well tolerated drug largely used for the treatment of post-menopausal osteoporosis.

The aim of our study was thus to investigate the response of myeloma cells to raloxifene. We also characterized the signalling pathways involved in this effect and performed a micro-array analysis to determine the transcriptional profile of raloxifene-treated multiple myeloma cells. These findings provide the framework for future studies of raloxifene activity in multiple myeloma.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

MATERIALS AND METHODS

Cell culture and reagents

JJN-3 and RPMI 8226 myeloma cells were kindly provided by Dr B. Van Camp (Free University Brussels, Brussels, Belgium). U266 myeloma cells were obtained from the European Collection of Cell Cultures (Salisbury, Wiltshire, UK).

Cells were grown in RPMI 1640 medium supplemented with 5% or 10% fetal calf serum, penicillin (100 IU/ml) and streptomycin (100 µg/ml). For all experiments, myeloma cells were plated in phenol-red free RPMI 1640 supplemented with 1% charcoal-treated FCS, penicillin (100 IU/ml) and streptomycin (100 µg/ml).

Raloxifene was a kind gift from Eli Lilly (Indianapolis, IN, USA). Tamoxifen and 17β-estradiol were purchased from Sigma-Aldrich (Schnelldorf, Germany). Raloxifene, tamoxifen and 17β-estradiol were dissolved in ethanol at a concentration of 1 mM. Vincristin sulfate was obtained from Teva Pharma (Wilrijk, Belgium). The broad spectrum caspase inhibitor (Z-VAD-FMK) (Alexis Corporation, San Diego, CA, USA) was dissolved in DMSO (10 mM). The NF-κB inhibitor BAY 11-7085 and the proteasome inhibitor MG-132 (Alexis Corporation) were dissolved in ethanol (20 mM) and in DMSO (20 mM), respectively.

MTS reduction assay

Myeloma cells were seeded at 2 x 10^4 cells/well in flat bottomed 96 well plates (VWR, Leuven, Belgium). Cell viability was assessed by reduction of the tetrazolium salt (MTS) to the formazan product in viable cells ("Cell Titer 96 ®Aqueous" Promega, Leiden, The Netherlands) as described (Olivier et al., 2005). The data were expressed as a percentage of absorbance observed in untreated cells.

Cell cycle analysis

The JJN-3 and U266 cells were synchronized at S phase by incubation with an inhibitor of DNA synthesis, thymidine (2.5 mM) for 24 h. The cells were then washed and seeded at 10^6

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

cells/well in 6 well plates. The cells were treated with raloxifene or tamoxifene for 16 or 24 hours, washed twice in PBS and fixed with 2 ml of ice-cold 70% EtOH before being centrifuged and incubated (at 37°C for 30 min) in PBS containing RNase (5 μg/ml) and propidium iodide (50 μg/ml). The cells were analyzed with a Coulter Epics XL flow cytometer equipped with an argon laser emitting at 488 nm (Beckman Coulter Inc., Miami, FL, USA). Red fluorescence intensity was measured using a 620 ± 15 nm band pass filter. Graphical and cell cycle analyses were performed with FlowJo version 6.01 software (TreeStar).

Western blotting assays

Total protein expression were estimated by Western blotting as described (Olivier et al., 2005). Anti-ERα, anti-ERβ (sc-543 and sc-8974, Santa Cruz Biotechnology, Santa Cruz, CA) anti-PARP, anti-Caspase 8, anti-Caspase 9 (all from BD Biosciences, Erembodegem, Belgium), anti-Bid (sc-6538, Santa Cruz Biotechnology), anti-Caspase 3 (Alexis corporation), anti-Myc (sc-40, Santa Cruz Biotechnology), anti-$p21^{WAF1}$ (Oncogene, Boston, MA) or anti-Actin (Sigma-Aldrich) antibodies were used as primary antibodies. Horseradish peroxidase-linked anti-rabbit, anti-goat or anti-mouse antibodies (DakoCytomation, Glostrup, Denmark) were used as secondary antibodies. The reaction was revealed with the enhanced chemiluminescence detection reagent (ECL kit, Amersham Pharmacia Biotech UK Limited, Buckinghamshire, UK).

Kinase assay

For endogenous IKK activity, JJN-3 cells (5 x 10^6 cells) were treated with raloxifene for the indicated times and subsequently lyzed with 1% Triton X-100 lysis buffer. Cellular extracts were incubated with 5 μl of anti-IKKγ antibody (sc-8330) or 5 μl of the control anti-HA antibody (sc-805) (both from Santa Cruz Biotechnology) for 2 h at 4 °C. Then, 100 μl of protein A-coupled Sepharose beads were added and incubated under gentle agitation overnight

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

at 4°C. After three washes in lysis buffer, the kinase activity was assayed by using 1μg of GST-IκBα as substrate.

Electrophoretic mobility shift assay

Electrophoretic mobility shift assays (EMSA) were performed as described (Bureau et al., 2002). The κB probe was: 5′-TTGGCAACGGCAGGGGAATTC-CCCTCTCCTTAGGTT-3′. Nuclear extract samples containing 5 μg of protein were mixed with 5 μl of binding buffer (HEPES, 20 mM, pH 7.6, NaCl 10 mM, $MgCl_2$ 1.5 mM, EDTA 0.2 mM, glycerol 20%, NP-40 0.1%, dithiothreitol 1 mM, and PMSF 0.5 mM), 2 μl bovine serum albumin, 3 μl polydIdC and the ^{32}P-labeled probe. The complexes were separated on a polyacrylamide gel. To confirm specificity, competition assays were performed with a 50-fold excess of unlabeled wild-type and non-specific probes. For supershifting experiments, 1μg of each antibody (p50: sc-114; p65: sc-109; RelB: sc-226; c-Rel: sc-6955 all from Santa Cruz Biotechnology and p52: 05-361 from Cell Signaling, Netherlands) was incubated with the extracts for 30 minutes on ice before addition of the ^{32}P-labeled probe.

Immunofluorescence

Myeloma cells (10^6 cells/well in 6 well plates) were cytocentrifuged and fixed in formaldehyde 4%. The slides were blocked with PBS containing 1.5% dry milk before incubation with a goat anti-p65 antibody (1/100 dilution, sc-372, Santa Cruz Biotechnology) for 45 minutes at 37°C. Slides were then washed in PBS and incubated with a secondary anti-goat antibody conjugated to fluorescein isothiocyanate (FITC) (1/50 dilution, DakoCytomation) for 45 minutes at 37°C. Slides were washed in PBS and analyzed under a fluorescence microscope (Nikon eclipse E800 with Sony DXC-9100P: magnification 40x).

Chromatin immunoprecipitation

Chromatin immunoprecipitation (ChIP) assays were performed with a ChIP assay kit (Upstate Biotechnology, Dundee, UK). 5 μl of anti-p65 antibody (sc-109), 5 μl of anti-

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

estrogen receptor antibody (sc-543) or 5 µl of the control anti-HA antibody (sc-805) (all from Santa Cruz Biotechnology) were used for the immunoprecipitation. The *mip-1α* promoter sequence was detected with the sense 5'-CTCTTCACACTCACAGGAGA-3' primer and the antisense 5'-TAGGCAGCCCTGGCGGAT-3' primer. PCR was performed using the AmpliTaq DNA polymerase (Applied Biosystems, Foster City, CA, USA) according to the following protocol: 99°C for 3 min followed by 30 amplification cycles (95°C for 1 min, 50°C for 1 min and 72°C for 1 min).

Immunoprecipitation

Nuclear and cytoplasmic protein extracts of JJN -3 cells (5 x 10^6 cells/dish) were prepared as previously described (Delhalle et al., 2002). For immunoprecipitation, nuclear extracts were incubated with 5 µl of anti-ERα antibody (sc-543) or 5 µl of the control anti-HA antibody (sc-805) (both from Santa Cruz Biotechnology) for 2 h at 4 °C. Then, 100 µl of protein A-coupled Sepharose beads were added and incubated under gentle agitation overnight at 4°C. After three washes in lysis buffer, bound proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Affymetrix microarray analysis

Total RNA was extracted from control or treated JJN-3 cells using RNeasy columns from Qiagen (Valencia, CA). The integrity of the RNA was checked with the Agilent 2100 Bioanalyzer. RNAs from three independent experiments were pooled and used as template to generate double stranded cDNAs using superscript II RT kit (Invitrogen, Merelbeke, Belgium). Subsequently, biotin labelled cRNA was generated using the Bioarray High Yield RNA Transcript labelling kit (Enzo Life Science, NY). The cRNAs were hybridized with the Human Genome U-133A array (Affymetrix). Data were processed using Gene Chip Operating Software (Affymetrix).

