PROTEASOME INHIBITORS INDUCE DEATH BUT ACTIVATE NF-kB ON ENDOMETRIAL CARCINOMA CELL LINES AND PRIMARY CULTURE EXPLANTS.
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Running title: Proteasome inhibitors and NF-kB in endometrial cancer.
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SUMMARY
Proteasome inhibitors are currently used as chemotherapeutic drugs because of their ability to block NF-kB, a transcription factor constitutively activated in many different types of human cancer. In the present study, we demonstrate that proteasome inhibitors induce cell death in endometrial carcinoma cell lines and primary explants but, instead of blocking NF-kB, they increase its transcriptional activity. Proteasome inhibitors induce phosphorylation of IKKα/β, phosphorylation and degradation of IκBα and phosphorylation of p65 NF-kB subunit on serine 536. Proteasome inhibitor-induced NF-kB activity can be blocked by a non-degradable form of IκBα or dominant negative forms of either IKKα or IKKβ. Lentiviral delivery of shRNAs to either IKKα or IKKβ cause blockade of NF-kB transcriptional activity and inhibit phosphorylation of p65 on serine 536, but has no effect on IκBα degradation. These results suggest a role for p65 phosphorylation in proteasome inhibitor-induced NF-kB activation. Accordingly, siRNA knock-down of p65 inhibits proteasome inhibitor-induced NF-kB transcriptional activity. Our results demonstrate that proteasome inhibitors, including bortezomib, induce cell death on endometrial carcinoma cells and primary explants. However, they activate NF-kB instead of blocking its transcriptional potential. Therefore, the concept that proteasome inhibitors are blockers of NF-kB activation should be carefully examined in particular cell types.

INTRODUCTION
The proteasome represents a novel putative target for cancer therapy. PS-341 (Velcade/bortezomib) is a dipeptidyl boronic acid inhibitor with high specificity for the proteasome (1,2). It is currently used in the treatment of patients with multiple myeloma (3,4,5,6,7). Preclinical studies have suggested that proteasome inhibitors show antitumour activity against solid tumours, including carcinomas of the breast (8), lung (9), colon (10), bladder (11), ovary, prostate (12), pancreas (13) and glioblastoma multiforme. Furthermore, evidence has shown that transformed cells appear to be more susceptible to proteasome inhibitor-induced apoptosis than nontransformed cells. Finally, these inhibitors can sensitize cancer cells to death induced by members of the TNF family such as TRAIL.

The antitumoural effects of bortezomib have been extensively studied in multiple myeloma, being inactivation of NF-kB one of the proposed mechanisms of action. NF-kB is a pleiotropic transcription factor, which is activated by a broad variety of stimuli such as growth factors, cytokines, ionizing radiation, ultraviolet light or chemotherapeutic drugs (14, 15) NF-kB regulates the expression of a large number of genes, which carry important functions in inflammation, apoptosis, proliferation, and angiogenesis. NF-kB shows constitutive or increased activity in a wide variety of tumours (16, 17), including endometrial carcinoma (18) and plays a crucial role in neoplastic transformation (16, 19). In resting cells, NF-kB is held inactive in the cytoplasm, bound to the
inhibitors of NF-kB (IkB). Activation of the canonical NF-kB pathway depends on stimuli-induced phosphorylation of the IkB kinase (IKK) complex, which includes the kinases IKKα, IKKB, and the regulatory subunit IKKγ. Activated IKKs induce phosphorylation of IkBα on serines 32 and 36, and the subsequent ubiquitination and degradation by a proteasome-dependent pathway. Free NF-kB is then translocated to the nucleus where it regulates the transcription of several sets of genes. Optimal activation of NF-kB transcriptional activity requires phosphorylation of NF-kB subunits such as p65. In addition, NF-kB activity can be further modulated by phosphorylation of p65 subunit. P65 sequence contains several serine residues that can be phosphorylated (20). One of such residues is the serine 536, which can be phosphorylated by several kinases in different signalling pathways. Phosphorylation of p65 at serine 536 is accomplished by several different stimuli such as lymphotixin beta (21), TNF (22), lipopolysaccharide (23) or IL-1 (24).

The main goal of the present study was to demonstrate that Bortezomib and other three proteasome inhibitors induce apoptosis in endometrial carcinoma cell lines and primary culture explants obtained from endometrial carcinoma tumour samples. However, we have found that such inhibitors activate NF-kB. Proteasome inhibitors induced phosphorylation and reduction of the levels of IkBα protein and phosphorylation of p65 subunit on serine 536, leading to increased NF-kB transcriptional activity. P65 phosphorylation and IkBα degradation depended on either IKKα and IKKB. Proteasome inhibitor-induced activation of NF-kB required degradation of IkBα, and also functional expression of IKKα, IKKB and p65 NF-kB subunit. Proteasome inhibitors have been widely used as pharmacological inhibitors of NF-kB. However, our results demonstrate that this may not always be the case. Therefore, an accurate study of the effects of proteasome inhibitors in signalling pathways may be needed in specific cell types. More importantly, our results may also have clinical relevance because proteasome inhibitors are currently used as anticancer drugs.

**EXPERIMENTAL PROCEDURES**

**Reagents, plasmids and antibodies** - 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide assay (MTT) and monoclonal antibody to Tubulin were from Sigma (St Louis, MO). Proteasome inhibitors MG-132, epoxomicin and ALLN and antibodies to IKKα and IKKB were from Calbiochem (La Jolla, CA). Bortezomib (Millenium Pharmaceuticals, Cambrigde, MA) was obtained from the Department of Pharmacy (Hospital Arnau de Vilanova, Lleida). The broad-specificity caspase inhibitor Boc-D-FMK (BAF) was purchased from Calbiochem (La Jolla, CA). Antibodies to anti-p65-phosphoserine 536, anti-phosho.IkBα and active caspase-3 were obtained from Cell Signalling (Beverly, MA). Antibody to anti-pan-cytokeratin and cytokeratin-7 were from DAKO (Glostrup, Denmark). Antibody to IkBα, p65, and siRNA targeting p65 or c-Rel were obtained from SantaCurz Biotechnology, Inc (SantaCruz, CA). Antibody to β-tubulin was from BDbiosciences (San Jose, CA). Peroxidase-conjugated anti-mouse and anti-rabbit antibodies were from Amersham-Pharmacia (Uppsala, Sweden).

