Introduction

Dysregulation of cell cycle and apoptosis is one of the major contributing factors in malignant transformation of normal cells. Cyclin-dependent kinases (CDK) are critical for cell growth because they regulate initiation, progression, and completion of cell cycle (1). Indeed, several lines of evidence suggest that transformed and normal cells differ in their requirement for cyclin/CDK complexes and in the amount of CDK inhibitors that tightly regulate these complexes. Moreover, uncontrolled CDK activities could generate cancer development (1), so it seems possible to develop novel antineoplastic agents by targeting CDK.

The Cip/Kip family of mammalian cyclin-dependent kinase inhibitors (CKI) is considered as a requirement for a rapid tumor cell growth considered as a requirement for a rapid tumor cell growth. In addition, p21 has been found to be involved in the regulation of the subcellular localization, through transcriptional and translational control mechanisms, or via modulation of protein stability (3). In fact, an elevation of proteasomal activity often observed in human cancers was suggested the ubiquitin–proteasome pathway as a regulator (2, 11, 12). The ubiquitin–proteasome system is critical for the regulated degradation of short-lived proteins, such as transcription factors, cell-cycle inhibitors, and signaling molecules (13). In fact, an elevation of proteasomal activity often observed in human cancers was considered as a requirement for a rapid tumor cell growth.

Abstract

Proteasome inhibitors such as bortezomib constitute novel therapeutic agents that are currently in clinical use and in clinical trials. In some neoplasms, cyclin-dependent kinase inhibitors (CKI) such as \( p21^{WAF1} \) have been proposed as key targets of proteasome inhibitors. \( p21^{WAF1} \) expression can be modulated by p53, a tumor suppressor, and especially in breast cancer cells, by estrogen receptor alpha (ER\( \alpha \)), which is highly relevant to cancer growth. We investigated the effects of bortezomib using a panel of six cancer cell lines with variable status of ER\( \alpha \) or p53 and found that bortezomib inhibited the growth of all cell lines in the same concentration range irrespective of the ER\( \alpha \) expression or the mutational status of p53. Bortezomib treatment significantly enhanced \( p21^{WAF1} \) protein levels in all cell lines but with different mechanisms according to ER\( \alpha \) status. In ER\( \alpha \)-positive cells, bortezomib treatment caused a strong increase in \( p21^{WAF1} \) mRNA, whereas in ER\( \alpha \)-negative cells it predominantly enhanced \( p21^{WAF1} \) protein levels suggesting a posttranslational mechanism of \( p21^{WAF1} \) regulation in the ER\( \alpha \)-negative cells. Moreover, the antiproliferative activity of bortezomib was prevented by ER\( \alpha \) silencing or \( p21^{WAF1} \) knockdown in ER\( \alpha \)-positive cells. Collectively, our results highlight the potential roles of ER\( \alpha \) and \( p21^{WAF1} \) in growth inhibition of cancer cells mediated by proteasome inhibitors, such as bortezomib.

Mol Cancer Res; 10(11); 1–9. ©2012 AACR.
degradation of target proteins such as p21, p27KIP1, p53, and Bax, which inhibit cell growth, protect genetic integrity, or induce apoptosis (17–19). Thus, proteasome inhibitors could be of potential interest in cancer treatment because they are expected to reverse these effects and to restore apoptosis. Moreover, the observation that cancer cells are more sensitive than normal cells to the effects of proteasome inhibition (20), also argues for the potential of proteasome inhibitors in cancer therapy.

A class of potent and specific compounds of threonine protease was designed with boronic acids to inhibit the proteasome (21). Bortezomib, a dipeptidyl boronic acid, is the first proteasome inhibitor that has entered in clinical use. Bortezomib selectively and reversibly inhibits the 26S chymotryptic site in the ubiquitin–proteasome system. Several studies showed that the treatment with such proteasome inhibitor mainly resulted in decreased proliferation and induction of apoptosis (22, 23). At present, the treatment with bortezomib has already been approved by the Food and Drug Administration in relapsed multiple myeloma. Another dipeptidyl proteasome inhibitor, CEP1612, was found to induce apoptosis in some breast cancer cells and this is accompanied by an accumulation of the p21, with the possible appearance of its ubiquitinated forms (24). Effects of proteasome inhibitors on cell cycle were also found in prostate and colorectal cancer cell lines, and were in part attributed to the p21 (25–29). Indeed, inhibiting the degradation of key cell-cycle proteins causes a disparity in the proliferative signaling and can eventually lead to cell-cycle arrest. However, the precise mechanisms by which proteasome inhibition induces growth arrest in cancer cells remain to be elucidated. Here, we investigated the effects of bortezomib in human cancer cells particularly in context to the ERα status and p21 regulation and collectively, our results reveal a unique ERα/p21 axis that seems to mediate a major part of the antiproliferative activity of bortezomib in ERα-positive breast cancer cells.