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

Real Time PCR

Real Time PCR was carried out using the TaqMan platform with the SYBR green master mix (Applied Biosystems), as described previously (Heid et al., 1996). Primers, whose sequences were available upon request, were designed using the Primers Express software.

Statistical analysis

Data are presented as mean values ± SEM (standard error of the mean). Data were analyzed by the Mann-Whitney test. In all analyses, $p < 0.05$ was considered statistically significant.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

RESULTS

Estrogen receptors (ERα and ERβ) expression in multiple myeloma cell lines

Before studying the effects of raloxifene on multiple myeloma cell growth and survival, we verified the expression of the two ER isoforms in JJN-3, U266 and RPMI 8226 cells. All cell lines expressed both ERα and ERβ, as previously shown (Figure 1A) (Treon et al., 1998).

Raloxifene and tamoxifen inhibit myeloma cell proliferation and induce cell cycle arrest

Raloxifene and tamoxifen (both at 5 μM) decreased JJN-3 cell viability after 48 or 72 hours of treatment whereas the same concentration of 17β-estradiol did not (Figure 1B). Both raloxifene and tamoxifen also decreased U266 cell viability (Figure 1B) but an equivalent cytotoxicity required a longer incubation in these cells. A weak effect of raloxifene and tamoxifen was also observed on RPMI 8226 cells after 72 hours of treatment (cell viability was decreased by 23 and 17% respectively) (data not shown).

The *in vitro* concentrations of raloxifene and tamoxifen required to induce apoptosis of breast cancer cells or glioma cells vary from 1 to 40 μM (Frasor et al., 2004; Hui et al., 2004). Based on these studies and on our dose-response data (not shown), all our experiments were carried out using a 5 μM concentration which inhibited JJN-3 cell growth by 50%.

Next, we determined whether the exposure of multiple myeloma cells to these SERMs potentiates the cytotoxic effects of other chemotherapeutic drugs, currently used in the treatment of multiple myeloma. Raloxifene or tamoxifen enhanced the cytotoxic effects of vincristin against JJN-3 and RPMI 8226 cells (Figure 1C). After 72 hours of treatment, vincristin (at 0,1 μg/ml), raloxifene or tamoxifene (at 5 μM) alone decreased JJN-3 cell viability by ~ 50%, whereas the addition of raloxifene or tamoxifen to vincristin induced, respectively, a 76% and 82% decrease in JJN-3 cell viability (Figure 1C). RPMI 8226 cells showed a similar response to vincristin alone and both raloxifene and tamoxifen again

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

enhanced vincristin-induced cell death (Figure 1C). A synergistic effect between SERM and arsenic trioxide (at 5 μM) was also observed in RPMI 8226 myeloma cells (Figure 1 D) but such an effect could not be investigated in JJN-3 cells as these cells are extremely sensitive to arsenic trioxide (data not shown). A weaker but reproducible additive cytotoxic effect was also observed when U266 cells were treated simultaneously with arsenic trioxide and SERMs (data not shown).

FACS analysis showed that both raloxifene and tamoxifen (both at 5 μM) blocked the cell cycle before G2/M in JJN-3 cells as shown by an increased proportion of cells in the S phase and a decrease of the number of cells in G2/M (Figure 1E). In U266 cells, raloxifene and tamoxifen also blocked cell cycle progression but in G2/M phase (data not shown).

Raloxifene and tamoxifen induce myeloma cell apoptosis

To verify that raloxifene and tamoxifen induced cell death by apoptosis, we studied the activation of caspases and the cleavage of PARP in JJN3 and U266 cells. Figure 2A shows that tamoxifen and raloxifene induced a proteolytic cleavage of caspase-8, caspase-3, and PARP (Figures 2A and 2B). The involvement of the mitochondrial apoptotic pathway was also shown by the cleavage of Bid and caspase-9 in JJN-3 cells after raloxifene and tamoxifen treatment (Figure 2C). To confirm that raloxifene and tamoxifen induced caspase-dependent apoptosis, we tested the effect of the caspase inhibitor, Z-VAD-FMK, on cell death and PARP cleavage induced by raloxifene or tamoxifen. Preatreatment of JJN-3 cells with Z-VAD-FMK (at 50 μM) for 1 hour prior the addition of raloxifene or tamoxifen (both at 5 μM) had a cytoprotective effect on myeloma cells (Figure 2D). In the same experimental conditions, the presence of the caspase inhibitor induced a marked decrease of raloxifene or tamoxifen-induced PARP cleavage in JJN-3 cells (Figure 2E).

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

SERMs inhibit NF-κB activity in myeloma cells.

As NF-κB is constitutively active in multiple myeloma cells and promotes myeloma cell growth, survival and drug resistance (Bharti et al., 2003; Feinman et al., 1999), we investigated the effects of the NF-κB inhibitor BAY 11-7085 or the proteasome inhibitor MG-132 on JJN-3 cell viability. Increasing concentrations of BAY 11-7085 (1-4 μM) or MG-132 (0.1-0.4 μM) decreased JJN-3 viability after 24 hours, as determined by the MTS reduction assay (Figure 3A).

We next investigated whether the SERMs inhibited the IKK activity and NF-κB binding activity in myeloma cells. As shown by kinase assay performed with a GST-IκBα substrate, a marked IKK activity was observed in untreated JJN-3 cells and raloxifene only weakly decreased this activity after 16 and 24 hours of treatment (Figure 3B). Consistent with these data, mobility shift assays also showed that NF-κB was constitutively active in the multiple myeloma cell lines. Raloxifene decreased NF-κB DNA-binding activity in JJN-3 or U266 cells (Figures 3C and 3D). In JJN-3 cells, the inhibition was already observed after 30 minutes and was complete after 16 hours of treatment. A similar, but less pronounced, inhibition was observed after treatment of JJN-3 or U266 cells with the same concentration of tamoxifen (Figures 3C and 3D). NF-κB DNA binding complexes contained various homo- or heterodimers of p50, p52, p65 and RelB subunits in JJN-3 cells whereas the NF-κB complex was mainly constituted by p52/RelB subunits in U266 cells as demonstrated by supershift experiments (Figures 3C and 3D). Competition experiments performed with a 50-fold excess of an unlabeled palindromic κb probe or a non-specific probe confirmed the specificity of NF-κB binding activity in JJN-3 or U266 cells (Figures 3C and 3D).

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

To determine the subcellular localization of NF-κB subunits, immunofluorescence staining was performed with an anti-p65 antibody. While p65 was exclusively observed in the nucleus of untreated cells, raloxifene or tamoxifen treatment rapidly induced p65 relocalization to the cytoplasm of JJN-3 (Figure 3E) and U266 cells (data not shown).

ERα interaction with p65

To study the mechanisms of raloxifene-mediated NF-κB inhibition in myeloma cells, we investigated whether ERα directly interacts with p65. Nuclear extracts from untreated or raloxifene-stimulated JJN-3 cells were immunoprecipitated with an anti-ERα antibody and the resulting precipitates were analyzed by an anti-p65 Western blotting analysis (Figure 4A). Whereas p65 was weakly associated with ERα in untreated cells, this interaction was strongly enhanced after 5 minutes of raloxifene treatment and became undetectable after longer stimuli (Figure 4A). These data suggested that raloxifene may induce a conformation change in ERα, resulting in decreased NF-κB DNA binding activity and consequently in the release and translocation of p65 from the nucleus to the cytoplasm.

