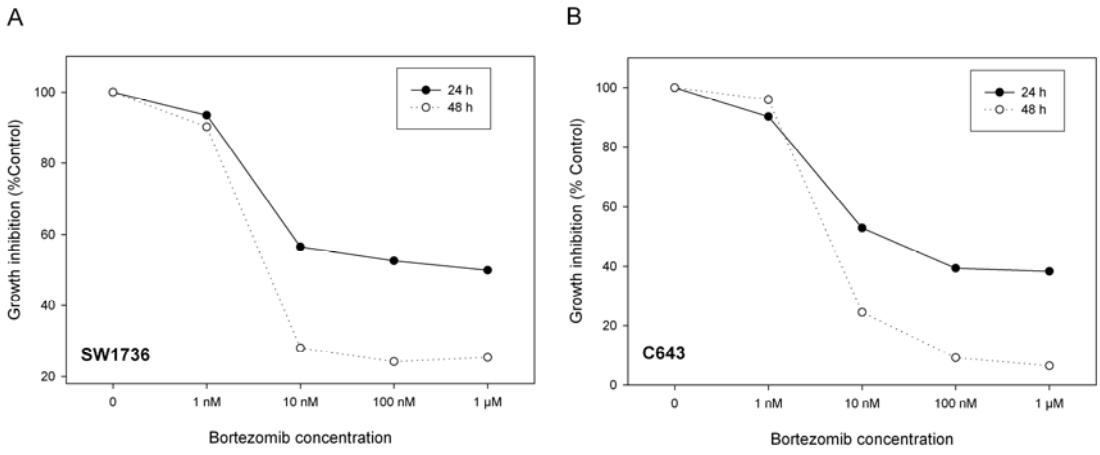
Growth Inhibition

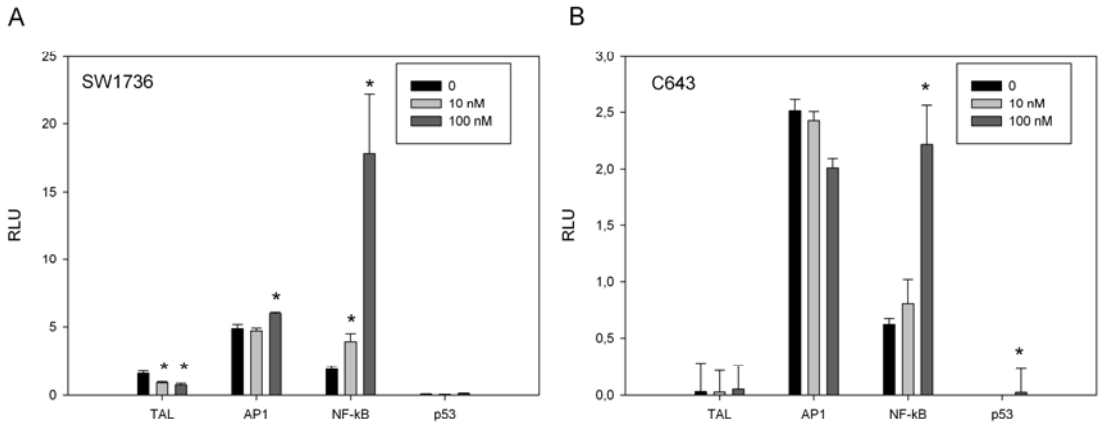
SW1736 (A) and C643 cells (B) treated with 10-fold dilutions (1 nM to 1 μM) bortezomib and growth inhibition was evaluated in by determination of the viable cell number in a Vi-Cell^{TM}XR cell viability analyzer. Data represent the percentage of viable bortezomib-treated relative to untreated cells.



Supplemental Figure 1: Growth inhibition was evaluated in SW1736 (A) and C643 cells (B) by determination of the viable cell number in a Vi-Cell^{TM}XR cell viability analyzer. Data represent the percentage of viable bortezomib-treated relative to untreated cells.