Dominant negative forms of IKKα and IKKB were a generous gift from Dr Alun M Davies. Plasmid containing 5 NF-kB sites and the luciferase reporter gene (NFkB-LUC) was a gift from Dr Giles Hardingham. Plasmid encoding beta-galactosidase was a gift from Mari Carmen Ruiz Ruiz.

**Cell lines, Culture Conditions and Transfection** - The Ishikawa 3-H-12 cell line was obtained from the American Type Culture Collection (Manassas, VA). KLE cells were a gift from Dr Palacios (Centro Nacional de Investigaciones Oncológicas, CNIO, Madrid). RL-95 and HEC-1-A cells were a gift from Dr Reventos (Hospital Vall d’Hebron, Barcelona). All cell lines were grown in Dulbeco’s modified Eagles Medium (DMEM) (Sigma) supplemented with 10% Foetal Bovine Serum (Invitrogen, Inc., Carlsbad, CA, USA), 1 mM HEPES (Sigma), 1 mM sodium pyruvate (Sigma), 2 mM L-glutamine (Sigma) and 1% of penicillin/streptomycin (Sigma) at 37°C with saturating humidity and 5% CO2.

Transfections of both plasmid constructs and siRNAs were performed by calcium phosphate or Lipofectamine 2000 reagent (Invitrogen) following the manufacturers instructions.
**Explant culture of endometrial adenocarcinoma** - Endometrial carcinoma samples were collected in the operating room of the Department of Gynecology, Hospital Universitari Arnuau de Vilanova of Lleida, by a pathologist (JP). A specific informed consent was obtained from each patient, and the study was approved by the local Ethic Committee. Tissue was collected in DMEM, chopped in 1 mm pieces and incubated with collagenase in DMEM for 1.5 hours at 37°C with periodic mixing. Digested tissue was mechanically dissociated through a 10 ml pipette and a 1 ml blue tip and resuspended in 2 ml of fresh DMEM medium. To separate endometrial epithelial cells from the stromal fraction, the dissociated tissue was seeded on top of 8 ml of DMEM medium and tissue was allowed to sediment by gravity for 5 minutes. This step was repeated three times. Finally, tissue explants were resuspended in DMEM supplemented with 10% Foetal Bovine Serum, 1 mM sodium pyruvate, 2 mM L-glutamine and 1% of penicillin/streptomycin (Sigma) and seeded on M24 multiwell plates. Explant cultures were incubated at 37°C with saturating humidity and 5% CO2. After two days in culture, explants were treated with the indicated concentrations of proteasome inhibitors.

**Lentiviral production and infection** - Oligonucleotides to produce plasmid based shRNA were cloned into the FSFSi vector using AgeI-BamHI restriction sites. shRNA target sequence to IKKα was GCAGGCTCTTTCAGGGACA and target sequence to IKKβ was AAAGTGTCAGCTGTATCCT. To produce infective lentiviral particles, 293T cells were co-transfected by calcium phosphate method with the virion packaging elements (VSV-G and Δ8.9) and the shRNA producing vector (FSPsi) or the expression vector (FCIV) on 293T human embryonic kidney. 293T cells were allowed to produce lentiviral particles during 3-4 days in same culture medium used for endometrial cell lines and explants. Culture medium was collected, centrifuged for 5 minutes at 1000 rpm and filtered through a 0.45 μm filter (Millipore). The medium was diluted 1:2 to 1:4 with fresh medium, and added to growing cell lines or primary explants. Cells were incubated for 24-48 hours in presence of medium containing lentiviral particles. After this period, medium was replaced for fresh medium and cells were incubated for two additional days to allow endogenous protein knock-down or protein overexpression.

**Cell viability assays and assessment of apoptosis** - Cell viability was determined by MTT assay. Endometrial adenocarcinoma cells were plated on M96 well plates at 15x10^3 cells per well. After the indicated treatments, the cells were incubated for 2-3 hours with 0.5 mg/ml of MTT reagent and lysed with DMSO. Absorbance was measured at 595 nm in a microplate reader (Bio-Rad, Richmond, CA).

Hoechst staining was performed by adding Hoechst dye to a final concentration of 0,5 mg/ml to each M96 well. Cells were counted under epifluorescence microscope (Leica Microsystems).

**Western blot analysis** - Endometrial adenocarcinoma cell lines were washed with cold PBS and lysed with lysis buffer (2% SDS, 125 mM Tris-HCL pH6.8). Protein concentrations were determined with the Protein assay Kit (Bio-Rad,). Equal amounts of proteins were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore, Bedford, MA). Non-specific binding was blocked by incubation with TBST (20 mM Tris-Hcl pH7.4, 150 mM NaCl, 0,1% Tween-20) plus 5% of non-fat milk. Membranes were incubated with the primary antibodies overnight at 4°C. Signal was detected with ECL Advance (Amersham-Pharmacia, Buckinghamshire, UK).