To confirm this hypothesis, we evaluated the binding of p65 to NF-κB-regulated promoters in the presence of raloxifene by ChIP assays. We selected the *mip-1α* gene promoter because *mip-1α* is a known NF-κB-regulated gene whose expression was significantly decreased after raloxifene treatment of JJN-3 cells (see Table 1 and Figure 5A). Raloxifene treatment led to the dissociation of p65 (Figure 4B, top left panel) and ERα (Figure 4B, middle left panel) binding to the *mip-1α* gene promoter. These data thus showed that p65 and ERα are released from the NF-κB binding site on specific gene promoters upon treatment with raloxifene.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

Gene expression after raloxifene treatment

Gene expression in JJN-3 cells either untreated or treated with raloxifene for 2 hours was compared by micro-array analyses. This study identified 29 genes that were transiently repressed and 75 genes that were induced upon raloxifene treatment. A representative list of raloxifene target genes is shown in Table 1. Interestingly, 14 of these raloxifene-regulated genes are known to be associated with cell cycle and apoptosis signalling pathways (Table 1). Indeed, raloxifene downregulated the transcription of the proto-oncogene *c-myc* and upregulated the expression of the transcriptional repressor *bcl-6* and of negative cell cycle regulators such as *p21, cyclin G2* and *e2f2* (Horne et al., 1997; Park et al., 2000; Shou et al., 2000; Tang et al., 2002; Zhu et al., 2001). Moreover, raloxifene suppressed the expression of other genes controlling multiple myeloma cell proliferation and/or survival including *hgf* and *mip-1*α (Anderson et al., 2002). Raloxifene also inhibited the expression of genes coding for the two MAPkinase phosphatases *dusp2* (*pac-1*) and *dusp4,* two mitogen-induced early responsive genes overexpressed in breast cancer (Wang et al., 2003), and of the ras homologue *arhe*.

In addition, raloxifene upregulated the expression of genes coding for the growth arrest and DNA damage-induced protein PP1R15A (Gadd34), the transcription factors ATF3 Retinoic Acid X Receptor beta (*rxrb*) and CCAAT/enhancer binding protein beta (C/EBPβ), the heat shock family protein 40 (DnaJB) and genes coding for the proteins involved in the response to endoplasmic reticulum stress (DnaJB, HERPUD1) (Table 1) (Fan et al., 2002; Hasegawa et al., 2000). It also repressed the transcription factor 8 (*tcf8*) expression.

Consistently with a recent study suggesting that tamoxifen-induced apoptotic response in glioma cells was mediated by an increase in cytosolic calcium (Hui et al., 2004), raloxifene upregulated the expression of genes controlling intracellular calcium and chloride flux such as *stc2, clic4* and *clcn6* (Table 1).

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

In order to validate the micro-array experiments, we confirmed by real time RT-PCR that raloxifene inhibited the expression of *mip-1α, c-myc, pac-1*/*dusp2* and although less significantly *hgf*, while it induced *gadd 34, cyclin G2* and *p21* gene expression in JJN-3 cells (Figure 5A). Western blotting analyses also confirmed that raloxifene inhibited c-Myc and induced p21 expression in these cells (Figure 5B). Similarly, in U266 cells, raloxifene up-regulated the expression of *gadd 34, cyclin G2* and *p21* genes and down-regulated *c-myc* but not *mip-1α* gene expression while the expression of *dusp2/pac-1* and *hgf* could not be detected by RT-PCR in these cells (Figure 5A).

As raloxifene inhibited constitutive NF-κB activity and as several of the raloxifene-regulated genes are known NF-κB targets, JJN-3 cells were treated with the NF-κB inhibitor BAY 11-7085 (20 μM and 40 μM) for 2 hours. In these conditions, real time PCR showed a decrease in *mip-1α, c-myc* and *hgf* gene expression thus indicating that the raloxifene-induced inhibition of these genes is most likely NF-κB-mediated while the drug did not influence *pac1*/*dusp2* expression (Figure 5C), indicating that raloxifene inhibited its expression through a pathway most probably NF-κB-independent.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

DISCUSSION

The SERM raloxifene has been approved for the treatment of post-menopausal osteoporosis on the basis of its estrogen-like activity in the bones. However, this drug also displays anti-estrogen activities and could therefore be effective for the treatment of ER-expressing breast cancers or multiple myelomas (Delmas et al., 2002; Martino et al., 2004). Indeed, multiple myeloma cells express ER and previous studies demonstrated that tamoxifen or toremifene could induce multiple myeloma cell death and cell cycle arrest (Otsuki et al., 2000; Treon et al., 1998). We therefore studied the activity of raloxifene on ER-positive multiple myeloma cells, considering that the raloxifene anti-resorptive effect on bones and its good clinical tolerance are additional arguments to justify such an investigation.

In our experimental conditions, we indeed observed that raloxifene blocked the cell cycle and induced apoptosis in several ER-positive multiple myeloma cell lines**.** Moreover, raloxifene also increased vincristin and arsenic trioxide cytotoxic response. To further characterize the mechanisms responsible for raloxifene *in vitro* activity, we first explored the NF-κB pathway. Indeed, the NF-κB transcription factor is constitutively active in refractory multiple myeloma and several novel treatments, such as bortezomib, successfully target this factor (Hideshima et al., 2002; Richardson, 2003). Raloxifene could indeed inhibit NF-κB activity, as demonstrated by the study of NF-κB cellular localization, DNA-binding and transcriptional activities. As ER had been demonstrated to interact with NF-κB (Kalaitzidis and Gilmore, 2005), we further showed that raloxifene treatment led to a transiently increased ER/NF-κB interaction, simultaneously with a loss of NF-κB binding to specifically regulated gene promoters. These data suggest that raloxifene could indeed block the NF-κB biological activity through a modulation of the ER association with p65 while raloxifene-induced inhibition of IKK activity was only partial.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

We then performed a large scale study of gene expression regulation in multiple myeloma cells treated with raloxifene. This experiment led to the identification of only 104 genes whose expression is modified in response to this treatment. More significantly, only 29 genes showed reduced expression levels in response to raloxifene. However, many of the identified genes could provide us with a new insight into the mechanisms of raloxifene action, as 14 genes are known to regulate cell cycle or apoptosis and several of them (*c-myc* or *mip-1α* for instance) had been previously identified as important regulators of multiple myeloma cell survival and disease progression. *Mip-1α* expression by tumor cells in the bone marrow of multiple myeloma patients correlates with an adverse prognosis (Terpos et al., 2003) and this gene is also a key regulator of bone resorption by osteoclasts and could therefore be responsible for the typical multiple myeloma bone lesions (Roodman and Choi, 2004).

It is very tempting to correlate the inhibition of NF-κB with the list of genes obtained from the micro-array experiment. Indeed, it is clear that some known NF-κB-regulated genes, such as *c-myc* and *mip-1α*, showed a decreased expression in response to raloxifene. We could also demonstrate that an NF-κB inhibitor had a similar effect on them and that raloxifene treatment was associated with a loss of NF-κB binding to the endogenous *mip-1α* promoter. However, as the number of downregulated genes is quite low, it is likely that raloxifene inhibits only a subset of NF-κB target genes. Indeed, although raloxifene blocked DNA-binding activities by several distinct NF-κB complexes in the investigated cell lines, this inhibition is quite slow and incomplete. It is thus not surprising that, after two hours of raloxifene treatment, only a part of NF-κB-regulated genes is affected. However, gene expression studies performed at later time points would identify many genes that are induced or repressed secondarily in the context of apoptosis or cell cycle arrest.

Obviously, raloxifene activity on multiple myeloma cells is not restricted to the inhibition of the NF-κB pathway. Indeed, the induction of cell cycle regulating genes (*p21,*

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

cyclin G2) or ion channels as well as the inhibition of *dusp* phosphatases gene expression are likely to be NF-κB-independent. Other specific anti-myeloma therapeutic agents also combine NF-κB-dependent and independent activities. For instance, the proteasome inhibitor bortezomib is highly efficient for the treatment of refractory MM (Richardson et al., 2005). This drug blocks IκB-α degradation and NF-κB activation but it also induces p53 and NOXA expression and increases the expression of cyclin-dependent kinase inhibitors including, as raloxifene, p21 (Hideshima et al., 2003; Mitsiades et al., 2002a; Qin et al., 2005).

In conclusion, raloxifene is a very promising drug for the treatment of multiple myeloma as it: 1) blocks cell cycle and induces apoptosis in multiple myeloma cells; 2) potentiates vincristin and arsenic trioxide cytotoxicity; 3) targets NF-κB, a key regulator of multiple myeloma cell survival, associated with the progression of refractory multiple myeloma; 4) decreases IL-6 production by osteoblasts (Cheung et al., 2003); 5) inhibits osteoclast function and could thus limit bone resorption and favor the reparation of lytic lesions and 6) is a well known and well tolerated compound. Given the poor prognosis of patients with refractory multiple myeloma and the current knowledge on raloxifene pharmacology, we believe that our data provide strong arguments for *in vivo* tests on animal models and a rapid onset of raloxifene clinical trials in these pathologies.