Cell Transfection and Reporter Gene Assays

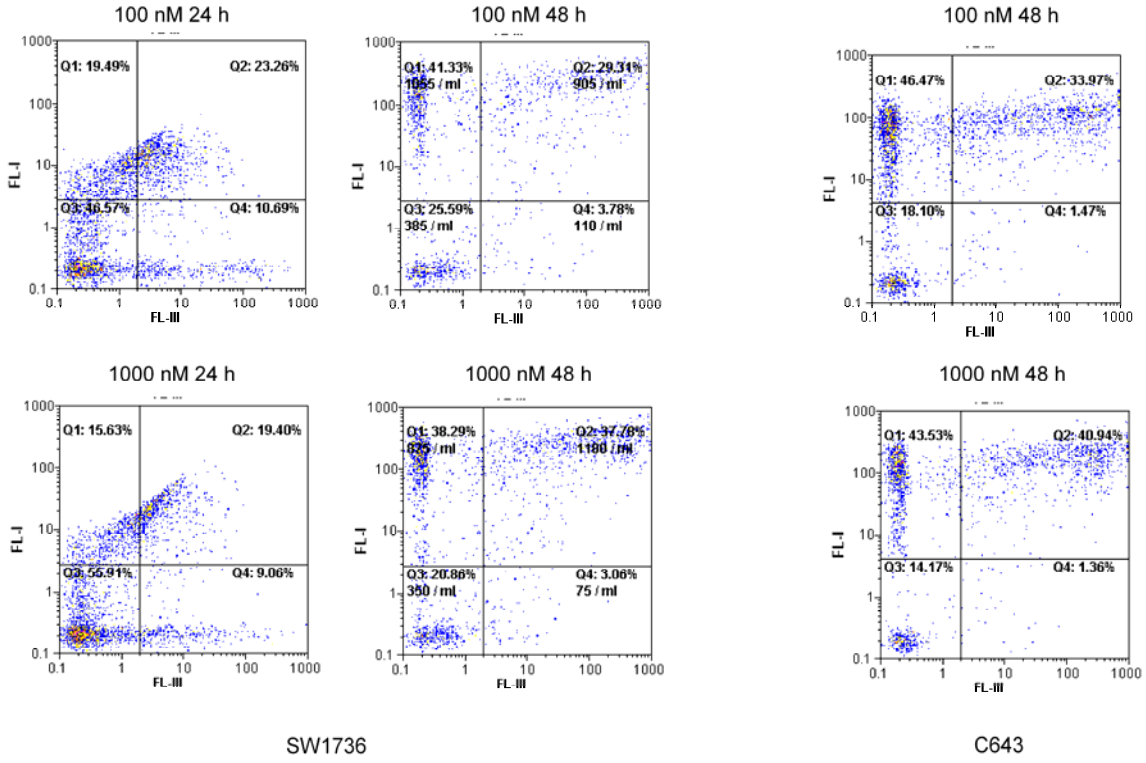
The following reporter gene constructs were purchased from Clontech: pNF-κB-TA-Luc and pp53-TA-Luc for monitoring the activation of the pNF-κB and p53 signal transduction pathways contain four tandem copies of the NF-κB consensus sequence or a p53 response element located upstream of the Herpes simplex virus thymidine kinase (HSVtk) minimal TA promoter fused with the firefly luciferase (FL) gene. As control, the pAP1-TA-Luc monitoring induction of the activator protein 1 (AP1) and pTA-luc were transfected bearing multiple copies of the AP1 enhancer and/or the TA promoter fused to FL. The phRL-TK vector (Promega) containing the HSVtk promoter upstream of the renilla luciferase (RL) gene was used as an internal control of transfection. SW1736 and C643 cells seeded in 24-well plates were transiently transfected for 3 h at 37°C with 0.4 μg reporter gene construct and 0.04 μg phRL-TK DNA plus 1 μl lipofectamine and 4 μl Plus reagent (Life Technologies) in Optimem medium followed by exposure of the cells to graded bortezomib-concentrations for 24 h. The reporter gene activities were correlated to the luminescence (relative light units; RLU) measured by use of the Dual-luciferase reporter assay system (Promega) in the luminometer.



Supplemental Figure 2: For determination of NF-κB- and p53 activity SW1736 (A) and C643 (B) cells were cotransfected with phRL-TK and one of the following constructs (see Materials and Methods): pNF-κB-TA-Luc, pp53-TA-Luc, pAP1-TA-Luc (positive control) or pTA-Luc (negative control). Subsequently, the cells were treated with graded concentrations of bortezomib for 24 h or left untreated. Data represent the activity of p53, NF-κB and AP1 correlated to the luminescence (relative luminescence units). The relative luminescence units (RLU) were calculated as follows: RLU = RLU FL activity/RLU RL activity). *p≤0.05