Materials and Methods

Cell culture
Cell lines selected in this study are ERα-positive MCF7, T47D, and ZR75.1, and ERα-negative MDA-MB-435, MDA-MB-231, and HCT116 cells. All cell lines were obtained from American Type Culture Collection, cultured in recommended media and checked periodically for micoplasma contamination by MycoProbe (R&D Systems). Among these cell lines, independently to ERα status, 3 cell lines expressed the wild-type (wt) p53 tumor suppressor (MCF7, ZR75.1, HCT116), whereas the other 3 displayed a mutated form of p53. The HCT116 cell line is a colon cancer cell line that was chosen because of its apparently intact DNA damage-dependent and spindle-dependent checkpoint (p53+/−; 30). MDA-MB-435 cells, which were previously considered as a model for metastatic breast cancer, were officially redefined as melanoma cells (31). Therefore, by including these 2 cell lines, the project presents symmetric groups for ERα and p53 status.

Human breast cancer cell lines MDA-MB-231, T47D, ZR75.1, and MCF7 were maintained in monolayer cultures in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% FBS (Invitrogen) and 50 μg gentamycin (Invitrogen) at 37°C under 5% CO2. The same culture conditions were applied to the human melanoma cell line, MDA-MB-435. Wild-type HCT116 human colon carcinoma cells, which express normal p53 and p21, and a derivative in which both p21 alleles have been deleted through homologous recombination (HCT116 p21−/−) were kindly provided by Dr. Bert Vogelstein (Johns Hopkins University, Baltimore, MD, USA) and were incubated in McCoy’s 5A medium supplemented with 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin, at 37°C under 5% CO2. MCF7 short hairpin RNA (shRNA) clones were grown in DMEM supplemented with 10% FBS, 100 U/mL penicillin, 100 μg/mL streptomycin, and 2 μg/mL puromycin to keep the knockdown stable in the infected cells.

In all experiments with ERα-positive cells, steroids were withdrawn by culturing cells for 5 days in phenol red-free DMEM supplemented with 10% dextran–charcoal–treated FBS (DCC). During the experiments, cells were treated with 1 nmol/L E2 and the indicated concentrations of bortezomib.

The bortezomib (or PS-341 or Velcade) was kindly given by Millennium Pharmaceuticals and stored at 4°C in aqueous solution. Treatments were always equilibrated for mannitol concentration.

Colony formation assay

These assays were carried out according to Prud’homme colleagues (32). Briefly, 10,000 cells were included in 0.3% agar (Sigma-Aldrich Chemie) and layered on preformed 0.7% agar layer in a 12-well plate. After plating, the cells were incubated in 2-day–conditioned medium prepared from the same cells and simultaneously treated with either bortezomib (25 and 50 nmol/L) or vehicle (mannitol). After 12 to 14 days, colonies were counted using a low magnification (×4) and photographed.