ACKNOWLEDGMENTS

The authors thank Aline Desoroux, Sophie Castermans, Andrée Villers and Miguel Lopez for their expert assistances. S. Olivier thanks Dr. J. Piette, Dr F. Lambert and Dr. B. Relic for their kind advices. The authors thank Eli Lilly and Co for providing raloxifene and for their kind help and discussion.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

REFERENCES

Anderson KC, Shaughnessy JD, Jr., Barlogie B, Harousseau JL and Roodman GD (2002) Multiple myeloma. *Hematology (Am Soc Hematol Educ Program)*:214-240.

Bharti AC, Donato N, Singh S and Aggarwal BB (2003) Curcumin (diferuloylmethane) down-regulates the constitutive activation of nuclear factor-kappa B and IkappaBalpha kinase in human multiple myeloma cells, leading to suppression of proliferation and induction of apoptosis. *Blood*, **101**:1053-1062.

Bureau F, Vanderplasschen A, Jaspar F, Minner F, Pastoret PP, Merville MP, Bours V and Lekeux P (2002) Constitutive nuclear factor-kappaB activity preserves homeostasis of quiescent mature lymphocytes and granulocytes by controlling the expression of distinct Bcl-2 family proteins. *Blood*, **99**:3683-3691.

Cheung J, Mak YT, Papaioannou S, Evans BA, Fogelman I and Hampson G (2003) Interleukin-6 (IL-6), IL-1, receptor activator of nuclear factor kappaB ligand (RANKL) and osteoprotegerin production by human osteoblastic cells: comparison of the effects of 17-beta oestradiol and raloxifene. *J Endocrinol*, **177**:423-433.

Delhalle S, Deregowski V, Benoit V, Merville MP and Bours V (2002) NF-kappaB-dependent MnSOD expression protects adenocarcinoma cells from TNF-alpha-induced apoptosis. *Oncogene*, **21**:3917-3924.

Delmas PD, Ensrud KE, Adachi JD, Harper KD, Sarkar S, Gennari C, Reginster JY, Pols HA, Recker RR, Harris ST, Wu W, Genant HK, Black DM and Eastell R (2002) Efficacy of raloxifene on vertebral fracture risk reduction in postmenopausal women with osteoporosis: four-year results from a randomized clinical trial. *J Clin Endocrinol Metab*, **87**:3609-3617.

Fan F, Jin S, Amundson SA, Tong T, Fan W, Zhao H, Zhu X, Mazzacurati L, Li X, Petrik KL, Fornace AJ, Jr., Rajasekaran B and Zhan Q (2002) ATF3 induction following DNA damage is regulated by distinct signaling pathways and over-expression of ATF3 protein suppresses cells growth. *Oncogene*, **21**:7488-7496.

Fassas AB, Rapoport AP, Bolanos-Meade J, Shanholtz C, Cottler-Fox M and Tricot G (2001) Tamoxifen-based treatment induces clinically meaningful responses in multiple myeloma patients with relapsing disease after autotransplantation. *Leuk Lymphoma*, **42**:1323-1328.

Feinman R, Koury J, Thames M, Barlogie B, Epstein J and Siegel DS (1999) Role of NF-kappaB in the rescue of multiple myeloma cells from glucocorticoid-induced apoptosis by bcl-2. *Blood*, **93**:3044-3052.

Frasor J, Stossi F, Danes JM, Komm B, Lyttle CR and Katzenellenbogen BS (2004) Selective estrogen receptor modulators: discrimination of agonistic versus antagonistic activities by gene expression profiling in breast cancer cells. *Cancer Res*, **64**:1522-1533.

Galien R and Garcia T (1997) Estrogen receptor impairs interleukin-6 expression by preventing protein binding on the NF-kappaB site. *Nucleic Acids Res*, **25**:2424-2429.

Gauduchon J, Gouilleux F, Maillard S, Marsaud V, Renoir JM and Sola B (2005) 4-Hydroxytamoxifen inhibits proliferation of multiple myeloma cells in vitro through down-regulation of c-Myc, up-regulation of p27Kip1, and modulation of Bcl-2 family members. *Clin Cancer Res*, **11**:2345-2354.

Greten FR and Karin M (2004) The IKK/NF-kappaB activation pathway-a target for prevention and treatment of cancer. *Cancer Lett*, **206**:193-199.

Harnish DC, Scicchitano MS, Adelman SJ, Lyttle CR and Karathanasis SK (2000) The role of CBP in estrogen receptor cross-talk with nuclear factor-kappaB in HepG2 cells. *Endocrinology*, **141**:3403-3411.

Hasegawa T, Xiao H, Hamajima F and Isobe K (2000) Interaction between DNA-damage protein GADD34 and a new member of the Hsp40 family of heat shock proteins that is induced by a DNA-damaging reagent. *Biochem J*, **352 Pt 3**:795-800.

Heid CA, Stevens J, Livak KJ and Williams PM (1996) Real time quantitative PCR. *Genome Res*, **6**:986-994.

Hideshima T, Chauhan D, Richardson P, Mitsiades C, Mitsiades N, Hayashi T, Munshi N, Dang L, Castro A, Palombella V, Adams J and Anderson KC (2002) NF-kappa B as a therapeutic target in multiple myeloma. *J Biol Chem*, **277**:16639-16647.

Hideshima T, Mitsiades C, Akiyama M, Hayashi T, Chauhan D, Richardson P, Schlossman R, Podar K, Munshi NC, Mitsiades N and Anderson KC (2003) Molecular mechanisms mediating antimyeloma activity of proteasome inhibitor PS-341. *Blood*, **101**:1530-1534.

Horne MC, Donaldson KL, Goolsby GL, Tran D, Mulheisen M, Hell JW and Wahl AF (1997) Cyclin G2 is up-regulated during growth inhibition and B cell antigen receptor-mediated cell cycle arrest. *J Biol Chem*, **272**:12650-12661.

Hui AM, Zhang W, Chen W, Xi D, Purow B, Friedman GC and Fine HA (2004) Agents with selective estrogen receptor (ER) modulator activity induce apoptosis in vitro and in vivo in ER-negative glioma cells. *Cancer Res*, **64**:9115-9123.

Kalaitzidis D and Gilmore TD (2005) Transcription factor cross-talk: the estrogen receptor and NF-kappaB. *Trends Endocrinol Metab*, **16**:46-52.

Martino S, Cauley JA, Barrett-Connor E, Powles TJ, Mershon J, Disch D, Secrest RJ and Cummings SR (2004) Continuing outcomes relevant to Evista: breast cancer incidence in postmenopausal osteoporotic women in a randomized trial of raloxifene. *J Natl Cancer Inst*, **96**:1751-1761.

Mitsiades CS, Mitsiades NS, McMullan CJ, Poulaki V, Shringarpure R, Hideshima T, Akiyama M, Chauhan D, Munshi N, Gu X, Bailey C, Joseph M, Libermann TA, Richon VM, Marks PA and Anderson KC (2004) Transcriptional signature of histone deacetylase inhibition in multiple myeloma: biological and clinical implications. *Proc Natl Acad Sci U S A*, **101**:540-545.

Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Fanourakis G, Gu X, Bailey C, Joseph M, Libermann TA, Treon SP, Munshi NC, Richardson PG, Hideshima T and Anderson KC (2002a) Molecular sequelae of proteasome inhibition in human multiple myeloma cells. *Proc Natl Acad Sci U S A*, **99**:14374-14379.

Mitsiades N, Mitsiades CS, Poulaki V, Chauhan D, Richardson PG, Hideshima T, Munshi N, Treon SP and Anderson KC (2002b) Biologic sequelae of nuclear factor-kappaB blockade in multiple myeloma: therapeutic applications. *Blood*, **99**:4079-4086.

Olivier S, Fillet M, Malaise M, Piette J, Bours V, Merville MP and Franchimont N (2005) Sodium nitroprusside-induced osteoblast apoptosis is mediated by long chain ceramide and is decreased by raloxifene. *Biochem Pharmacol*, **69**:891-901.

Otsuki T, Yamada O, Kurebayashi J, Moriya T, Sakaguchi H, Kunisue H, Yata K, Uno M, Yawata Y and Ueki A (2000) Estrogen receptors in human myeloma cells. *Cancer Res*, **60**:1434-1441.

Panwalkar A, Verstovsek S and Giles F (2004) Nuclear factor-kappaB modulation as a therapeutic approach in hematologic malignancies. *Cancer*, **100**:1578-1589.

