Abstract. Velcade (also known as PS-341 or Bortezomib) is a highly selective and reversible inhibitor of the 26S proteasome and is approved for the treatment of patients with advanced multiple myeloma. Here we investigated the anti-proliferative effect of Velcade on 4T1 breast cancer and B16F10 melanoma cells and evaluated the mechanism of action. It was found that two cell lines are differentially sensitive to proteasome inhibitor Velcade. The IC50 concentrations for B16F10 and 4T1 were 2.5 nM and 71 nM, respectively, indicating that B16F10 cells are more sensitive to proteasomal inhibition. Velcade was equally potent in inhibiting the chymotrypsin-like activity of the proteasome in both cell lines. It was determined that B16F10 cells proliferate more rapidly than 4T1 cells; doubling time ($T_d$) = 14.2 h versus $T_d$ = 22.9 h, suggesting that a rapid proliferation rate may be an important factor in cellular resistance towards proteasomal inhibition. We observed for the first time that p53 and p21 proteins were increased in B16F10 cells but not in 4T1 following Velcade-treatment, demonstrating that p53 and p21 may enhance Velcade sensitivity. Furthermore, it was observed that caspase-3 proenzyme was reduced by ~20% in B16F10 melanoma cells, but not in 4T1 cells in response to 26S proteasomal inhibition by Velcade. Altogether, we concluded that p53 protein plays a central role in higher sensitivity of B16F10 cells to Velcade by inducing the accumulation of p21, a cell cycle inhibitor, as well as by stimulating the mitochondrial pathway of apoptosis through caspase-3 activation.

Introduction

The 26S proteasome, is an intracellular ATP-dependent multicatalytic protease involved in the degradation of short-lived proteins under normal metabolic conditions. It is also involved in the degradation of long-lived proteins, the processing of certain proteins (e.g., transcription factor NF-κB), and antigen presentation (1,2). The 26S proteasome has a molecular mass of ~2.5 MDa and is formed by the assembly of one 20S proteasome complex (the core particle) with 2 molecules of the 19S complex, the regulatory complex. The 19S regulatory complexes are attached to both ends of the 20S proteasome. The eukaryotic 20S proteasome is made up of 2 copies of 7 distinct $\alpha$ and 7 distinct $\beta$ type subunits. The $\alpha$ subunits make up the 2 outer layers, and the $\beta$ subunits the 2 inner rings of the structure [i.e. $(\alpha_1-\alpha_7)(\beta_1-\beta_7)(\beta_1-\beta_7)(\alpha_1-\alpha_7)]$ (3,4).

Studies of the proteasome inhibitors MG132 and lactacystin indicated that the proteasome is responsible for the degradation of >80% of intracellular proteins. Among the key regulatory proteins degraded by the 26S proteasome are the transcription factor c-Fos, M-, S-, and G1-phase specific cyclins, cyclin-dependent kinase inhibitors, p53, polyamine biosynthetic enzymes ornithine decarboxylase, S-adenosylmethionine decarboxylase, and a variety of oncoproteins (1,5-8). For targeting the 26S proteasome, substrate proteins are usually conjugated to a 76 amino acid protein ubiquitin, a multistep process. First, ubiquitin is transferred to the ubiquitin activating enzyme (E1), from there it is transferred to one of the ubiquitin conjugating enzymes (E2). Finally, the ubiquitin molecule is transferred to the ubiquitin ligase (E3), which performs the final transfer of the activated ubiquitin to the specific proteins. After the first attachment of the ubiquitin molecule, a poly-ubiquitin chain is formed on the substrate protein; each additional ubiquitin is linked to the lysine 48 of the previous ubiquitin molecule (9-11).