Immunoblotting

For protein expression analysis of whole cell lysates, cells were harvested after 3 freeze–thaw cycles in buffer containing 50 mmol/L HEPES, 150 mmol/L NaCl, 0.1% NP40, 10% glycerol, 2.5 mmol/L EGTA, and protease inhibitors (Complete, Roche Diagnostics), and centrifuged at 10,000 × g for 15 minutes at 4°C. Proteins were quantified by Bradford assay and a constant amount was analyzed by immunoblotting, as previously described (5). Afterward, samples were mixed with equal amounts of sample buffer (125 mmol/L Tris/HCl, 288 mmol/L β-mercaptoethanol, 20% glycerol, 2% SDS, 10 μg/mL bromphenol blue), boiled for 3 minutes, and separated by SDS-PAGE using 12% gels. Immunostaining was done using mouse monoclonal p21WAF1 (Oncogene Research Products) and rabbit polyclonal ERα antibody (Santa Cruz Biotechnology). Polyclonal rabbit antibody against actin was purchased from Sigma-Aldrich Chemie (A2066). Immunoblotting was conducted...
with goat antirabbit or sheep antimouse secondary antibodies, purchased from Amersham. The bound immunoglobulins were visualized using the ECL detection system (Amersham). Densitometries of immunoblotting films were analyzed with the PC-Bas 2.0 software (Fuji).

Quantitative real-time PCR
Total RNA from MCF7 cells was isolated using the Rneasy Mini Kit from Qiagen. For analyzing the transcription of p21, 1 μg of total RNA was reverse transcribed in 20 μL of reaction mix by using the "SuperScript II First-Strand Synthesis System" (Invitrogen).
Quantitative real-time PCR (qRT-PCR) was carried out in Roche Lightcycler using DNA double-strand-specific SYBR green I dye for detection (Roche). P21 primer sequences were 5'-CTG GTG ACT CTC AGG GTC GAA-3' (sense primer) and 5'-GGA TTA GGG CCT CTT GGA-3' (antisense primer). The relative mRNA levels in cells were calculated by using the ΔΔCt method with endogenous RS9 mRNA as a control.

Immunostaining
For all immunofluorescence experiments, cells were grown on coverslips. Cells were fixed and permeabilized with 3.7% paraformaldehyde for 12 minutes, cold methanol for 4 minutes, cold acetone for 2 minutes, and then stained with 2.5% goat serum in PBS containing 4% bovine serum albumin (BSA) overnight at 4°C. Immunostaining was conducted using mouse monoclonal p21WAF1 (Oncogene research Products) antibody. Alexa Fluor 568 antimouse (Fisher Bioblock Scientific) was used as secondary antibody. Then, cells were incubated for 10 minutes with 4',6-diamidino-2-phenylindole (DAPI; 0.5 μg/mL; Sigma-Aldrich Chemie) for nuclei staining.

Cell-cycle analysis
The effect of bortezomib on the cell cycle was determined by fluorescence-activated cell sorting (FACS) analysis using propidium iodide (PI) staining. Cells were seeded in 60 mm Petri dishes at 40% confluency for 24 hours and then treated with vehicle or bortezomib (25 or 50 nmol/L) for the next 24 hours. Adherent cells were harvested with trypsin (5 mg/mL; Invitrogen), fixed in 70% cold ethanol overnight at 4°C and finally stained with a solution of 100 μg/mL PI (Sigma-Aldrich Chemie) at 4°C overnight. DNA content analysis was done in a fluorocytometer Cytomics FC500 (Beckman and Coulter) with a minimum of 10,000 events collected and data were analyzed with 7.5.5 FlowJo software (TreeStar).

p21 shRNA knockdown
Clones of MCF7 were knocked down for p21 with lentiviral-based shRNA approach. The pLKO.1 lentiviral vectors containing the scrambled or p21-specific shRNAs were purchased from Open biosystem. p21 shRNA #123 sequence is CGCTTCTACATCTCTGCCTTA and shRNA #124 sequence is GCTGATCTTCTCAAGAGGAA. Viral particles were produced per the manufacturer’s protocol. MCF7 cells were infected with the corresponding lentivirus at a multiplicity of infection of 1.0 for 5 days and Western blot analyses were used for testing the suppression efficiency of endogenous p21 protein in different clones.
siRNA analysis

Specific oligomers of ERα siRNA have been described previously (5). A 19-nt oligomer siRNA specific for luciferase (5'-AACGTACGCGGAATACTTCGA-3', sense) was used as a negative control to check nonspecific effects of transfection. For ERα silencing, cells were plated on 6-well plates after reverse transfection. After cell adhesion, bortezomib or its vehicle was added in medium for 24 hours and then cells were lysed according to protein extraction protocols.

Statistical analysis

Statistical analysis was done using the Student t test. A P value of less than 0.05 was considered statistically significant.