**Luciferase assays** - Endometrial carcinoma cell lines were plated in M24 multiwell plates and transfected using either Calcium phosphate or Lipofectamine 2000 following manufacturer’s instructions, with the reporter NFκB-LUC construct together with a plasmid encoding Beta-galactosidase. After 24 hours, cells were treated as indicated in each experiment and cells were lysed with 60 μl of luciferase lysis buffer (25 mM Glyciglycine pH7.8, 15 mM Mg2SO4, 1% Triton X-100, 5 mM EGTA) and rocked on ice for 15 min on ice. 30 μl of lysates were transferred to M96 multiwell plates and 30 μl of luciferase assay buffer was added to a final concentration of (25 mM Glyciglycine, 15 mM Glyciglycine).
KHPO₄ pH7.8, 15 mM Mg₂SO₄, 1% Triton X-100, 5 mM EGTA, 1mM DTT containing, 2mM ATP, 100μM Acetyl-coenzyme A and 100μM Luciferine). Luciferase was measured using a microplate luminometer. After luciferase measuring 60 μl of 2X β-galactosidase buffer (200 mM NaPO₄, 20 mM KCl, 2mM MgSO₄, 4 mg/ml ONPG) was added to each well and was measured on a microplate reader at 415 nm.

**Electrophoretic Motility Shift Assay (EMSA)**

After appropriate treatment, nuclear and cytoplasmic extracts were obtained from 2 x10⁶ IK and HEC cells using the NE-PER nuclear and cytoplasmic extraction kit (Pierce, Rockford, IL). EMSA was carried out using the Light-Shift Chemoluminiscent kit (Pierce, Rockford, IL), 5-10 μg of nuclear protein extracts were incubated with a 20 fmol of 5’ biotin-labelled NF-kB consensus oligonucleotide (MWG-Biotech, Ebersberg, Germany) for 20 min at room temperature. DNA complexes were electrophoresed on a 5% acrilamide gel, and transferred to a nylon membrane (Amersham Pharmacia Biotech, Uppsala, Sweden). Light signal was developed following manufacturers’ instructions. For supershift experiments, 2 μg of p65 antibody were added to the binding reaction and incubated for additional 45 minutes.

**RESULTS**

**Proteasome inhibitors induce cell death on endometrial adenocarcinoma cell lines.**

Bortezomib is currently used as chemotherapeutic agent in patients with relapsed multiple myeloma. It is under ongoing clinical trials for evaluation of efficacy in the treatment of some solid tumours. However, some tumoural cell types undergo apoptosis or cell growth arrest after proteasome inhibitor treatment whereas others are insensitive to them, or require cotreatment with other drugs or factors. To investigate whether bortezomib and other three proteasome inhibitors were able to induce cell death on endometrial adenocarcinoma cells; we treated four endometrial carcinoma (EC) cell lines, Ishikawa (IK), KLE, RL-95 (RL) and HEC-1-A (HEC) with bortezomib and three other different proteasome inhibitors (MG-132, Epoxomicin or ALLN). All proteasome inhibitors tested caused a dose-dependent decrease on cell viability as assessed by MTT (Fig 1A, 1B). The reduction on viability was accompanied by an increase of apoptotic nuclei as seen by Hoechst staining (Fig 1C) suggesting apoptotic cell death. Treatment with MG-132 resulted in processing of caspase-3 and caspase-9. (Fig 1D). Co-treatment of IK with bortezomib or MG-132 plus BAF, a broad specificity caspase inhibitor almost completely abolished cell death (Fig 1E). Altogether these data indicate that proteasome inhibitors are effective in inducing apoptotic cell death on endometrial carcinoma cell lines.

**Proteasome inhibitors induce phosphorylation and degradation of IkBα.**

Inhibition of proteasome prevents NF-kB activation and causes cell cycle arrest or cell death on many different types of cell. The antitumoural effects of bortezomib have been proposed to involve NF-kB inhibition as a main mechanism of action. To investigate whether this was the case in our cell lines, we assessed the levels of IkBα, after treatment with proteasome inhibitors. Time course treatment of IK, HEC or RL cells with 25 nM of bortezomib resulted in a marked increase on phosphorylation of serine 32, followed by reduction of IkBα protein levels over time (Fig 2A). To rule out the possibility that IkBα phosphorylation and reduction were non-specific effects of bortezomib in our particular cell lines, we treated IK cells with the other three proteasome inhibitors. Treatment of IK cells with MG-132 resulted in phosphorylation and reduction of IkBα, similar to that observed with Bortezomib (Fig 2B). Although each cell line showed different basal levels of IkBα, treatment with MG-132 resulted in a similar reduction on IkBα levels in all of them (Fig 2C). We also observed a significant reduction of IkBα with epoxomicin or ALLN (Fig 2D).

**Proteasome inhibitors induce NF-kB nuclear translocation, DNA binding, transcriptional activity, and phosphorylation of p65 on serine 536.**

The results described above stimulated us to investigate whether the reduction of IkBα resulted in an activation of NF-kB transcriptional activity. To address this point, we used different experimental approaches. We carried out a transcriptional activity assay of NF-kB by
luciferase reporter assay. Cells were transfected with NFκB-dependent luciferase reporter construct and treated with the indicated doses of proteasome inhibitors for 14-16 hours. IK cells treated with 10 or 25 nM bortezomib displayed a marked increase on the basal luciferase activity (Fig 3A). Such increase was also observed when IK cells were treated with MG-132, epoxomicin or ALLN. Consistent with this result, we also observed that treatment with MG-132 or bortezomib resulted in the formation of a DNA/NF-κB complex as assessed by EMSA (Fig 3B). This complex showed similar pattern of migration to that observed with TNF.

