Reagents
Bortezomib (BTZ)/Velcade® was kindly provided by Millennium Pharmaceuticals (Cambridge, USA). The proteasome-inhibitors MG132 (Z-Leu-Leu-Leucinal) and MG262 (Z-Leu-Leu-Leu-boronate) were purchased from Calbiochem/Merck (Nottingham, UK). The cytotoxic peptide 4A6 (Ac-Thr(tBu)-His(Bzl)-Thr(Bzl)-Nle-Glu(OtBu)-Gly-Bza) was synthesized as described previously.26 The tripeptidylpeptidase II inhibitor H-Ala-Ala-Phe-chloromethylketone was obtained from Bachem (Germany). Chloroquine, mitoxantrone, cisplatin (CDDP), geldanamycin, doxorubicin, sulfasalazine, 5-FU, NP-40, trimethylrhodamine-ethyl ester (TMRE), ALLN (N-acetyl-Leu-Leu-norleucinal) and leupeptin (Ac-Leu-Leu-Arg-al) were obtained from Sigma-Aldrich (St. Louis, MO, U.S.A.). Bleomycin was obtained from Dagra Pharma (Diemen, The Netherlands). Methotrexate was from Pharmachemie (Haarlem, The Netherlands). Methylprednison was purchased from Pfizer (New York, NY, U.S.A.), cyclosporin A was kindly provided by Novartis (Arnhem, The Netherlands) and Iressa®/gefitinib was a gift from AstraZeneca (Zoetermeer, The Netherlands). Protease Inhibitor Cocktail (PIC) was obtained from Roche Diagnostics (Almere, The Netherlands). RPMI-1640 tissue culture medium and fetal calf serum were obtained from Gibco Boco, Co (Grand Is., NY, U.S.A.). All fluorogenic peptide substrates (Suc-Leu-Leu-Val-Tyr-amc, Ac-Arg-Leu-Arg-amc and Z-Leu-Leu-Glu-amc), the proteasome inhibitor Ac-APnLD-al and proteasome-related antibodies (β1, β2, β5, α7, α/β core were purchased from Biomol (Plymouth Meeting, PA, U.S.A.). Antibody 20S X (NB120-3330) was obtained from Novus Biologicals, Littleton, USA). Antibodies to Hsp27 (#2402) and Hsp90 (#4875) were from Cell Signaling Technologies (Danvers, MA, USA), Antibodies to XIAP (M044-3) and P21 (OP68) were from MBL, Int. Co (USA) and Calbiochem (Germany), respectively. Anti-ubiquitin antibody (sc-8017) was purchased from Santa Cruz Biotechnology (Santa Cruz, Ca, USA).

RT-PCR/siRNA proteasome subunits
mRNA expression levels of proteasome subunits PSMB5 (β5), PSMB6 (β1), PSMB7 (β2) and the endogenous housekeeping gene β-glucuronidase (GUS) as a reference were quantified using real-time PCR analysis (Taqman) on an ABI Prism 7700 sequence detection system (PE Applied Biosystems). All probes were labeled with 5’-FAM and 3’-BHQ1 as a reporter. Primers and probes were designed using Primer Express software (Applied Biosystems). Primers, probe combinations and concentrations used for the quantitative real-time PCR were as follows: PSMB5 forward (50 nM): CTTCAGGGTTCCCGCATGGA; PSMB5 reverse (300 nM): CCGTCTGGAGGGCAA GTGAA; PSMB5 probe (200 nM): TTGCAGCTGACTC; PSMB6 forward (300 nM): AGGCATGACCAAGGAAGAGTGT; PSMB6 reverse (50 nM): GAGCCATCGCTCCAT; PSMB6 probe (200 nM): TGCAATTACTGGCCAAAT GCTCTCG; PSMB7 forward (300 nM): TCGGTCTCACCTTCAACGAG; GUS forward (300 nM): GAAAATATGTGGTTG GAGAGCTCATT; GUS reverse (300 nM): CCGA GTGAAGATCCCCTTTTTA; GUS probe (200 nM): CCAGCCTCAGTGCATGGTGAC TGTTCA. Real-time PCR was performed in a total reaction volume of 50 μl containing TaqMan buffer A (Applied Biosystems, Nieuwerkerk a/d IJssel, The Netherlands), 4mM MgCl2, 0.25 μM of each dNTP (Amersham Pharmacia Biotech) and 1.25 U AmpliTaq Gold DNA polymerase (Applied Biosystems). Samples were heated for 10 min at 95°C to activate the AmpliTaq Gold DNA polymerase and amplified during 40 cycles of 15 s at 95°C and 60 s at 60°C. Relative
mRNA expression levels of the target genes in each sample were calculated using the comparative cycle time (Ct) method. Briefly, this PCR Ct value is the cycle number at which emitted fluorescence exceeds 10× the standard deviation (S.D.) of baseline emissions as measured from cycles 3–15. The Ct of the target gene is normalised to the GUS PCR Ct value by subtracting the GUS Ct value from the target Ct value. The mRNA expression level for each target PCR relative to GUS was calculated using the following equation: mRNA expression = $2^{(Ct \text{ target} - Ct \text{ GUS})} \times 100\%$.