Proteasome inhibitors exhibit antitumor activities in a number of cancer models by triggering apoptosis. The mechanism of action generally involves inhibition of the
processing of the proenzyme form of NF-kB (important for cell survival), and by accumulation of pro-apoptotic proteins Bid, Bax, tumor suppressor p53 and cyclin-dependent kinase inhibitors p21 and p27 (12). Due to their manageable cellular toxicity and significant antitumor activities, they are currently tested in clinical studies. The dipeptide boronic acid analogue Velcade (formerly known as Bortezomib or PS-341) is the first proteasome inhibitor used in clinical trial for the treatment of a number of malignancies, e.g., multiple myeloma, non-Hodgkin lymphoma, non-small cell lung cancer (NSCLC), and other solid tumors (13-15). In contrast to peptide aldehyde inhibitors (e.g., MG132), Velcade is a more potent and selective proteasome inhibitor. This boronic acid inhibitor blocks threonine protease activity by inhibiting the chymotrypsin activity of the 26S proteasome (16). Although promising results were obtained in several clinical studies, Velcade was not found to be effective as a single agent in the treatment of patients with metastatic melanoma (17). In addition, co-treatment with Velcade did not overcome the resistance of mammary tumor cells to antitumor drugs, such as cyclophosphamide and cisplatin under in vitro conditions (18). Recently, it was demonstrated that resistance to proteasome inhibitor treatment was associated with increased expression of proteasomal subunits in NSCLC cell lines, leukemia and lymphoma cells (15,19). Additionally, upon exposure to proteasome inhibitor, increased resistance to apoptosis may result from the accumulation of anti-apoptotic protein, Bcl2 family member proteins and Heat shock proteins (20). These studies indicated that the 26S proteasome, whose many functions are still unknown, needs to be investigated in detail. In this study, we investigated the growth inhibitory effect of Velcade on 4T1 breast cancer and B16F10 melanoma cells. We observed that both cell lines are differentially sensitive to 26S proteasome inhibition by Velcade.

Materials and methods

Materials. Enhanced chemiluminescence (ECL) detection reagent for Western blotting and Bio-Rad protein assay reagents were purchased from Amersham Pharmaceuticals (now GE, Arlington Heights, IL). RPMI-1640 cell culture media, fetal bovine serum (FBS), trypsin, penicillin/streptomycin, MG132 and Bio-Max X-ray film were obtained from Sigma-Aldrich Inc. Anti-p21 and anti-p27 antibodies (Novocastra Laboratories) were a kind gift from Dr David J. Feith (Penn State University College of Medicine, Hershey, PA) and anti-p21 antibody (Santa Cruz) was kindly provided by Lisa M. Shantz (Penn State University College of Medicine, Hershey, PA). MTI 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] was kindly provided by Dr Diane McCloskey (Penn State University College of Medicine, Hershey, PA). Rabbit polyclonal anti-caspase-3 antibody was purchased from Santa Cruz Biotechnology Inc. 20S proteasome activity assay kit was obtained from Chemicon International. Velcade (PS-341) was generously provided by Dr Engin Ulukaya (Uludag University, Bursa, Turkey). All other reagents were purchased from Sigma-Aldrich Inc unless otherwise mentioned.

Cell culture maintenance. 4T1 and B16F10 cells were maintained in RPMI-1640 (plus 4.5 g/l glucose, 10 mM HEPES, 1 mM sodium pyruvate, 0.15% sodium bicarbonate, 100 μg/ml streptomycin and 100 U/ml penicillin) supplemented with 10% FBS. Cells were incubated in a humidified atmosphere at 37°C with 5% CO2. Stock cultures were maintained in 25 cm2 Corning flasks, and experimental cultures were grown in 35x10 mm Corning plates. The cells were subcultured when they were 70-80% confluent.

Growth curve. Cells (100,000) were plated in 35x10 mm dishes. The growth rate of each cell was determined by counting the number of cells using a hemacytometer as a function of time. Using the Prism 3.03 program, data from the exponential phase of growth (data points at 24, 48 and 72 h) were used to obtain an exponential growth curve (\( N_t = N_0 e^{\text{Kt}^2} \) where \( N_t \) is the cell number at time \( t \), \( N_0 \) is the cell number at the initial time, and \( K \) is the constant rate. Doubling time \( T_d \) was then obtained using the formula: \( T_d = \ln 2/K \).