To provide a control of proteasome inhibitors action, we stimulated a melanoma cell line (M16) and the endometrial carcinoma cell lines RL-95 with TNF. TNF treatment triggers the canonical, proteasome-dependent, IκBα degradation and p65 NF-κB binding activity in both cell lines. As expected, both bortezomib and MG-132, reduce NF-κB binding to DNA in M16 cells. Similar results are obtained in RL cells with MG-132 (Fig 3C). A supershift analysis using a p65 antibody caused a marked reduction on the NF-κB complex and the appearance of a supershifted band in both MG and TNF stimulated cells (Fig 3B). Accordingly, western blot analysis on nuclear and cytoplasmic extracts revealed that treatment with either MG-132 or bortezomib induced p65 nuclear translocation on IK cells. As control we treated HEC cells with TNF, which is known to induce p65 nuclear translocation (Fig 3C). Finally, we also analyzed phosphorylation of p65 subunit of NF-κB on serine 536, which has been associated with increased transactivation potential. Treatment with bortezomib or other proteasome inhibitors resulted in increased phosphorylation on serine 536 of p65 in all the cell lines that were tested (Fig 3D)

**SR-IκBα- blocks proteasome inhibitor-induced NF-κB activity.**

To ascertain whether IκBα degradation was dependent on serine phosphorylation, we transfected IK and HEC cells with a construct encoding a form of IκBα carrying serine-to-alanine mutations at residues 32 and 36, named SR-IκBα. These mutations prevent IκBα phosphorylation and its subsequent proteasome-mediated degradation, thereby preventing release and nuclear translocation of NF-κB (Rodriguez et al., 1996). Expression of SR-IκBα caused a marked reduction on the activation of NF-κB transcriptional activity by either bortezomib or MG-132 in HEC and IK cells (Fig 4). This data supports the hypothesis that proteasome inhibitors require phosphorylation and degradation of IκBα.

**NF-κB activity induced by proteasome inhibitors requires functional IκKα and IκKβ.**

The major upstream kinases involved in NF-κB activation are IκKα and IκKβ. We investigated whether these kinases were involved in activation of NF-κB by proteasome inhibitors. Treatment of IK, HEC or RL cells with bortezomib caused increased phosphorylation of IκKα/β, as assessed by western blot analysis of lysates with an antibody that specifically recognizes the phosphorylated forms of both proteins (Fig 5A).

To determine the requirement of either or both IκKα or IκKβ subunits, we co-transfected IK or HEC cells with NF-κB-luciferase reporter construct plus dominant negative forms of either IκKα (DN−IκKα) or IκKβ (DN-IκKβ). Both DN-IκKα and DN-IκKβ blocked NF-κB transcriptional activity on IK cells treated with 25 nM of bortezomib (Fig 5B, left) and in IK and HEC cells treated with MG132 (Fig 5B, right).

To further demonstrate the role of IκKα or IκKβ subunits, we designed lentiviral shRNAs targeting each subunit, to knock-down endogenous expression. We designed shRNAs targeting two sequences for each kinase. Infection of IK cells with lentiviruses carrying shRNAs to either IκKα or IκKβ subunits revealed that each shRNA selectively knocked-down the expression of the corresponding protein (Fig 5C). These two shRNAs were selected for subsequent experiments. We infected IK cells with the functional IκKα or IκKβ shRNAs for 3 days to allow protein knock-down; and cells were subsequently treated with bortezomib. As shown in figure 5D, both IκKα shRNA, and IκKβ shRNA blocked NF-κB activity with similar efficiency to the dominant negative forms. Our data suggest that phosphorylation and expression of both IκKα and IκKβ subunits are required for proteasome inhibitor-induced NF-κB activity. Neither IκKα nor IκKβ knock down...
increased the cell viability in proteasome inhibitor treated cells (data not shown).

**IKKα and IKKβ are required for p65 phosphorylation but not for IkBα degradation after proteasome inhibitor treatment**

To determine whether IKK was responsible for proteasome inhibitor-induced p65 phosphorylation, HEC cells were infected for three days with lentiviruses carrying either IKKα or IKKβ shRNAs. HEC cells were treated for 6 or 24 hours with 25 nM of bortezomib and cell lysates were analysed by western blot (Fig 6A). As shown in figure 6, both IKKα and IKKβ shRNA strongly inhibited p65 phosphorylation. The cells transfected with IKKβ shRNA showed a reduction of phosphorylated IkBα, but also a decrease on total IkBα protein. However, the molecular weight shift on migration of IkBα and degradation of IkBα by bortezomib remained unaffected (Fig 6A). Our findings suggest that p65 phosphorylation is mediated by either IKKα or IKKβ, but also show that IkBα degradation is independent of IKKs. Similar results were obtained with IK cells (data not shown).

These results suggest that proteasome inhibitors increase NF-κB activity, by involving the p65 subunit of NF-κB. To determine if transcriptional activity of NF-κB was really dependent of p65 subunit, we co-transfected p65 siRNA or c-Rel siRNA with the NF-κB reporter construct, and cells were exposed to bortezomib. Transfection of p65 siRNA resulted in a significant decrease of NF-κB transcriptional activity in both IK and HEC cells (Fig 6B). Such result was not observed when c-Rel siRNA was transfected.

**Proteasome inhibitors induce cell death and activate NF-κB in primary culture explants from endometrial adenocarcinoma samples.**

To further demonstrate the effect of proteasome inhibitors on endometrial carcinoma, we assessed the effects of such inhibitors on primary explants obtained from human endometrial carcinomas. After enzymatic digestion, glandular-like structures were plated on 24-well dishes and the epithelial origin of the samples was assessed by immunofluorescence to a wide spectrum cytokeratin, cytokeratin-7 and β-catenin, two proteins that are only expressed on endometrial cells of epithelial origin, but not on stromal cells (Fig 7A). To ascertain whether proteasome inhibitors induced apoptosis on primary explants as well as on cell lines, we treated endometrial explant cultures with 0.5 μM MG-132 or 25 nM bortezomib and we assessed caspase-3 activation by immunofluorescence with an antibody that recognizes its active form (Fig 7B). Both MG-132 and bortezomib increased the number of cytokeratin positive cells displaying active caspase-3. These results suggest that bortezomib and MG-132 induce apoptotic cell death on primary endometrial carcinoma explants. To determine whether MG-132 and bortezomib also were able to activate NF-κB, we analysed IkBα phosphorylation and degradation after treatment with these two inhibitors at doses that induced IkBα degradation and phosphorylation on endometrial carcinoma cell lines. As shown in figure 7C, both MG-132 and bortezomib induced phosphorylation and degradation of p65 at serine 536 in a similar way to that observed in all cell lines tested. Altogether, these results demonstrate that proteasome-inhibitors are able to activate NF-κB signalling in primary endometrial carcinoma explants.