Results

We investigated the effect of bortezomib on 3 ERα-positive and 3 ERα-negative human cancer cell lines and noted a significant cytostatic activity toward these cells. As shown in dose–response curves, the 50% growth inhibition (GI50) value for a 4-day treatment with bortezomib among ERα-positive cell lines including T47D, MCF7, and ZR75.1 was 22, 35, and 52 nmol/L, respectively (Fig. 1A). In ERα-negative cell lines, HCT116, MDA-MB-435, and MDA-MB-231 the GI50 values of bortezomib treatment varied from 12, 26, and 40 nmol/L, respectively (Fig. 1B). We then compared the effects of bortezomib on colony formation in soft agar on MCF7 (Fig. 1C) and MDA-MB-231 (Fig. 1D). Treatments with 50 and 25 nmol/L bortezomib were found to prevent drastically colony formation in a dose-dependent manner as previously observed in cell monolayers. This effect seemed independent of ERα expression because a significant inhibition of colonies was observed with the lower bortezomib concentration in ERα-positive cells (MCF7 and T47D; Fig. 1E) and ERα-negative cells (MDA-MB-231 and HCT116; Fig. 1F). This confirmed,
in anchorage-independent condition, the growth inhibition showed by MTT assays.

Previous studies have suggested that bortezomib may affect cell growth through altered expression of cell-cycle regulatory proteins (25). Therefore, we explored the effect of bortezomib on p21 protein in these cell lines. Treatment by 100 nmol/L bortezomib for 24 hours significantly increased p21 levels in all cell lines independently of the ERα or p53 status (Fig. 2A). Indeed, MDA-MB-435, MDA-MB-231, and T47D harbor mutant p53, whereas MCF-7, ZR75.1, and HCT116 harbor wt p53. The increases in p21 levels ranged from 3.7-fold in HCT116 cells to 11.7-fold in MDA-MB-435 cells. A dose–response experiment indicated that 10 to 25 nmol/L bortezomib were sufficient to significantly increase p21 levels in MDA-MB-231 and MCF7 cells (Fig. 2B and C). Bortezomib (100 nmol/L) increased p21 levels in a time-dependent manner from 4 hours to a maximal effect at 16 hours in both cell lines (Fig. 2D and E). Moreover, cell-cycle studies indicated that the percentages of MCF7 cells (Fig. 2H) and MDA-MB-231 cells (Fig. 2I) in G0–G1 phase were significantly elevated by 24-hour incubation in the presence of 25 or 50 nmol/L bortezomib. These findings indicate that the increase in p21 expression is associated with a cell-cycle arrest induced by bortezomib.

As pathologic tissues including breast tumors frequently exhibit aberrant p21 localization, the nuclear localization of p21 was determined by immunofluorescence in the presence or absence of bortezomib. Figure 2F and G firstly confirmed the increase in p21 expression by bortezomib treatment in MCF7 and MDA-MB-231 cells. The large majority of the p21 staining was located in the nuclei (stained with DAPI), suggesting that bortezomib treatment did not alter p21 subcellular distribution.

To investigate the potential mechanism by which bortezomib increases p21, we investigated its effect on p21 mRNA levels using qRT-PCR assays. Results in Fig. 3 show that bortezomib strongly enhanced p21 mRNA levels in all ERα-positive cells, whereas, in the ERα-negative cells, the p21 mRNA increase was not significant. This enhanced in p21 mRNA level observed in ERα-positive cells suggested that bortezomib might mediate accumulation of certain Cip/Kip coactivator transcription proteins. To assess the relevance of coactivator transcription protein, MCF7 cells were cotreated with the proteasome inhibitor and actinomycin D, a transcription inhibitor. We found that actinomycin D was able to reduce by 2-fold bortezomib-induced p21 increase (data not shown). This result suggested that bortezomib involved a neosynthesis or stabilization of p21 mRNA in ERα-positive cells.