MTT-based cytotoxicity assay. Cells (50,000) were seeded in each plate (35x10 mm). Cells in the exponential phase of the growth were treated with various doses of Velcade (0.001, 0.01, 0.1, 1, 10 or 100 μM) or MG132 (0.01, 0.1, 0.5, 1, 10 or 50 μM) for 24 h. After the inhibitor exposure period, cells were fed fresh media. At 48 h after the inhibitor removal, cells were treated for 4 h with RPMI-1640 media containing 0.5% FBS + 0.5 mg/ml MTT at 37°C with 5% CO2 to determine the number of surviving cells. After removing the medium and MTT, cells were incubated with 3% SDS (200 μl) + 1 ml 40 mM HCl/isopropanol for 15 min at room temperature with intermitten shaking to dissolve the MTT-formazan crystals. The homogenate in each plate was pipetted well to dissolve the MTT-formazan crystals completely and centrifuged at 12,000 rpm for 5 min. The absorbance at 570 nm was recorded with a Bio-Rad Smartspec Plus spectrophotometer (21). The IC50 values were then obtained by fitting the data with a Prism 3.03 program to a Sigmoidal dose-response curve.

Assay of proteasome activity. The Chemicon 20S Proteasome Activity Assay Kit was used for evaluation of proteasome activity in 4T1 and B16F10 cell lysates. Cells (100,000) were plated in 35x10 mm petri dishes and treated with various doses of Velcade (0, 0.01, 0.1, 1 or 10 μM) for 6 h in the logarithmic phase of the growth. After treatment, cells were washed with 1 ml PBS buffer and lysed in 150 μl of ice-cold buffer (50 mM HEPES pH 7.5, 1 mM EDTA, 0.1% NP-40, 0.002% SDS (w/v), 1 mM 2-mercaptoethanol and 20% glycerol (v/v)) for 30 min on ice with occasional agitation. After repeated pipetting, cell lysates were centrifuged at 13,000 rpm for 15 min at 4°C. The supernatants were stored at -80°C until use. A total of 10 μg protein sample was added to each tube containing 25 mM HEPES pH 7.5, 0.5 mM EDTA, 0.05% NP-40, 0.001% SDS and 50 μM proteasome substrate LLVY-AMC specific for chymotrypsin-like activity. The total volume of cell protein, HEPES buffer and proteasome substrate amounted to 100 μl. The samples were incubated for 90 min at 37°C. At the end of incubation, 2 ml of ice-cold dH2O was added to each sample and the fluorescence was
Western blotting. The ECL detection system was utilized for Western blotting according to manufacturer's instructions. Proteins were separated on 10 or 12% polyacrylamide gels under denaturing conditions in 0.1% sodium dodecyl sulfate (SDS) (23). The proteins were then transferred to PVDF membrane at 70 V for 2 h at room temperature in the transfer buffer (25 mM Tris base, 192 mM glycine, 7% methanol and 0.007% SDS). After the transfer membrane was removed, washed with methanol and left to dry for 15 min at room temperature to enhance the protein binding. After drying, membranes were reactivated by methanol. Reactivated membranes were then blocked with 5% skim milk overnight at 4°C. Hsp70 level was determined by incubating the membranes with a mouse monoclonal anti-Hsp70 (1:5000 dilution) followed by HRP-conjugated anti-mouse secondary antibody (1:5000 dilution) in Tris-buffered saline-Tween-20 (TBS-T). The relative amount of p53 was determined by a rabbit polyclonal anti-p53 antibody (1:5000 dilution), p27 was detected by probing the membranes with a purified mouse monoclonal anti-p27 antibody (1:2500 dilution). The induction of p21 was detected by a mouse monoclonal anti-p21 antibody (1:500 dilution). To determine the amount of caspase-3, the membranes were probed with a rabbit polyclonal anti-caspase-3 antibody (1:500 dilution). After ECL substrate incubation for 2 min, the membranes were wrapped with plastic stretch and exposed to a Bio-Max X-ray film in a dark room for 1 min followed by manual film developing.