**DISCUSSION**

Bortezomib and other proteasome inhibitors trigger cell growth arrest or apoptosis on several tumours (2). Bortezomib is a modified dipeptidyl boronic acid that creates compounds that form covalent complexes with the proteasome. It is used as a chemotherapeutic agent for treatment of relapsed and refractory multiple myeloma (3, 4, 5, 6, 7). It is also a promising anti-cancer agent for treatment of solid tumours. In many different types of tumour cells, proteasome inhibition cause cell death by blocking NF-κB activity. In this article, we demonstrate for the first time, that bortezomib and other three proteasome inhibitors (MG-132, Epoxomicin and ALLN) may induce apoptosis in endometrial cancer cell lines and primary culture explants from endometrial carcinomas. Cell death was accompanied by activation of caspases and apoptotic nuclear morphology. However, in contrast to that observed on other cancer cells, this cell death is not related with NF-κB blockage. In endometrial cancer cells, bortezomib and other proteasome inhibitors increase NF-κB activity rather than its inhibition. NF-κB is a family of transcription
factors, involved in the regulation of genes encoding cytokines, cytokine receptors, and cell adhesion molecules, that drive immune and inflammatory responses (14, 15, 16). However, NF-κB is also related to carcinogenesis, by regulating genes involved in apoptosis, the cell cycle, differentiation, invasion and cell migration (16, 19). Therefore, inhibition of NF-kB is a promising target for treatment of cancer. Because of their ability to block IkBα degradation, proteasome inhibitors have been widely used as inhibitors of NF-kB. In fact, inhibition of NF-kB activity has been reported as the main mediator of cytotoxic effects of bortezomib. One of the main goals of this study was to investigate the effect of proteasome inhibition on NF-kB in endometrial carcinoma. Treatment of endometrial carcinoma cell lines with bortezomib did not stabilize or inhibit IkBα, but induced phosphorylation and reduction of IkBα levels. Similar results were obtained when the endometrial adenocarcinoma cell lines were exposed to the other three proteasome inhibitors (MG-132, epoxomicin or ALLN) which inhibit the proteasome by other chemical mechanisms. Although these results seem to contradict the well-established mechanism of IkBα degradation by proteasome inhibition, it is worth mentioning that some recent evidences suggest that proteasome inhibitors may activate NF-kB in some cancer cell lines. This was the case of the colon adenocarcinoma cell line HT-29 after treatment with MG-132 (25). In agreement with this report, we have found IkBα degradation, IKK phosphorylation and increased transcriptional activity of NF-kB, as a result of exposure to the four different proteasome inhibitors.

Phosphorylation and degradation of IkBα is followed by increase of NF-kB activity. We found that proteasome inhibitors induced the formation of a DNA/NF-kB complex displaying similar migration properties than the observed after stimulation with TNF. TNF is a well-known activator of the canonical NF-kB pathway which results in nuclear translocation of p65 subunits. Therefore, this result suggests that p65 may be involved in NF-kB activity induced by such inhibitors. Accordingly, we have found supershift of the NF-kB complex using p65 antibody, nuclear translocation of p65 and, more importantly, p65 siRNA reduced the transcriptional activity induced by proteasome inhibitors. Moreover, we have found that all proteasome inhibitors tested, including bortezomib increase p65 phosphorylation on serine 536. Recent evidences suggest that phosphorylation on this serine is critical for p65 transcriptional activity after different stimuli such as lymphotoxin beta (21), TNF (22), lipopolysaccharide (23) or IL-1 (24) stimulation. Moreover, a recent report also shows that MG-132 can also enhance serine 536 phosphorylation on HeLa cells (24). Therefore, phosphorylation of p65 is consistent with the increased transcriptional activity observed after treatment with proteasome inhibitors.

Recent reports describe IKK-independent mechanisms that can also activate NF-kB upon certain stimuli. Recently, it has been shown that DNA damaging agents such as topoisomerase inhibitors require IKK complex to induce NF-kB (26, 27) whereas others suggest that NF-kB activation can take place independently of IKKs (28). To address the involvement IKKα or IKKβ in proteasome inhibitor effects on endometrial carcinoma cell lines, we transfected IK or HEC cells with the dominant negative forms of IKKα or IKKβ or with lentiviral transduced shRNAs, and we assessed NF-kB activity after bortezomib or MG-132 exposure. These experiments clearly demonstrated that, in contrast to topoisomerase inhibitors, NF-kB activation by proteasome inhibitors in endometrial carcinoma cell lines requires functional IKKs.