Park WH, Seol JG, Kim ES, Hyun JM, Jung CW, Lee CC, Kim BK and Lee YY (2000) Arsenic trioxide-mediated growth inhibition in MC/CAR myeloma cells via cell cycle arrest in association with induction of cyclin-dependent kinase inhibitor, p21, and apoptosis. *Cancer Res*, **60**:3065-3071.

Qin JZ, Ziffra J, Stennett L, Bodner B, Bonish BK, Chaturvedi V, Bennett F, Pollock PM, Trent JM, Hendrix MJ, Rizzo P, Miele L and Nickoloff BJ (2005) Proteasome inhibitors trigger NOXA-mediated apoptosis in melanoma and myeloma cells. *Cancer Res*, **65**:6282-6293.

Richardson P (2003) Clinical update: proteasome inhibitors in hematologic malignancies. *Cancer Treat Rev*, **29 Suppl 1**:33-39.

Richardson PG, Sonneveld P, Schuster MW, Irwin D, Stadtmauer EA, Facon T, Harousseau JL, Ben-Yehuda D, Lonial S, Goldschmidt H, Reece D, San-Miguel JF, Blade J, Boccadoro M, Cavenagh J, Dalton WS, Boral AL, Esseltine DL, Porter JB, Schenkein D and Anderson KC (2005) Bortezomib or high-dose dexamethasone for relapsed multiple myeloma. *N Engl J Med*, **352**:2487-2498.

Roodman GD (2004) Pathogenesis of myeloma bone disease. *Blood Cells Mol Dis*, **32**:290-292.

Roodman GD and Choi SJ (2004) MIP-1 alpha and myeloma bone disease. *Cancer Treat Res*, **118**:83-100.

Shou Y, Martelli ML, Gabrea A, Qi Y, Brents LA, Roschke A, Dewald G, Kirsch IR, Bergsagel PL and Kuehl WM (2000) Diverse karyotypic abnormalities of the c-myc locus associated with c-myc dysregulation and tumor progression in multiple myeloma. *Proc Natl Acad Sci U S A*, **97**:228-233.

Tang TT, Dowbenko D, Jackson A, Toney L, Lewin DA, Dent AL and Lasky LA (2002) The forkhead transcription factor AFX activates apoptosis by induction of the BCL-6 transcriptional repressor. *J Biol Chem*, **277**:14255-14265.

Terpos E, Politou M, Szydlo R, Goldman JM, Apperley JF and Rahemtulla A (2003) Serum levels of macrophage inflammatory protein-1 alpha (MIP-1alpha) correlate with the extent of bone disease and survival in patients with multiple myeloma. *Br J Haematol*, **123**:106-109.

Treon SP, Teoh G, Urashima M, Ogata A, Chauhan D, Webb IJ and Anderson KC (1998) Anti-estrogens induce apoptosis of multiple myeloma cells. *Blood*, **92**:1749-1757.

Valentine JE, Kalkhoven E, White R, Hoare S and Parker MG (2000) Mutations in the estrogen receptor ligand binding domain discriminate between hormone-dependent transactivation and transrepression. *J Biol Chem*, **275**:25322-25329.

Wang HY, Cheng Z and Malbon CC (2003) Overexpression of mitogen-activated protein kinase phosphatases MKP1, MKP2 in human breast cancer. *Cancer Lett*, **191**:229-237.

Zhu JW, Field SJ, Gore L, Thompson M, Yang H, Fujiwara Y, Cardiff RD, Greenberg M, Orkin SH and DeGregori J (2001) E2F1 and E2F2 determine thresholds for antigen-induced T-cell proliferation and suppress tumorigenesis. *Mol Cell Biol*, **21**:8547-8564.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

FOOTNOTES

A) S. Olivier was supported by a TELEVIE grant from The Belgian National Fund for Scientific Research (FNRS). M.P. Merville, A. Chariot and N. Franchimont are Research Associates at the F.N.R.S. (Belgium). We thank TELEVIE (F.N.R.S.), the F.I.R.S. (CHU Liège), the Centre AntiCancéreux (University of Liège) and the Belgian Federation against cancer for their financial support.

B) Address for reprint requests : Vincent Bours

Laboratory of Medical Chemistry and Human Genetics

CHU B35,

University of Liège, Sart Tilman,

4000, Liège

BELGIUM

Tel : +32 43 66 81 44

Fax : +32 43 66 81 46

Email : vbours@ulg.ac.be

C) [1]The two senior authors share equal responsibility.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

FIGURE LEGENDS

Figure 1 : Levels of ERα and ERβ in myeloma cells and SERMs effect on multiple myeloma cell viability and cell cycle: (A) Estrogen receptors expression. Cellular extracts from JJN3 (1), U266 (2), RPMI 8226 (3) and human breast cancer MCF7-AZ (4: positive control) cells were analyzed by Western blotting for ERα (upper panel) and ERβ (lower panel) expression. (B) SERMs reduce myeloma cell viability. JJN3 cells or U266 cells were treated with control media (CT), raloxifene (RAL), tamoxifen (TAM) or 17β-estradiol (βE) at 5μM for 48, 72 or 96 hours (hrs). Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± SEM in 12 samples pooled from 3 independent experiments. **: $p < 0.01$ significantly different from untreated control. (C and D) SERMs effect on vincristin or Arsenic trioxide cytotoxicity. (C) JJN3 cells were treated with either control media (CT), raloxifene (RAL, 5μM), tamoxifen (TAM, 5μM), vincristine (VINC, 0,1 μg/ml) or vincristin + raloxifene (RAL+VINC) or vincristin + tamoxifen (TAM+VINC) for 48 or 72 hours (hrs). RPMI 8226 cells were treated as JJN-3 cells but with vincristin at 0,001 μg/ml. (D) RPMI 8226 cells were treated with either control media (CT), raloxifene (RAL, 5μM), tamoxifen (TAM, 5μM), arsenic trioxide (As203, 5 μM) or arsenic trioxide + raloxifene (RAL+ As203) or arsenic trioxide + tamoxifen (TAM+ As203) for 48 or 72 hours (hrs). Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± SEM in 4 samples from 1 representative experiment. (E) Cell cycle analysis after raloxifene or tamoxifen treatment. JJN-3 cells were treated with thymidine at 2,5 mM for 24 hours. Synchronized cells were replated in 6 wells and treated with control media (CT), raloxifene (RAL) or tamoxifen (TAM) at 5μM for 16 hours (hrs). After treatment, cell cycles were analyzed by FACS. The data are representative of at least 3 distinct experiments.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

Figure 2 : SERMs induce myeloma cell apoptosis through caspase activation. (A and B) JJN-3 (A) and U266 (B) cells were treated with raloxifene or tamoxifen at 5 μM for the indicated times. Total protein extracts were analyzed by Western blotting with anti-Caspase 8, anti-Caspase 3, anti-PARP or anti-Actin antibodies. (C) JJN-3 cells were treated as in (A). Total protein extracts were analyzed by Western blotting with anti-Bid, anti-Caspase 9 or anti-Actin antibodies. Black arrows indicate full length proteins while white arrows show cleaved proteins. (D and E) Caspase inhibitor effect on SERMs-induced myeloma cell apoptosis. (D) JJN3 cells were pretreated with the caspase inhibitor Z-VAD-FMK at 50 μM for 1 hour prior to treatment with control media (CT), raloxifene (RAL) or tamoxifen (TAM) at 5μM for 48 or 72 hours (hrs). Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± SEM in 14 samples pooled from 3 independent experiments. **: $p< 0.01$ significantly different from untreated control. (E) JJN-3 cells were treated as in (D). Total protein extracts were analyzed by Western blotting with anti-PARP antibody. Black arrows indicate full length proteins while white arrows show cleaved proteins.