Measurement of Apoptosis

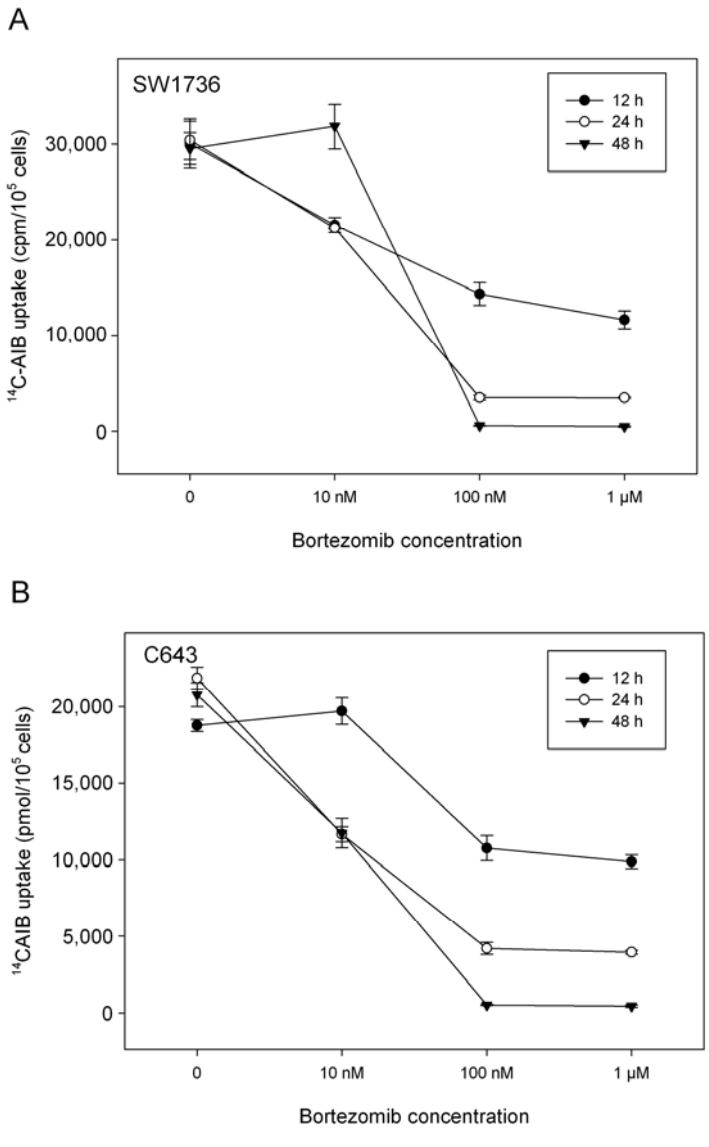
Apoptosis was measured by the Annexin V/Propidium Iodide assay. Samples of the bortezomib-treated and untreated cells were harvested after indicated time points, centrifuged for 5 min at 800 rpm, washed with cold PBS, and suspended in 100 μL cold annexin-binding buffer (10 mmol/L *N*-(2-hydroxyethyl)-piperazine-*N'*-(2-ethanesulfonic acid), 150 mmol/L NaCl, 5 mmol/L KCl, 1 mmol/L MgCl2, 1.8 mmol/L CaCl2, pH 7.4). After the addition of 1.5 μL annexin V-Alexa Fluor 488 (Molecular Probes) and an incubation period of 15 min, the suspensions were mixed with 300 μL cold annexin-binding buffer. Flow cytometry measurements were performed with mixtures of 100 μL cell suspension, 900 μL cold annexin-binding buffer, and 10 μL propidium iodide solution (250 μg/mL). The fluorescence emissions were adjusted to 530 (FL-I) and >575 nm (FL-III) and measured in triplicate.



Supplemental Figure 3.