Reporter experiments performed with the IkBα suppressor indicate that IkBα phosphorylation and degradation are required for proteasome inhibitor-induced NF-kB activity. However, shRNA to either IKKα or IKKβ did not block IkBα degradation by proteasome inhibitors. IKKβ shRNA diminished IkBα phosphorylation but such reduction can be explained by the reduction of total IkBα protein levels. Although both IKKα and IKKβ shRNAs did not block IkBα degradation, they markedly reduced NF-kB activity. These results suggest that NF-kB activation by proteasome inhibitors is further
regulated downstream of IkBα degradation. Increasing evidence supports that NF-kB phosphorylation may be an important mechanism of NF-kB regulation (20). As mentioned above, we found that all proteasome inhibitors phosphorylated p65 at serine 536. Such phosphorylation can be achieved by different upstream kinases. Phosphorylation on serine 536 is triggered by IKKα after TNFα (29) and lymphotoxin β (30) or by IKKβ after T cell co-stimulation (Mattioli et al., 2004) or lipopolysaccharide (23) but also by TBK1, Akt or IKKe (20, 24). In this study, we have found that shRNA inhibition of either IKKα and IKKβ blocked p65 at serine 536 and NF-kB transcriptional activity, suggesting a major role for p65 subunit in proteasome inhibitor-induced activation. Consistent with this hypothesis, we have found p65 nuclear translocation and reduced NF-kB transcriptional activity in p65 siRNA transfected cells, indicating a main role for p65 in NF-kB activation. As discussed above, IkBα is degraded even after IKK knock-down by shRNA, but both serine phosphorylation of p65 and NF-kB transcriptional activity is inhibited. Altogether the mechanism by which proteasome inhibitors induce NF-kB activity seems to be tightly regulated. Such activation requires phosphorylation and degradation of IkBα but also an IKK-mediated phosphorylation of p65. Despite the activation of NF-kB, proteasome inhibitors are able to induce cytotoxic effects on endometrial carcinoma cell lines. Although it may be surprising, these features are also observed after treatment of cancer cells with other stress signals such as ionizing radiation, hypoxia or ultraviolet light and chemotherapeutic drugs such as vincristine, vinblastine, etoposide, adriamycin, cisplatin, daunorubicin, etc (31). For example, topoisomerase inhibitors such as camptothecin or doxorubicin activate NF-kB but induce cell death (32, 33, 34). However, the NF-kB activation observed after proteasome inhibitors treatment has a particular relevance because they are widely used as pharmacological blockers of IkBα degradation and subsequent NF-kB inhibition. In fact, as a control of correct proteasome inhibition, we have found that such inhibitors are effective to block NF-kB activation by TNF in both a melanoma cell line and the RL-95 endometrial cell lines, indicating that such drugs are inhibiting proteasome and, as a consequence, the NF-kB activation by the canonical pathway. These results indicate that proteasome inhibitors are effective to block NF-kB by TNF, but long treatment (hours) lead to induction of NF-kB by themselves.

NF-kB inhibition has been proposed as the main mechanism by which proteasome inhibitors are used as anticancer-drugs. Although in some cell types NF-kB activation trigger cell death (Farhana et al, Jin et al.,), NF-kB does not mediates cytotoxic effects of proteasome inhibition. To test this hypothesis endometrial cancer cell lines were infected with lentiviruses carrying shRNA either to IKKα or IKKβ and treated with cytotoxic doses of bortezomib or MG-132 and we assessed cell viability by MTT assay. Neither IKKα nor IKKβ shRNA increased cell viability after treatment with such inhibitors (supplementary Figure 1). Rather, we observed a slightly decrease. We observed that IKKα or IKKβ knock-down reduced basal proliferation and viability of endometrial cancer cells (data not shown), which is consistent with an anti-apoptotic role of NF-kB in endometrial carcinoma cell lines. Because of the use of bortezomib on treatment of multiple myeloma and the promising effects on some solid tumours we decided to test the ability of this inhibitor to induce apoptosis on primary explants from tumour samples from patients with endometrial carcinoma. Both MG-132 and bortezomib, induced activation of caspase-3, suggesting that both inhibitors triggered apoptotic cell death. Similar to that observed on endometrial cancer cell lines, bortezomib and MG-132 caused IkBα phosphorylation and degradation which suggests that such inhibitors also activate NF-kB on primary endometrial carcinoma explants. During the last years, other molecular mechanisms, not related to NF-kB have been involved in proteasome inhibitor cell death. Among them, it has been reported an involvement of the endoplasmic reticulum stress (ER stress) (37, 38, 39) proteins or increases in expression of death receptors (40). Other recent
findings suggest that proteasome inhibitors may induce apoptosis by up-regulating BH3 only family members such as NOXA (41, 42, 43), Bik (44, 45) or PUMA (46). To determine whether proteasome inhibitors produce ER stress in endometrial cancer cells we have performed RT-PCR analysis of GADD153 and heme-oxygenase, two stress genes that have been previously demonstrated to increase after ER stress after bortezomib treatment (37, 38, 39). We have alsoanalysed NOXA, BIK and Puma expression after treatment with bortezomib. (Supplementary Figure 2). In agreement with previous reports, we have found up-regulation of both GADD153 and heme-oxygenase, suggesting that ER stress may be involved in apoptosis triggered by proteasome inhibitors. We have not found significant changes in either Puma or Noxa, but a slightly and transient increase in Bik levels. Future experiments will determine whether BH3 only proteins such as Bik and ER stress regulate apoptosis induced by proteasome in endometrial carcinomas.

In summary, we have demonstrated that bortezomib and other three proteasome inhibitors induce cell death in four endometrial carcinoma cell lines as well as in primary culture explants obtained from endometrial carcinoma samples. However, all proteasome inhibitors tested activated NF-kB, a signalling pathway strongly associated in oncogenesis in many types of cancer. Our findings may have important biochemical and clinical relevance. First, because proteasome inhibitors are widely used as pharmacological inhibitors of NF-kB on the basis of their ability to block IκBα degradation but, as we show here for endometrial carcinomas, they can rather lead to activation of NF-kB. Therefore, the general use of such inhibitors as NF-kB blockers has to be carefully analysed for particular cell types. Second, if proteasome inhibitors may activate of NF-kB, administration of these drugs may increase the expression of genes involved with proliferation, apoptosis resistance or angiogenesis. The activation of these genes could explain the development of adverse effects in patients under treatment with proteasome inhibitors. Additional studies should be performed to demonstrate that the NF-kB activation shown in this report in endometrial carcinoma cell lines, and in a previous study on colonic adenocarcinoma cell lines, is also taking place in other types of tumour cells.

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FOOTNOTES

Key words: Endometrial carcinoma, proteasome inhibitors, NF-kB, molecular pathology, Apoptosis.