Figure 3 : NF-κB inhibition induces myeloma cell death and raloxifene inhibits NF-κB activity in myeloma cells. (A) JJN-3 cells were treated with BAY 11-7085 (BAY) or MG 132 at the indicated concentrations for 24 hours. Cell viability was measured with the MTS reduction assay. Data are expressed as mean percentage of control levels ± SEM in 16 samples pooled from 3 independent experiments. **: $p< 0.01$: significantly different from untreated control. (B) IKK activity after raloxifene treatment. JJN-3 cells were untreated or treated with raloxifene (RAL, 5 μM) for the indicated times. Whole extracts were immunoprecipitated (IP) with anti-IKKγ or anti-HA (negative control) antibodies and kinase assay was performed with a GST-IκBα substrate. The presence of IKKγ in the extracts prior

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

to the immunoprecipitation is illustrated by western blotting. (C and D) NF-κB binding activity after raloxifene or tamoxifen treatment. JJN-3 (C) and U266 (D) cells were untreated or treated with raloxifene (RAL) or tamoxifen (TAM) at 5 μM for the indicated times and nuclear extracts were analyzed by EMSA for NF-κB DNA binding activity. Competition experiments were performed with a 50-fold excess of an unlabeled non-specific (c ns) or specific (c wt) probe. Supershift experiments were performed with antibodies against p50, p65, p52, RelB and c-Rel. Arrows indicate the NF-κB complexes. (E) Subcellular distribution of p65 in response to raloxifene or tamoxifen treatment. Immunofluorescence staining of p65 was performed on JJN-3 cells either untreated (0) or treated with raloxifene (RAL, 5 μM) or tamoxifen (TAM, 5 μM) for the indicated times.

Figure 4 : ERα interaction with p65. (A) Immunoprecipitation assay. JJN-3 cells were untreated (0) or treated with raloxifene (RAL) (5 μM) for the indicated times. Nuclear extracts were immunoprecipitated (IP) with anti-ERα or anti-HA (negative control) antibodies followed by anti-p65 Western analysis. The presence of p65 and ERα in the nuclear extracts prior to the immunoprecipitation is illustrated by western blotting. (B) p65 and ERα are released from the *mip-1α* promoter after raloxifene treatment. JJN-3 cells were untreated (0) or treated with raloxifene (RAL) at 5 μM for the indicated times and ChIP assays were performed. The immunoprecipitation was done with antibodies against p65 (upper panel), ERα (middle panel) or HA (negative control). The promoter DNA fragments were amplified by PCR using primers flanking the NF-κB sites of *mip-1α* gene promoter. The input panels represent chromatin fragments that were reversed cross-linked and amplified by PCR (lower panel). Positive control (C+) lane represents DNA of JJN-3 cells directly amplified by PCR. There was no amplified fragment in the absence of DNA (-).

Figure 5 : Raloxifene-modulated genes in myeloma cells. (A) Expression of selected raloxifene target genes : *c-myc*, *mip-1α*, *hgf*, *pac-1*, *gadd34*, *p21* and *cyclinG2*. RNA was

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

extracted from JJN-3 or U266 cells untreated or treated with raloxifene at 5 μM for 2 hours. Levels of transcripts were measured by real-rime RT-PCR. The number of transcript copies for control cells is set to 100%. The data were normalized by quantification of the β2-microglobulin transcripts (β2M) and are expressed as mean percentage of control levels ± SEM in 6 samples pooled from 3 independent experiments. (B) c-Myc and p21 protein expression. Total cellular extracts from raloxifene-treated JJN-3 cells were analyzed by Western blotting with anti-p21, anti-c-Myc or anti-actin antibodies. (C) Expression of selected target genes *c-myc* (Myc), *mip-1α* (Mip-1), *hgf* (Hgf) and *dusp2*/*pac-1* (Pac). RNA was extracted from JJN-3 cells untreated or treated with BAY 11-7085 at 20 μM or 40 μM for 2 hours. Levels of transcripts were measured as in (A). The data are expressed as mean percentage of control levels ± SEM in 4 samples pooled from 2 independent experiments.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

Affymetrix no.	GENE NAME		REGULATION (fold change over control)	
myeloma cell death or cell cycle-related				
202431_s_at	v-myc myelocytomatosis viral oncogene homolog (avian)	MYC	- 4,3	- 3,5
205114_s_at	chemokine (C-C motif) ligand 3	CCL3	- 1,9	- 2,0
210755_at	hepatocyte growth factor (hepapoietin A; scatter factor)	HGF	- 1,6	- 1,6
204014_at	dual specificity phosphatase 4	DUSP4	- 1,5	- 1,7
204794_at	dual specificity phosphatase 2/ pac1	DUSP2	- 2,8	- 7,0
212724_at	ras homolog gene family, member E	ARHE	- 1,6	- 1,6
202284_s_at	cyclin-dependent kinase inhibitor 1A (p21, Cip1)	CDKN1A	1,6	1,5
202769_at	cyclin G2	CCNG2	3,5	4,0
211559_s_at	cyclin G2	CCNG2	3,5	2,8
202770_s_at	cyclin G2	CCNG2	3,7	3,2
201702_s_at	protein phosphatase 1, regulatory subunit 10	PPP1R10	1,6	1,6
201703_s_at	protein phosphatase 1, regulatory subunit 10	PPP1R10	2,0	1,9
202014_at	protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	2,0	2,0
37028_at	protein phosphatase 1, regulatory (inhibitor) subunit 15A	PPP1R15A	1,9	1,5
211527_x_at	vascular endothelial growth factor	VEGF	1,6	1,5
204912_at	interleukin 10 receptor, alpha	IL10RA	1,5	1,5
203140_at	B-cell CLL/lymphoma 6 (zinc finger protein 51)	BCL6	1,6	1,7
207042_at	E2F transcription factor 2	E2F2	2,0	1,9
endoplasmic reticulum stress and translation				
201123_s_at	eukaryotic translation initiation factor 5A	EIF5A	- 2,1	- 2,1
200664_s_at	DnaJ (Hsp40) homolog, subfamily B, member 1	DNAJB1	1,5	1,5
212225_at	putative translation initiation factor	SUI1	2,3	2,8
217168_s_at	homocysteine-inducible, endoplasmic reticulum stress-inducible	HERPUD1	2,1	2,1
202842_s_at	DnaJ (Hsp40) homolog, subfamily B, member 9	DNAJB9	2,0	2,1
202843_at	DnaJ (Hsp40) homolog, subfamily B, member 9	DNAJB9	2,5	2,5
203810_at	DnaJ (Hsp40) homolog, subfamily B, member 4	DNAJB4	1,9	2,0
203811_s_at	DnaJ (Hsp40) homolog, subfamily B, member 4	DNAJB4	1,9	2,8
solute carrier				
200924_s_at	solute carrier family 3 , member 2	SLC3A2	1,5	1,5
221881_s_at	chloride intracellular channel 4	CLIC4	1,7	1,9
220924_s_at	solute carrier family 38, member 2	SLC38A2	1,5	1,6
218041_x_at	solute carrier family 38, member 2	SLC38A2	1,6	1,7
203950_s_at	chloride channel 6	CLCN6	1,6	1,5
217678_at	solute carrier family 7, (cationic amino acid transporter) member 11	SLC7A11	1,7	1,5
209921_at	solute carrier family 7, (cationic amino acid transporter) member 11	SLC7A11	1,7	1,9
214963_at	nucleoporin 160kDa	NUP160	- 3,2	- 4,9

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

transcription factors						
202672_s_at	activating transcription factor 3	ATF3		2,3		2,6
206175_x_at	zinc finger protein 222	ZNF222		2,1		2,0
210282_at	zinc finger protein 198	ZNF198		1,0		1,7
209102_s_at	HMG-box transcription factor 1	HBP1		1,6		1,7
212501_at	CCAAT/enhancer binding protein (C/EBP), beta	CEBPB		1,5		1,5
215099_s_at	retinoid X receptor, beta	RXRB		1,6		1,5
208078_s_at	transcription factor 8 (represses interleukin 2 expression)	TCF8	-	1,7	-	1,6
others						
201625_s_at	insulin induced gene 1	INSIG1		1,6		1,5
202067_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR		1,7		1,6
202068_s_at	low density lipoprotein receptor (familial hypercholesterolemia)	LDLR		2,0		2,1
221638_s_at	syntaxin 16	STX16		1,5		1,5
203439_s_at	stanniocalcin 2	STC2		2,3		1,9
204285_s_at	phorbol-12-myristate-13-acetate-induced protein 1	PMAIP1		1,9		1,9
205417_s_at	dystroglycan 1 (dystrophin-associated glycoprotein 1)	DAG1		1,5		1,6
210836_x_at	phosphodiesterase 4D, cAMP-specific	PDE4D		1,6		1,6
222238_s_at	polymerase (DNA directed), mu	POLM		1,9		2,3
217221_x_at	RNA binding motif protein 10	RBM10		1,5		1,5
200758_s_at	nuclear factor (erythroid-derived 2)-like 1	NFE2L1		1,7		1,5
202126_at	PRP4 pre-mRNA processing factor 4 homolog B (yeast)	PRPF4B	-	1,5	-	1,7
201129_at	splicing factor, arginine/serine-rich 7, 35kDa	SFRS7	-	1,5	-	1,6
213229_at	Dicer1, Dcr-1 homolog (Drosophila)	DICER1	-	2,0	-	1,6

TABLE 1

Genes regulated by raloxifene. Micro-array analysis were carried out using RNA extracted from JJN-3 cells, either untreated or treated with raloxifene at 5μM for 2 hours. The table shows a list of selected raloxifene modulated genes with the ratio of gene expression in raloxifene-treated cells versus untreated JJN-3 cells (fold change over control) from 2 independent experiments.