Statistical analyses. Data were analyzed and graphed by GraphPad Prism 3.03 program. Where appropriate, Student t-test was used to evaluate the statistical significance. P<0.05 was considered significant.

Results

Morphological changes in 4T1 breast cancer and B16F10 melanoma cells exposed to various concentrations of Velcade for 24 h, were examined in detail using a light microscope. As seen in Fig. 1, cells underwent characteristic morphological changes commonly observed in apoptotic cells (24), such as shrinkage, rounding and detachment. These marked morphological changes were observed in B16F10 cells with a Velcade concentration as low as 0.001 μM; however, in 4T1 cells, such morphological changes were observed with 0.01 μM or higher concentrations, suggesting that B16F10 cells are more sensitive to proteasome inhibitor Velcade. The anti-proliferative and cytotoxic effects of a concentration range of Velcade in both cell lines were also determined by MTT assay. As seen in Fig. 2A, in agreement with the morphological changes observed, the two cells were found differentially sensitive to Velcade-induced growth inhibition. The IC50 concentration of B16F10 was 2.5 nM and that of 4T1 was 71 nM, indicating again that B16F10 cells are more sensitive to proteasome inhibitor Velcade. The anti-proliferative and cytotoxic effects of a concentration range of Velcade in both cell lines were also determined by MTT assay. As seen in Fig. 2B, MG132 decreased cell viability in a dose-dependent manner. The IC50 of MG132 for B16F10 cells was 0.62 μM and for 4T1 was 1.22 μM. Together, these measured using the excitation and emission filters of 380 and 460 nm respectively, using a Bio-Rad VersaFluor Fluorometer.

Protein determination. The protein concentration was determined using the Bio-Rad dye-binding assay. Bovine serum albumin was used as a standard (22).

Protein determination. The protein concentration was determined using the Bio-Rad dye-binding assay. Bovine serum albumin was used as a standard (22).
data indicated that a) B16F10 cells are more sensitive to proteasome inhibition compared to 4T1 cells and b) Velcade is more potent than MG132.

Initially, it was hypothesized that the mechanism of resistance to Velcade may be due to the differential activity of the proteasome in each cell line. Therefore, we measured the chymotrypsin-like proteolytic activity of the proteasome in 4T1 and B16F10 cells upon treatment with various concentrations of Velcade for a short time period (6 h). As seen in Fig. 3, the baseline activity of the proteasome was higher by ~49% in B16F10 cells than in 4T1 cells. However, treatment with 10 nM Velcade led to 70 and 82% inhibition of proteasome activity in 4T1 and B16F10 cells, respectively. Furthermore, increasing concentrations of Velcade were equally potent in both cell lines, indicating that resistance to Velcade in 4T1 compared to B16F10 cells is not due to intrinsic differences in the sensitivity of the 26S proteasome to Velcade (Fig. 3).

It has been previously reported that proteasome inhibitors preferentially induce cell death in proliferating HL60 cell, but not in quiescent, differentiated cells. Therefore, the 26S proteasome appears to be required for cell survival and cell cycle progression (25). To investigate whether cell proliferation status was one of the determinants of cellular sensitivity to proteasome inhibition, the doubling time ($T_d$) of each cell was determined. As can be seen in Fig. 4, B16F10 cells are more rapidly proliferating than 4T1 cells ($T_d = 14.2$ h versus $T_d = 22.9$ h), suggesting that the proliferative status may be an important factor in cellular sensitivity to 26S proteasome inhibition.