For RNA interference experiments all targeted and non-targeted siRNA constructs were obtained from Dharmacon (Lafayette, USA) and all experiments were performed in 24 well plates or T25 flasks. THP1/WT, THP1/BTZ$_{-100}$ and THP1/BTZ$_{200}$ cells were cultured following the suppliers’ protocol for THP1 cells. Briefly, prior to transfection, cells were cultured overnight at a density of $5 \times 10^4$ cells/ml in RPMI 1640 medium supplemented with 7.5% FCS and 20mM HEPES. Cells were transfected using Dharmafect 2 (DF2) and 100nM of PSMB5 On-Targetplus SmartPool siRNA. As negative control 100nM On-targetplus siControl non-targeting and GAPD pool were used. To assess transfection-efficiency siGLO was used as a transfection-indicator. After 24 hours, transfection-efficiency (usually > 85%) was determined by flow cytometry using siGlo as transfection indicator. At this stage different concentrations of bortezomib were added and induction of apoptosis and cell growth-inhibition was determined after 24 hours bortezomib exposure. Western blot experiments to assess protein knock-down were performed after 72 hours siRNA incubations.

**Sequence analysis**
DNA was isolated from THP1/WT, THP1/BTZ$_7$, THP1/BTZ$_{30}$, THP1/BTZ$_{100}$ and THP1/BTZ$_{-100}$ cells using a Qia amp DNA blood mini kit (250) (Qiagen, Valencia, CA, USA). Subsequently, part the second exon of the PSMB5 gene was amplified by PCR. The primers were designed using Vector NTI (Invitrogen) software (forward : TTCCGCCATGGAGTCATA, reverse : GTTGGCAAGCAGTTTGGA). PCR products were directly sequenced by dideoxy chain-termination method using a kit ABI PrismTM BigDye Terminator (Perkin Elmer, Foster City, CA, USA) and analyzed by an autosequencer ABI Prism Genetic Analyser 3100 automatic DNA sequencer (Perkin Elmer).

**Growth inhibition assays**
Evaluation of drug sensitivity was carried out as described previously. Cells were seeded at an initial density of $1.25 \times 10^5$ cells/ml in individual wells of a 24-well plate containing up to 50 µl of drug solutions. Inhibition of cell growth was determined after 72 hour incubation at 37°C by counting viable cells based on trypan blue exclusion. The drug concentration required to inhibit cell growth by 50% compared to untreated controls was defined as the IC$_{50}$.