To gain further insight into the role of p21 in bortezomib-mediated effects, we investigated the effect of bortezomib in p21-deficient cells. For this, MCF7 cells stably infected with different p21-specific shRNA (nos. 123 and 124) and a HCT116 p21−/− derivative in which both p21 alleles have been deleted were used. In MCF7 cells, p21 protein (Fig. 4A) and mRNA (Fig. 4C) expressions were largely reduced by shRNA even under bortezomib treatment. This partial p21 silencing seemed to block by 50% the bortezomib-mediated growth inhibition as compared with control MCF7 clones (Fig. 4D). Conversely, the deletion of p21 in HCT116 p21−/− cells that totally prevented p21 expression (Fig. 4B) was found to inhibit by at least 50% the growth effect induced by bortezomib treatments as compared with wild-type HCT116 cells (Fig. 4E). Taken together, these results indicate that p21 seems to be an important protein implicated in the response to bortezomib.

As we and others have previously showed (3, 5) that ERα was able to regulate p21 protein, we investigated the implication of ERα in the bortezomib actions on p21 expression and cell growth. First, we investigate the effect of bortezomib on ERα expression in Fig. 5A. An ERα expression elevation up to 300% was found from 4 to 16 hours of bortezomib treatment that is consistent with p21 upregulation observed between 8 and 24 hours (Fig. 2D). We also found a 50% decreased of ERα expression at 24 hours as previously showed by Powers and colleagues and Periyasamy-Thanadan and colleagues (33, 34). To evidence the ERα role in p21 regulation, we knocked down ERα with specific siRNA. As shown in Fig. 5B, the ERα silencing in MCF7 cells impairs bortezomib effect on p21 accumulation. We further investigated the effect of bortezomib on growth behavior of control and ERα siRNA transfected cells. Although bortezomib effectively inhibited by 50% the growth of scrambled control transfected

![Figure 3](image-url)
cells, its effect on ERα knocked down cells was near totally abrogated (Fig. 5C).

**Discussion**

Proteasome inhibitors are novel anticancer agents currently used in multiple myeloma and under evaluation for non–small cell lung and androgen-independent prostate carcinomas (35). However, the exact mechanisms by which bortezomib induce growth arrest and apoptosis in cancer cells remain to be elucidated. Here, we investigated whether the molecular effects of bortezomib involve p21 regulation and its relationship to ERα presence.

Our results show that bortezomib prevents the growth of ERα-positive or ERα-negative cancer cells isolated from breast, melanoma, or colorectal cancers within the same range of concentrations. Interestingly, cells harboring wild-type or mutant p53 also display a similar sensitivity to bortezomib. These results are in agreement with previous findings in myeloid leukemia cells, melanoma, mantle lymphoma, and myeloma cells in which bortezomib activity occurs in a p53-independent manner (36–38). It has however, been reported that in some cases, bortezomib activity could occur in a p53-dependent manner, for example, in response to DNA damage (39).

This study also shows a major role of p21 in bortezomib antiproliferative action. In all cancer cell lines, growth arrest induced by this proteasome inhibitor is correlated to a significant increase in p21 levels and these results are consistent with previously reported findings (2, 40, 41). Moreover, we have defined the contribution of p21 in the bortezomib action through experiments on p21-deficient MCF7 and HCT116 cell lines. In these 2 cell types, the p21 deficiency seems to be sufficient to prevent, by approximately 50%, the growth inhibitory effects of bortezomib. To our knowledge, this is the first evidence of a direct implication of p21 in the antiproliferative action of bortezomib in breast and colorectal cancer cells. The fact that p21 deficiency did not completely abrogate the growth inhibitory effects of bortezomib suggests that p21-independent pathway(s) seem to also exist. In fact, previous studies indicated that cell growth inhibition by proteasome inhibitors is probably because of multiple proteins acting in concert (14). For examples, growth-inhibitory doses of bortezomib upregulated p27 and c-myc in Burkitt’s lymphoma (42), or activated JNK/c-Jun signaling in multiform glioblastoma cells (43), which led to the initiation of apoptotic pathways.

Our present report also indicate that bortezomib controls p21 by distinct mechanisms in ERα-positive and ERα-negative cancer cells, its effect on ERα knocked down cells was near totally abrogated (Fig. 5C).