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

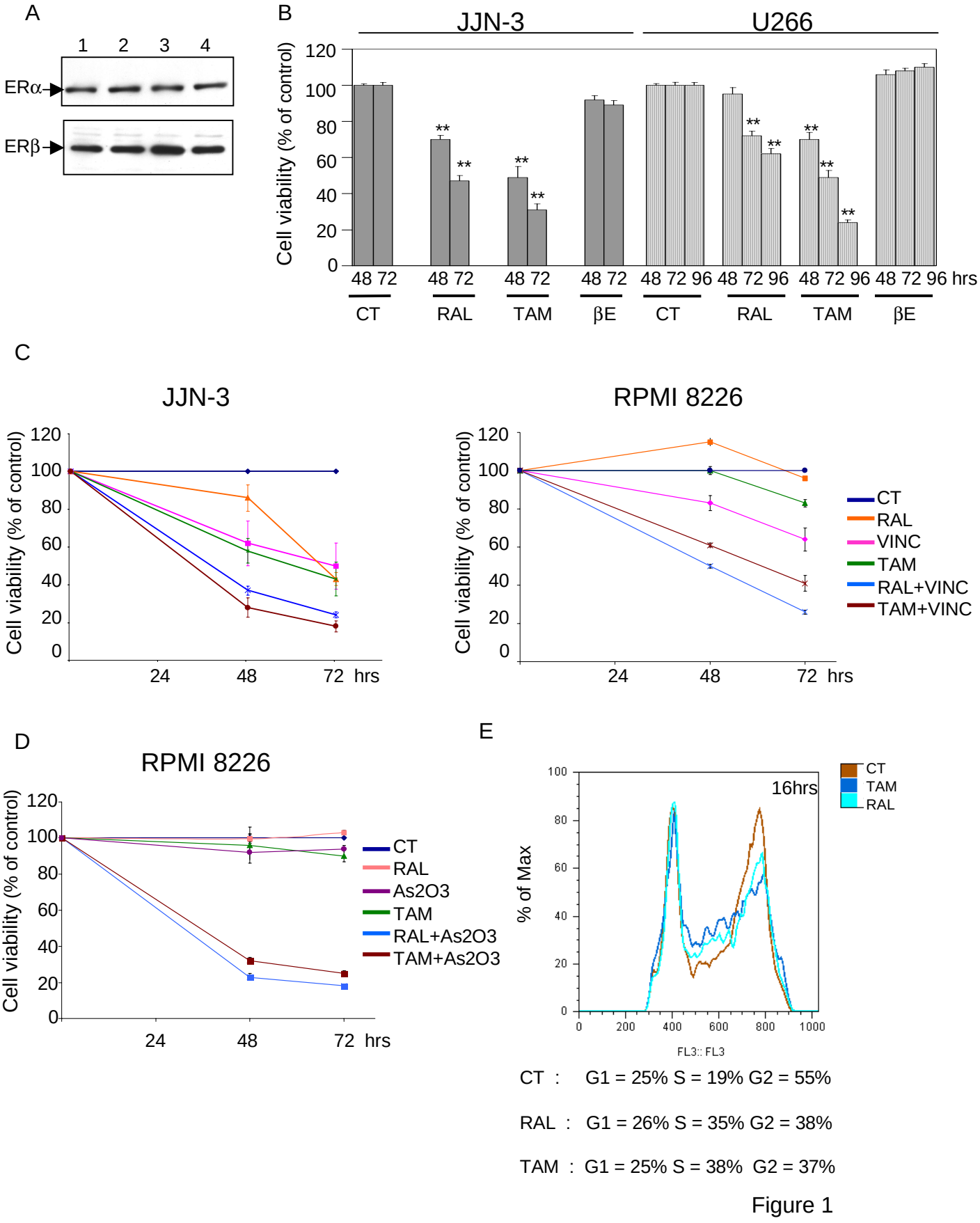


Figure 1

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

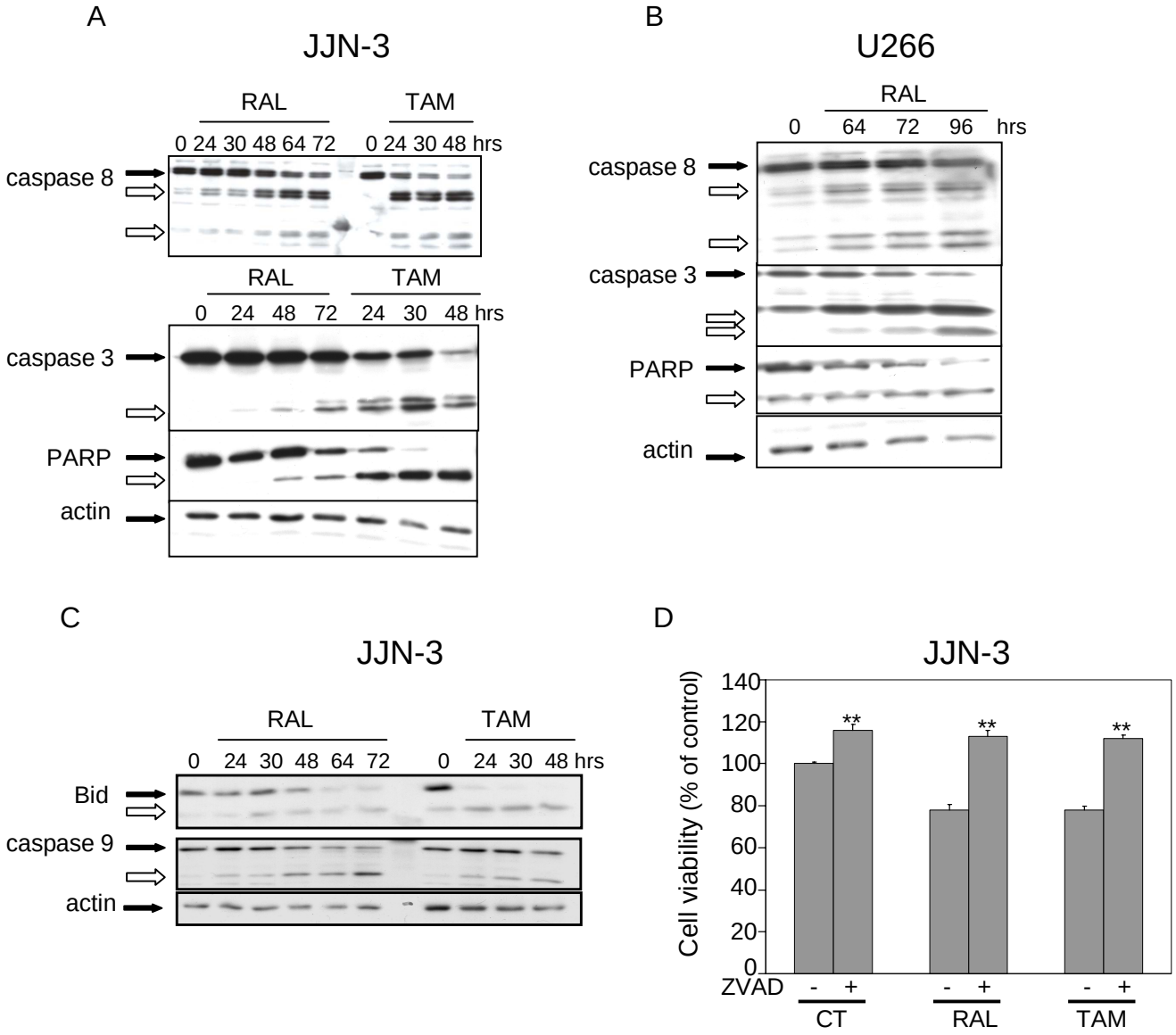


Figure 2

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

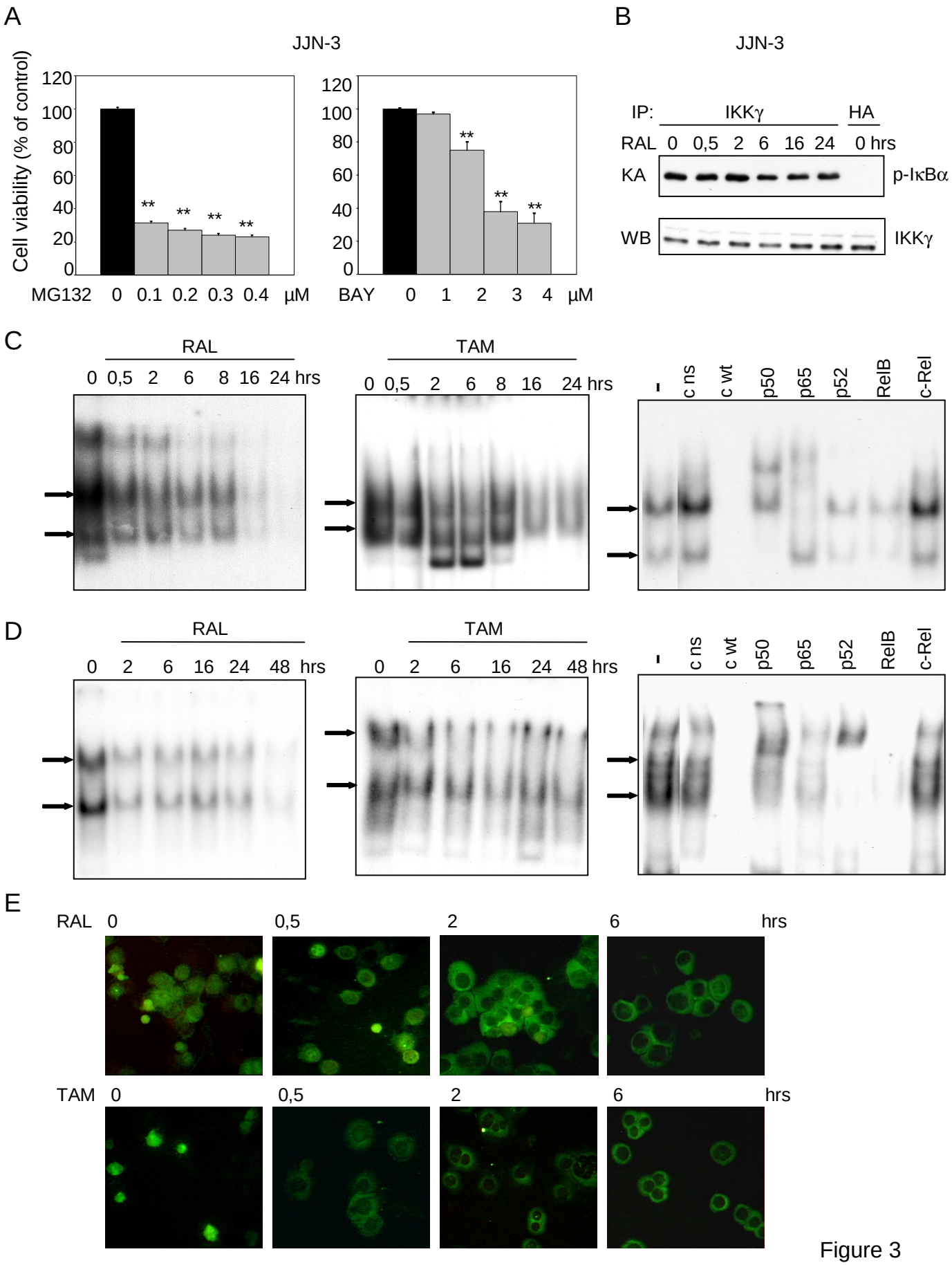


Figure 3

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

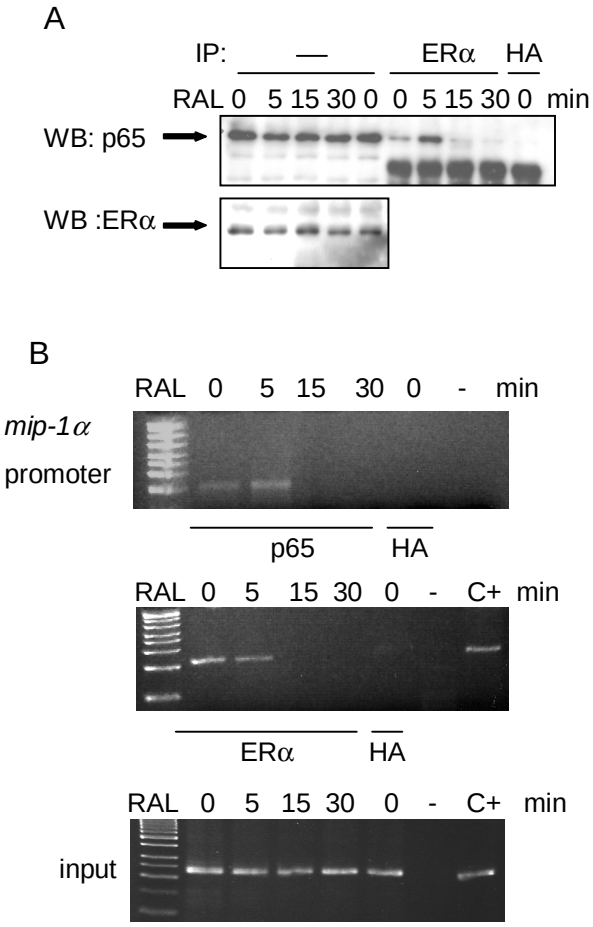


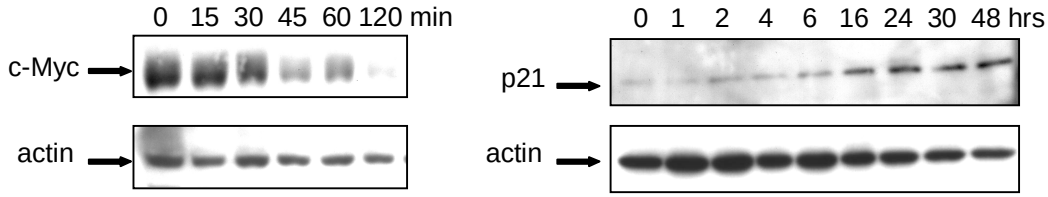
Figure 4