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This work is dedicated to Eric and Laura.

FIGURE LEGENDS

Fig1. Proteasome Inhibitors trigger apoptosis on endometrial carcinoma cell lines. A, Ishikawa (IK), KLE, RL-95 (RL) AND HEC-1-A (HEC) cells were treated with increasing doses of Bortezomib.
for 24 or 48 hours and cell viability was assessed by MTT. Results are expressed as percent of the control values. B, Ishikawa (IK), KLE, RL-95 (RL) AND HEC-1-A (HEC) cells were treated for 48 hours with the indicated doses of MG-132, Epoxomicin or ALLN and cell viability was assessed by MTT. Results are expressed as percent of the control values. C, Micrographs of IK cells left untreated (UN) or treated for 36 hours with 25 nM of Bortezomib, 1 μM MG-132, 50 nM Epoxomicin or 50 μM ALLN and stained with Hoechst dye. D, Quantification of apoptotic nuclei after 36 hour treatment with 25nM bortezomib or 0.5 μM of MG-132 (left graph). Quantification of Hoechst stained apoptotic nuclei of IK cells treated with 25nM Bortezomib alone (Bort), 100 μM of BAF or the combination (Bort+BAF) (right graph). E, IK, RL, KLE or HEC cells were treated with 0.5 μMMG-132 for 24 hours and lysates were subjected to western blot with antibodies to active caspase-3, or antibody to caspase-9.

**Fig2. Proteasome inhibitors induce phosphorylation and degradation of IkBα.** A, Ishikawa (IK), HEC-1-A (HEC), RL-95 (RL) cells were treated for the indicated times with 25 nM Bortezomib and cell lysates were subjected to western immunoblot (IB) with antibodies to phosphorylated IkBα, IkBα or tubulin. B, IK cells were treated for 6 or 24 hours with 0.5mM of MG-132 and cell lysates were analyzed by western blot with antibodies to phosphorylated IkBα, IkBα or tubulin. C, IK, RL, KLE or HEC cells were treated with 0.5 μM MG-132 or left untreated for 24 hours and cell lysates were subjected to western blot with antibodies to IkBα (top panel) or tubulin (bottom panel). D, RL and HEC cells were treated for 24 hours with 0.5 μM of MG-132 (MG), 50 nM of Epoxomicin (EPO) or 50 μM ALLN (ALL) and cell lysates were subjected to western blot antibodies to IkBα (top panel) or tubulin (bottom panel).

**Fig3. Proteasome inhibitors increase NF-kB nuclear translocation, DNA binding, transcriptional activity and phosphorylate p65 subunit at serine 536.** A, IK cells were transfected with NFkB-luciferase reporter construct and treated with 10 and 25 nM of bortezomib (left graph), or were treated with 0.5 μM of MG-132 (MG), 50 nM of Epoxomicin (EPO) or 50 μM ALLN (ALL) (right graph) for 16 hours and cell lysates were assayed for luciferase activity. Results are expressed as relative luciferase units. B, IK cells were treated with bortezomib MG-132(MG) for 16 hours and HEC cells were treated with MG-132 for 16 hours or with TNF for 30 minutes and nuclear lysates were incubated with 20 fmol of labelled probe. A 200 hundred molar excess of unlabelled competitor was included in the indicated lanes to demonstrate the specificity (upper panel). For supershift experiments HEC cells were treated with MG-132 or TNF and nuclear lysates were incubated with 200 molar excess of the unlabelled competitor an antibody to p65 as indicated (lower panel) C, RL endometrial carcinoma cells and the melanoma cell line M16 were treated for 30 min in presence or absence of proteasome inhibitors. and nuclear lysates were incubated with 20 fmol of biotin labelled probe. Arrows indicate the proteasome inhibitor-induced NF-kB complex and the free probe. A 200 hundred molar excess of unlabelled competitor was included in the indicated lanes to demonstrate the specificity. D, HEC cells were treated with 50ng/ml TNF for 30 minutes or with 0.5μM MG-132 for 16 hours. Cytoplasmic and nuclear cell lysates were analyzed by western blot with antibodies to p65 NF-kB subunit. To determine the purity of cytoplasmic and nuclear lysates, membranes were reprobed with anti-ERK antibody (marker of cytoplasmic fraction) or with histone H1 antibody (nuclear fraction). E, IK cells were treated for 16 hours with either 25 nM bortezomib or 0.5 mM MG-132 and nuclear and cytoplasmic lysates were analyzed with p65 antibodies. Membranes were reprobed with antibodies to ERK and Histone H1. F, IK cells were treated for 0, 6, 12, or 24 hours with 25 nM bortezomib (left panel) and IK, RL, HEC and KLE cells were treated for 16 h with 0.5 μM of MG-132, 50 nM of Epoxomicin or 50 μM ALLN (right panel) and cell lysates were subjected to western blot with antibodies that specifically recognize serine 536 only when it is phosphorylated. Membranes were also incubated with antibodies to total p65 to ensure equal amounts of protein.

**Fig4. IkBα superrepressor blocks bortezomib and MG132-induced NF-kB transcriptional activity.** IK or HEC cells were co-transfected with NFkB-luciferase reporter construct and either the
empty vector (PCDNA3), or a IkBα superrepressor which can not be phosphorylated on serines 32 and 36 (SR-IkBα). After 48h, cells were stimulated with Bortezomib 25 nM or MG132 0.5 μM and luciferase activity was assayed 16 h later. Results are expressed in Relative Luciferase Units.