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negative cells and further highlight the role of ERα in bortezomib action. The 3 ERα-negative cell lines from melanoma, breast, and colorectal cancers exhibit a highly significant increase of p21, but this augmentation is not correlated to p21 mRNA variations. The p21 protein accumulation is probably because of the inhibition of a high proteosomal activity as described previously. Because ERα-negative tumors, and more precisely triple-negative breast cancers (ERα, PR, and HER2) have more aggressive features and lack of identified therapeutic targets, proteasome inhibitor may become a key topic of research and clinical interest in these cells.

In contrast, in ERα-positive breast cancer cells, a new mechanism of bortezomib action is showed, which implicates ERα and a p21 upregulation. The strong elevation of p21 transcripts seems to be a general phenomenon because it was observed in 3 ERα-positive breast cancer cell lines. To our knowledge, only one study from Baiz and colleagues indicated a transcriptional regulation of p21 by bortezomib in hepatocellular cells (44). Moreover, we provide evidence indicating that silencing of ERα prevents the bortezomib effects on both p21 expression and cell growth inhibition. This data indicate, for the first time, a major role of ERα in bortezomib action. However, the mechanism of ERα action on p21 gene remains unknown. ERα could stabilize p21 mRNA or could activate the p21 promoter either through DNA binding to specific estrogen response elements, or indirectly through protein–protein interaction with other transcription activators bound to p53-responsive elements or Sp1/Sp3 binding sites (45). Another hypothesis is that ERα can activate an uncharacterized transcriptional factor that will further activate p21 promoter (46). It should be noted that the total ERα detected by immunoblotting is upregulated from 4 to 16 hours and then downregulated by bortezomib treatment. This is consistent with previous studies of Powers and colleagues and Periyasamy-Thandavan and colleagues, who showed that the chymotrypsin-like activity of the proteasome is not implicated for ERα degradation (33, 34). We show that, in presence of bortezomib, ERα retains the capacity to activate p21 expression and this overexpression is responsible for a significant part of the bortezomib-induced growth arrest. However, it should be noted that the knockdown of ERα impairs more efficiently the effect of bortezomib than p21 knockdown, suggesting that, in addition to p21, ERα could regulate other growth-regulatory proteins.

In this study, the potential influence of unliganded ERα in bortezomib action was not evaluated because all studies were done in the presence of estradiol. However, the presence of a part of unliganded receptor could also influence the proteasome-induced growth arrest. The fact remains that, our data, along with that from other preclinical models (49, 50) support the potential for proteasome inhibition as a viable route for development of new therapeutics in ERα-positive and ERα-negative breast cancers. Moreover, bortezomib is now licensed for the treatment of newly diagnosed breast cancers and is now being tested in new clinical trials.
as well as relapsed/progressive multiple myeloma and has had a major impact on the improvement in the treatment of this cancer in the last few years (35). Examination of bortezomib effects alone or in combination with other therapeutics in early breast cancer lesions would lead further insight into the clinical use of bortezomib. In this context, our present study highlighting a new mechanism of bortezomib action that involves an ERa-dependent regulation of p21 mRNA provides strong rationales for the therapeutic evaluation of bortezomib in early stages of breast cancer.

Disclosure of Potential Conflicts of Interest
No potential conflicts of interest were disclosed.

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Acquisition of data (provided animals, acquired and managed patients, provided facilities, etc.): M. Maynadier, O. Vaillant, M. Gazy-Bobo, M. Gleizes, A.-M. Cathiard, J. Lee Tin Wah, M. S. Sheikh
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Study supervision: M. Garcia

Acknowledgments
The authors thank N. Vie for cytometry advices and J.Y. Cance for artwork. M. Maynadier is a fellow of the Montpellier II University and O. Vaillant from the Ligue Nationale contre le Cancer.

Grant Support
This work is supported in part from the University of Montpellier I and the “Association pour la Recherche contre le Cancer,” Grant no. SFU2010-120096.

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Received March 6, 2012; revised August 22, 2012; accepted August 22, 2012; published OnlineFirst September 10, 2012.

References


Molecular Cancer Research

Roles of Estrogen Receptor and p21Waf1 in Bortezomib-Induced Growth Inhibition in Human Breast Cancer Cells

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Mol Cancer Res  Published OnlineFirst September 10, 2012.

Updated version  Access the most recent version of this article at:
doi:10.1158/1541-7786.MCR-12-0133