To further examine the molecular determinants of cellular sensitivity to proteasome inhibition, the accumulation of cell cycle modulators p21, p27 and p53, and the expression of anti-apoptotic protein Hsp70 in 4T1 and B16F10 cells were analyzed in response to proteasome inhibition using the Western blot technique. Morphological analysis of 4T1 and B16F10 cells treated with 10 μM Velcade as a function of time indicated cell rounding, shrinkage and detachment as early as 8 h (data not shown). In addition, the proteasome activity assay indicated that almost complete inhibition of the proteasome activity was achieved in both cell lines after 6 h of exposure to 10 μM Velcade (Fig. 3). On the basis of these experiments, it was decided to treat the cells with 10 μM...
Velcade for 6 h in the subsequent experiments to compare the differences in the induction of pro-apoptotic and anti-apoptotic proteins, preceeding the morphological changes observed between the two cells under investigation. The stress protein Hsp70 is known to be stimulated and has a cytoprotective role following the treatment of cells with proteasome inhibitors (26). Although a 30-40% increase in Hsp70 level was detected following the inhibition of the 26S proteasome, the degree of change did not differ between two cell lines, indicating that Hsp70 is probably not involved in the differential sensitivity of B16F10 and 4T1 cells to proteasome inhibitor Velcade (Fig. 5). Then, we examined the changes in the level of cell cycle inhibitor p27 following Velcade treatment. During a short period of 26S proteasome inhibition, the increase in expression level of p27 was similar in B16F10 and 4T1 cell lines (~30% increase was detected), eliminating the fact that p27 determines Velcade-sensitivity in B16F10 cells (Fig. 5).

As mentioned in the Introduction, the tumor suppressor protein p53 is degraded by the ubiquitin-proteasome pathway in *in vitro* and *in vivo* models and is commonly known to be linked to the control of cell proliferation and cell cycle progression (3,27). Stabilization of p53 leads to apoptosis in a number of cell culture models. Therefore, we examined whether there was a differential induction of p53 following a brief inhibition of the 26S proteasome in both cell lines. As can be seen in Fig. 6A, treatment of B16F10 cells with 10 μM Velcade for 6 h led to significant accumulation of p53 protein compared to vehicle-treated (NaCl) cells. In contrast, Velcade-treatment did not result in accumulation of p53 in 4T1 cells. The tumor suppressor protein p53 is a transcriptional factor that induces the expression of the cyclin-dependent kinase inhibitor p21. To determine whether p53 accumulation in B16F10 in response to proteasome inhibition was functional, p21 expression after Velcade treatment was assayed in both cell lines. Similarly, a brief treatment with Velcade induced a very significant accumulation of p21 in B16F10 cells but not in 4T1 cells (Fig. 6B). Although there is contrasting evidence regarding the presence of p53 in 4T1 cells (28,29), the results here show that this mammary cell line is most likely a p53-null cell. Collectively, these results indicated that inhibition of the chymotrysin-like activity of the 26S proteasome by Velcade resulted not only in stabilization of p53 but also remarkable induction of p21 in
B16F10 cells. While the signaling cascade induced by p53 during apoptosis depends on the cell type used, it is well documented that p53-induced apoptosis is commonly mediated by pro-apoptotic proteins Bax, Bad and PUMA, which are essential proteins for the release of cytochrome c from the mitochondrial membrane and subsequent activation of caspase-3 (30,31). Caspase-3 is an enzyme activated in almost every apoptotic process (32) and is therefore considered a hallmark of apoptosis activation. Therefore, we examined whether the proenzyme form of this apoptosis executor is cleaved in response to proteasomal inhibition by Velcade. As can be seen in Fig. 7, we observed a significant 20% reduction in the proenzyme form of caspase-3 upon inhibition of the 26S proteasome by Velcade in 6 h (p=0.0117). However, no change was detected in caspase-3 level in 4T1 cells, suggesting that the sensitivity of B16F10 cells is probably associated with caspase-3 mediated apoptosis induction following p53 and p21 accumulation after inhibition of the 26S proteasome.