Aminoisobutyric Acid Uptake

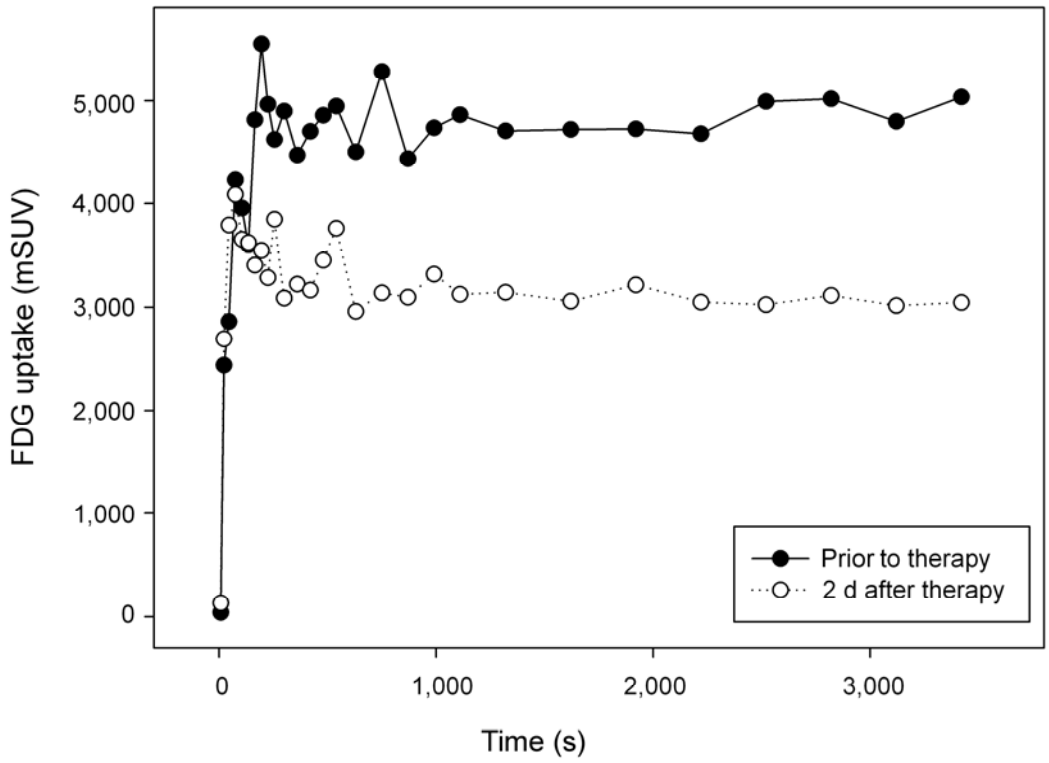
For the AIB uptake the cells were pre-incubated in Earle's Solution medium (Gibco) for 15 min, before 74kBq 2-[1-^{14}C]aminoisobutyric acid (1,9GBq/mmol=51mCi/mmol in 10ml 0,01N HCl; 37,0MBq=1,0mCi) per well was added. After incubation for 10 min the cells were washed twice with ice-cold PBS and lysed with 500µl 0.3 N NaOH on ice. The radioactivity in 0.3ml lysate was quantified by scintillation counting.



Supplemental Figure 4: Uptake of ^{14}C-AIB in SW1736 (A) and C643 cells (B) after the treatment with 10-fold dilutions of bortezomib for 12 h to 48 h. After incubation of

the ATC cell lines with 74kBq ^{14}C-AIB for 10 min the radioactivity in the lysate of each sample was quantified by scintillation counting and normalized to 10^5 cells. The data represent the mean values and standard deviations of triplicate samples.

Time activity curves of tumor SUVmax values in a SW1736 tumor prior and after treatment; C and D.



Supplemental Figure 5.

Affymetrix Gene Chip Analysis

Synthesis of double-stranded (ds) cDNA, in vitro transcriptions (IVT) of ds cDNA into cRNA and hybridizations to GeneChip Human Genome U133A 2.0 arrays (Affymetrix) were carried out in an in-house gene array core facility according to the manufacturer's one-cycle target labelling protocol (GeneChip Expression Analysis, Affymetrix) using reagents and kits provided by Affymetrix. Briefly, after spiking with the GeneChip eukaryotic poly-A RNA control kit total RNA was reverse transcribed using the one-cycle cDNA synthesis kit with T7-oligo(dT) primer and superscriptII reverse transcriptase. Second strand cDNA was synthesized by adding E. coli DNA polymerase I, ribonuclease H, E. coli DNA ligase and T4 DNA polymerase. After clean-up using cDNA spin columns from the sample cleanup module ds cDNA was in vitro transcribed using the IVT labelling Kit (Affymetrix). Biotin-labelled-cRNA was purified by cRNA cleanup spin columns provided in the sample cleanup module and fragmented with RNA fragmentation buffer. GeneChip HG U133A 2.0 arrays were hybridized with fragmented cRNAs for 16h, stained with streptavidin/R-phycoerythrin (SAPE) conjugate from the hybridization wash and stain kit and scanned in a GeneArray scanner (Agilent Technologies). Quality of total RNA, ds cDNA, IVT cRNA synthesis and fragmentation was checked using an agilent 2100 bioanalyzer or agarose gel electrophoresis. The relative quantification of transcriptional changes was performed by comparison of gene expression in bortezomib-treated versus untreated cells.