**Quantification of ubiquitinated proteins/proteasome subunits**
Western blot analysis to determine the accumulation of ubiquitinated proteins upon bortezomib exposure was performed essentially as described previously. In short, cells were harvested in the mid-log phase of growth and washed 3 times with ice-cold buffered saline pH 7.4. Total cell lysates of $5 \times 10^6$ cells were prepared by resuspending in 0.5 ml lysis buffer containing: 50 mM Tris-HCl (pH 7.6), 5 mM dithiotreitol (DTT), 20 µl PIC (Protease Inhibitor Cocktail; 1 tablet/ml H$_2$O), 20% glycerol and 0.5% NP-40. The suspension was sonicated (MSE sonicator, amplitude
7, for 3 × 5 seconds with 20 seconds time intervals at 4°C) and centrifuged in an Eppendorf micro-centrifuge (5 min, 12,000 rpm, 4°C). Protein content of the supernatant was determined by the Bio-Rad protein assay. Twenty to thirty microgram of total cell lysates were fractionated on a 10% polyacrylamide gel containing SDS and transferred onto a PVDF membrane. The membranes were pre-incubated overnight at 4°C in blocking buffer (5% Bio-Rad Blocker in TBS-T; 10 mM Tris-HCl, pH 8.0, 0.15 M NaCl, 0.1% Tween-20) to prevent non-specific antibody binding. After blocking, the membranes were incubated for 1 hour at room temperature with an ubiquitin-specific antibody (1:1000, Santa-Cruz, SC-8017). After 3 washing steps with TBS-T, the membranes were incubated for 1 hour with HRP-labelled goat-anti-mouse (1:6000, Dako, Glostrup, Denmark) secondary antibody. Detection of antibody binding was followed by chemoluminescence using Supersignal (Pierce Biotechnology, Rockford, USA) according to the manufacturers’ instructions. Digital Image acquisition was performed using the Versadoc Imaging System (Biorad Lab., Veenendaal, The Netherlands).

Expression of β1, β2, β5, α7 and α/β-core proteasome subunits were determined essentially as described above. Primary anti-bodies from Biomol for β1 (1:1000, PW8140), β2 (1:1000, PW8145), β5 (1:1000, PW8895 or 1:1000 20S X), α7 (1:1000, PW8110) and α/β (1:1000, PW8155) were incubated for 1 hour at room temperature. An antibody to α-tubulin was used (1:1000, Santa Cruz, sc-8035) to check and normalize for any loading differences. HRP-labelled Donkey-anti-rabbit (1:6000, Amersham, UK) or goat-anti-mouse (1:6000, DAKO) secondary anti-bodies were used. The signal intensity was determined densitometrically using Quantity One software (Bio-Rad) and was expressed relative to the intensity of the α-tubulin signal.

Native gel electrophoresis/gel filtration chromatography
Characterization of the proteasome (detection of core/regulatory particles and suc-Leu-Leu-Val-Tyr-amc hydrolysis activity), in wild type and bortezomib-resistant THP1 cells by native gel electrophoresis was performed essentially as described by Elsasser et al. using acid washed glass beads (Sigma) for the preparation of lysates.

Gel filtration chromatography of proteasome complexes and proteasome subunits was performed as described by Chondrogianni et al using a Superdex 200 HR 10/30 column (Amersham Pharmacia Biotech, The Netherlands) connected to an Gynkotek Model 300 HPLC system and calibrated with a with a mixture of purified proteins in a MW range of 16.6 kD to 669 kD. Briefly, 1.5 × 10⁷ THP1/WT and THP1/BTZ cells were washed three times in PBS and then lysed in 200 µl ice-cold 20 mM Tris-HCl, pH 7.5, 5 mM ATP and 0.2% Nonidet-P40. Cell extracts were then centrifuged at 4°C for 15 min at 14,000 rpm in an Eppendorf centrifuge. Hundred microliters of the supernatant fraction were applied on the Superdex column equilibrated in 20 mM Tris-HCl, pH7.5, 5 mM ATP and 120 mM NaCl. The column was then eluted with the same buffer at a flow rate of 0.4ml/min. Fractions of 0.4 ml were collected for western blot analysis of proteasome β5 and α7 subunit expression.