Discussion

In this study, the differential effects of proteasome inhibitors on the viability of B16F10 melanoma and 4T1 cancer cells were examined. B16F10 melanoma cells originate from C57BL-6 mice, form large tumors in vivo and are used commonly as an in vivo melanoma model (33). 4T1 breast carcinoma cells are also widely used as a metastatic mammary tumor model (34). The findings presented in this study demonstrate for the first time that B16F10 melanoma cells are more sensitive towards proteasomal inhibition than 4T1 cells as determined by morphological analysis and MTT assay. It was found that proteasome inhibitor Velcade is more potent and cytotoxic than proteasome inhibitor MG132 in both cell lines tested. Although the basal level of the proteasome activity was higher in B16F10 cells compared to 4T1 cells, both cell lines showed marked inhibition of the 26S proteasome upon exposure to various doses of Velcade, eliminating the potential differences in the proteasome-inhibition as the determinant of the Velcade-resistance. Using a number of mantle cell lymphoma (MCL) cell lines, Rizzatti et al also concluded that the intrinsic resistance to Velcade is not due to the basal activity of the 26S proteasome or compensatory increases in the proteasome activity after drug exposure (35). It was previously shown that 26S proteasome inhibition is pro-apoptotic in rapidly dividing HL60 leukemic and endothelial cells (25,36). Similarly, Lopes et al reported that proteasome inhibitor MG115 failed to induce apoptosis of quiescent Rat-1 cells. In contrast, the proteasome inhibitor tested in their study was still able to induce the apoptosis of non-proliferating differentiated PC12 cells and they concluded that cell proliferation was not the sole determinant of sensitivity to proteasomal inhibition (27). The results presented herein support the fact that there is a correlation between the cellular sensitivity to proteasome inhibitors and rapid proliferation rate since B16F10 cells are rapidly dividing and are more sensitive to Velcade as compared to 4T1 cells.

The present study indicates that the cellular sensitivity or resistance of cells examined here toward proteasomal inhibition, may not result from the differences in the accumulation of anti-apoptotic protein Hsp70 or Cdk-inhibitor p27. In agreement with these results, expression of Hsp70 was found to be similar in Velcade-sensitive cell line SW1573 and in resistant cell line H460 (both are NSCLC cells). In contrast, the expression of anti-apoptotic proteins Hsp27 and Hsp90 were higher in SW1573 cells than in H460 cells. The author concluded that the apoptosis resistance of SW1573 cells may result from increased expression of Hsp27 (15). Chauhan et al also reported that Hsp27 confers Velcade-resistance in lymphoma cells (20). It is therefore of interest to determine the expression level of Hsp27 as well as Hsp90 in B16F10 and 4T1 cells upon exposure to proteasome inhibitors.

Remarkable increases in tumor suppressor p53 level and cell cycle inhibitor p21 observed very early in 6 h after the addition of Velcade, clearly suggest that p53 and the p53-inducible gene product p21 are important factors in B16F10 cellular sensitivity towards proteasomal inhibition. In addition, we observed a statistically significant 20% decrease in caspase-3 proenzyme in B16F10 but no apparent changes in 4T1 cells. The reduction in caspase-3 may thus be stimulated by p53-induced pro-apoptotic protein (e.g., Bax) and through subsequent release of cytochrome c from mitochondria in B16F10 cells. Nevertheless, it is known that 26S proteasome inhibition induces growth inhibition or apoptosis in a p53-independent manner (37,38). Therefore, the growth inhibition observed in 4T1 cells may be mediated through a p53-independent mechanism since the results presented here and in a prior study (28) prove that 4T1 cells are p53-null cells. In future studies we will design experiments to further investigate the mechanism of growth inhibition in 4T1 cells to delineate further the intracellular functions of the 26S proteasome.

A better understanding of the molecular link between cellular proliferation, apoptosis and 26S proteasome is crucial for understanding the differences between Velcade-responsive and non-responsive patients in multiple myeloma and possibly other cancer patients. Further experiments will certainly help to define novel molecular targets and therapeutic strategies to overcome the clinical resistance to proteasome inhibition in some cancer patients.

Acknowledgements

This work was funded by the Scientific and Technological Research Council of Turkey (TUBITAK) with Project No 105S351 to A. Yerlikaya. The study was supported in part by funds from Akdeniz University Research Units, Antalya, Turkey. We would like to thank graduate student Harun Dokudur (Dumlupinar University, Department of Biology) for assistance with experiments carried out in this study.

References