Molecular Pharmacology Fast Forward. Published on February 23, 2006 as DOI: 10.1124/mol.105.020479
This article has not been copyedited and formatted. The final version may differ from this version.

A

	JJN3 % of control means (± SEM)	U266 % of control means (± SEM)
c-myc	14,7 (3,4)	34,2 (4,6)
mip-1α	37,0 (6,0)	91,4 (20,8)
hgf	69,7 (8,9)	NE
pac 1	38,8 (7,1)	NE
gadd 34	203,8 (16,1)	204,8 (60,1)
p21	209,5 (31,5)	204,8 (60,8)
cyclin G2	373,2 (94,0)	274,1 (73,5)

B



C

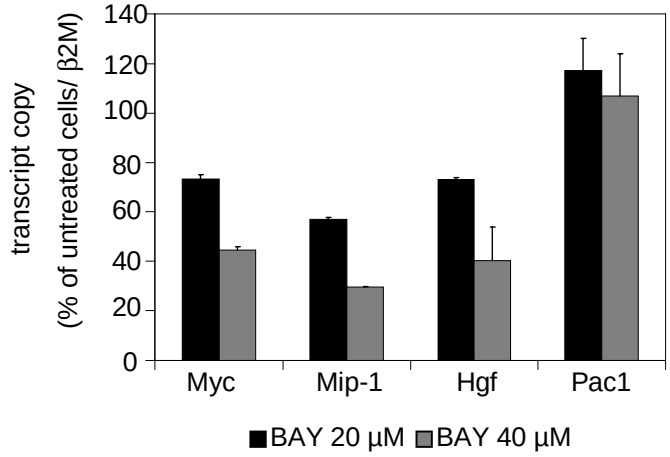


Figure 5