Proteasome subunit affinity probing
Affinity labelling of functional proteasome subunits in intact cells was performed with the BodipyFL-Ahx₃L₃VS probe essentially as described by Berkers et al. Prior to affinity labelling experiments THP1/BTZ cells were cultured in the absence of bortezomib for 4 days.
Mitochondrial trans-membrane potential ($\Delta \psi_m$)
The effect of bortezomib on the mitochondrial trans-membrane potential ($\Delta \psi_m$) was measured as described by Ling et al. Briefly, $1 \times 10^5$ cells were resuspended in 0.5 ml culture-medium and incubated for 15 minutes at 37°C with 25 nM tetramethylrhodamine ethyl ester (TMRE), after which cells were washed 3 times with ice-cold PBS supplemented with 0.1% BSA. TMRE accumulation was measured by flow cytometry using a B&D FACScalibur apparatus. Data were analyzed with FCS-express V3 software as described above.

Microarray analysis
THP1/WT cells, THP1/BTZ10, THP1/BTZ100 and THP1/BTZ (-100, 6mo) cells were harvested in the mid-log phase of growth. The amount and integrity of isolated RNA was measured using a spectrophotometer (NanoDrop ND100 (Wilmington, DE, USA)) and a Bioanalyzer (Agilent Technologies, Palo Alto, CA, USA).

Microarray expression analysis was carried out as described by Cloos et al., using the Human Release 2.0 oligonucleotide library (designed by Compugen (San Jose, CA, USA) and obtained from Sigma-Genosys (Zwijndrecht, The Netherlands)) for printing the slides.

The arrays were scanned using an Agilent Microarray Scanner (Agilent Technologies, Amstelveen, The Netherlands). Spot analysis and feature extraction were fully automated using BlueFuse version 3.4 (BlueGnome, Cambridge, UK). Spots were excluded when the Confidence value <0.1. Subsequently, Lowess block normalization was performed within the same program on the non-flagged spots and exported to Excel (Microsoft). Ratio’s of resistant divided by the untreated reference sample were used for further analysis.

Immunofluorescence microscopy
Freshly prepared cytospins from THP1/WT and THP1/BTZ200 cells were used at a density of 10,000 cells per spin. Cells were fixed with 3.5% paraformaldehyde for 15 min at RT and then washed twice with PBS and permeabilized by saponin (PBS + 2% saponin, 2 min at RT). Following two successive washes with PBS, slides were incubated for 2 hours at RT with either PBS (control), rabbit anti-\(\beta_5\) subunit antibody (20S X) diluted 1:50 and mouse anti-\(\alpha_7\) subunit antibody (Biomol, PW8110) diluted 1:100 in PBS + 4% BSA. Double staining detection was performed with a FITC-conjugated goat anti-mouse antibody (Sanquin, Amsterdam, The Netherlands) diluted 1:100 and TRITC-conjugated swine anti-rabbit antibody (Dako, Glostrup, Denmark) diluted 1:100. The slides were mounted with Vectashield (Vector Laboratories, Burlingame, CA, USA) and examined using a Leica fluorescence microscope.

Apoptosis assay
Bortezomib-induced apoptosis was analyzed by measuring AnnexinV-FITC/7-aminoactinomycin D (7-AAD) staining (APOPTEST™-FITC A700, VPS Diagnostics, Hoeven, the Netherlands) according to the instructions of the manufacturer. Briefly, induction of apoptosis was determined after 24 hours exposure by bortezomib. One million cells were harvested and washed 3 times with ice-cold PBS. The cell pellet was incubated for 30 minutes with 7-Amino-actinomycin D (7-AAD) on ice followed by incubation with Annexin-V according to the instructions of the manufacturer. Annexin-V and 7-AAD expression was measured by flow cytometry (Beckton &
Dickinson, FACScalibur) and analysed using FCSexpress V3 software (Denovo software, Thornhill, Canada).

**Statistics**
Statistical analysis was performed using Analysis of Variance between groups (ANOVA) in Graphpad prism. \( P \) values < 0.05 were considered to be statistically significant.