**Fig5.** **IKKα and IKKβ are required for bortezomib and MG132- induced NF-kB activity.** A, western blot showing IK, HEC and RL cell lines stimulated for 6, 12 or 24 hours with 25nM bortezomib and incubated with an antibody that recognizes IKKα/IKKβ phosphorylated or with tubulin to ensure equal protein loading (bottom panel). B, Bar charts showing NF-kB transcriptional inhibiton by either IKKα or IKKβ dominant negative forms on IK cells treated with bortezomib (left chart) or in IK and HEC cells treated with MG-132 (right charts). C, Western blot analysis of IKKα and IKKβ expression on IK cells infected for 3 days with lentiviruses carrying two different shRNAs targeting IKKα or IKKβ. D, IK cells infected with lentiviruses carrying functional shRNAs to IKKα or IKKβ for 3 days to allow protein knock-down and then transfected with NFkB-luciferase reporter construct. 24 hours later they were treated with 25 nM bortezomib and luciferase activity was assayed.

**Fig6.** **Downregulation of IKKα and IKKβ inhibit p65 phosphorylation and activity but not IkBα degradation.** A, HEC cells were infected with lentiviruses carrying functional shRNAs to IKKα or IKKβ to allow protein knock-down and then stimulated for 6 or 24 hours with 25 nM of bortezomib, and lysates were analysed by western blot. Blots were incubated with antibodies to IkBα, phospho- IkBα, phospho-s536-p65. Membranes were also blotted with antibodies to IKKα or IKKβ to ensure down-regulation of target proteins by shRNAs. B, Bar chart showing HEC co-transfected with NFkB-luciferase reporter construct and either siRNAs targeting p65 (RelA) or c-Rel subunits of NF-kB. After 48h to allow siRNA to down-regulate protein expression, cells were stimulated with Bortezomib 25 nM and luciferase activity was assayed 16 h later. Results are expressed in Relative Luciferase Units.

**Fig7.** **Proteasome inhibitors induce cell death and activate NF-kB on primary endometrial carcinoma explants.** A, endometrial carcinoma explants after 3 days in culture stained with antibodies against pan-cytokeratin, cytokeratin-7 or β-catenin. Nuclei were visualized by Hoechst staining. B, endometrial carcinoma explants were treated with bortezomib 25 nM, MG-132 0.5 mM or left untreated for 24 hours, fixed and stained with anti-active caspase-3 antibody (green), citokeratin-7 (red) and hoechst dye to visualize nuclei (blue). C, Two different endometrial carcinoma explants were treated with 25 nM bortezomib or 0.5 μM MG-132 and cell lysates were analyzed by western blot by IkBα, phosphorylate IkBα, phosphorylated p65 or tubulin.
Figure 1

A 24h

Viability (% of CONTROL)

B Bortezomib (nM)

Viability (% of CONTROL)

C

D

% APOPTOTIC NUCLEI

E

MG-132
0.5 μM

Pro-Caspase-9

CF

Active Caspase-3

IK HEC RL KLE

MG-132
0.5 μM

Pro-Caspase-9

CF

Active Caspase-3

IK HEC RL KLE

MG-132
0.5 μM

Pro-Caspase-9

CF

Active Caspase-3

IK HEC RL KLE

MG-132
0.5 μM

Pro-Caspase-9

CF

Active Caspase-3

IK HEC RL KLE

MG-132
0.5 μM

Pro-Caspase-9

CF

Active Caspase-3

IK HEC RL KLE

MG-132
0.5 μM

Pro-Caspase-9

CF

Active Caspase-3

IK HEC RL KLE

MG-132
0.5 μM

Pro-Caspase-9

CF

Active Caspase-3
**Figure 2**

A

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<th>RL</th>
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**IB: IκBα**

**IB: p-IκBα**

**IB: Tubulin**

B

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**IB: IκBα**

**IB: p-IκBα**

**IB: Tubulin**

C

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**IB: IκBα**

**IB: Tubulin**

D

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<th>UN</th>
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**IB: IκBα**

**IB: Tubulin**

**IB: IκBα**

**IB: Tubulin**
Figure 4

IK

- UN
- Bortezomib

PECNA3               IkB\textsubscript{\(\alpha\)}-SR

HEC

- UN
- Bortezomib

PECNA3               IkB\textsubscript{\(\alpha\)}-SR

PCDNA3                   IkB\textsubscript{\(\alpha\)}-SR

MG132

- UN
- MG132

PECNA3               IkB\textsubscript{\(\alpha\)}-SR

PCDNA3               IkB\textsubscript{\(\alpha\)}-SR
Figure 5

A

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</table>

IB: P-IKKα/β
IB: Tubulin

B

Relative Luciferase Units

Vector          IKKαDN          IKKβDN

IK

Relative Luciferase Units

Vector          IKKαDN          IKKβDN

HEC

Relative Luciferase Units

Vector          IKKαDN          IKKβDN

C

shRNA  FSPsi  IKKα  IKKα  IKKβ  IKKβ

IB: IKKα
IB: IKKβ
IB: Tubulin

D

Relative Luciferase Units

Vector          IKKα shRNA          IKKβ shRNA

Bortezomib 25 nM

UN          Bortezomib

Vector          IKKαDN          IKKβDN

IK

Downloaded from http://www.jbc.org/ on August 17, 2017
Figure 6

A

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<th>IKKβ shRNA</th>
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<td>24</td>
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IB: IkBα

IB: P- IkBα

IB: P- p65

IB: p65

IB: IKKα

IB: IKKβ

B

IK

Relative Luciferase Units

HEC

Relative Luciferase Units
Figure 7

A

Pan-Citokeratin
Citokeratin-7
β-catenin

B

UN
MG-132
bortezomib

C

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<th>Bortezomib</th>
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IB: IkBα

IB: P-IkBα

IB: P-p65

IB: p65

EC1

EC2
Proteasome inhibitors induce death but activate NF-κB on endometrial carcinoma cell lines and primary culture explants
Xavier Dolcet, David Llobet, Mario Encinas, Judit Pallares, Albert Cabero, Joan Antoni Schoenenberger, Joan X. Comella and Xavier Matias-Guiu

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