Supplemental Table 1: Transcriptional Profile of C643 cells

	12h	24h	48h
Apoptosis-related			
growth arrest and DNA-damage-inducible34	11.36	22.5	1.79
growth arrest and DNA-damage-inducible, alpha	8.36	18.57	1.56
Bcl2-associated athanogene 3 (BAG3)	6.36	6.93	0.96
Heat shock proteins			
heat shock 70kD protein 1A	100.85	112.94	1.49
heat shock 70kD protein 6 (Hsp70B)	81.81	315.73	1.55
heat shock 70kD protein 1B (HspA1B)	38.03	45.64	1.11
heat shock 105kD	27.33	33.07	1.45
heat shock 40kD (hspF1)	15.77	17.91	1.03
heat shock protein (hsp110)	13.82	12.99	1.32
heat shock 105kD (hsp105B)	10.02	11.8	1.27
stress-induced-phosphoprotein	8.34	8.84	1.55
stress-induced-phosphoprotein 1	7.79	8.27	1.56
heat shock 90kD	6.96	7.68	1.08
heat shock protein apg-2	6.54	5.81	1.34
heat shock DNAJ-like 2 (HsJ2)	5.96	6.22	1.19
heat shock DNAJ-like 1	5.25	6.56	0.95
heat shock 70kD protein 9B (HspA9B)	5.01	6.6	1.37
Proteasome pathway			
ubiquitin specific protease 9	181.25	0.75	4.5
proteasome 26S subunit	6.4	5.89	5.29
ubiquitin-conjugating enzyme E2H	5.89	8.81	1.62
proteasome 26S subunit, ATPase 4	5.7	7.0	1.79
proteasome 26S subunit, non-ATPase	5.69	4.36	3.02
proteasome 26S subunit, non-ATPase 12	5.63	3.83	4.65
Growth signaling/Cell cycle			
insulin-like growth factor 2	165.35	14.2	10.95
serinethreonine protein kinase	99.33	0.5	16.67
protease inhibitor 12	73.9	11.3	9.6
serinethreoninekinase17b	24.33	11.37	14.97
insulin-like growth factor 1	23.76	1.51	0.8
serine (or cysteine) proteinase inhibitor, clade E	8.81	4.27	10.52
tyrosine kinase	7.87	4.01	2.54
protein kinase inhibitor α	6.44	1.5	0.65
cyclin-E binding protein 1	5.14	3.54	6.16

C643 cells were treated with 100 nM bortezomib for indicated time periods. Values represent the ratio of transcriptional changes in bortezomib-treated relative to untreated cells measured by gene chip analysis.

Supplemental Table 2: Transcriptional Profile of SW1736 cells

	12h	24h	48h
Apoptosis-related			
death-associated protein kinase3	15.89	14.2	3.62
Bcl2-associated athanoge3 (BAG3)	8.82	8.48	5.08
$TRAIL_{R4B}$	8.5	7.67	4.26
growth arrest and DNA-damage-inducible, beta	7.56	3.84	4.47
Fas- (TNFRSF6) associated factor 1	6.55	7.05	3.88
Fas-interacting serinethreonine kinase	5.66	4.97	1.84
Heat shock proteins			
heat shock 70kD protein 6 (Hsp70B)	480.9	439.33	233.42
heat shock 70kD protein Hsp70B	364.88	329.77	120.25
heat shock 70kD protein (HspA1A)	89.39	79.98	48.75
heat shock 70kD protein 1B (HspA1B)	55.94	48.11	27.67
heat shock 40kD protein 1 (HspF1)	27.58	17.23	8.38
heat shock 105kD	19.44	13.71	8.82
DnaJ-like heat shock protein 40	18.92	14.53	9.05
stress-induced-phosphoprotein 1	14.83	11.75	6.68
heat shock 27kD protein (HspB1)	9.66	2.41	2.28
heat shock protein (Hsp110 family)	9.62	4.03	2.55
heat shock protein, neuronal DnaJ-like	8.88	6.06	4.5
dnaJ (Hsp40) homolog, subfamily B member 4	7.92	6.04	3.3
heat shock 70kDprotein5	7.62	9.11	2.77
heat shock 105kD (Hsp105B)	7.31	5.78	4.09
DnaJ (Hsp40) homolog, subfamily A member 1	5.61	2.98	2.1
Proteasome pathway			
ubiquitin specific protease 9	99	92	46
ubiquitin-conjugating enzyme E2H	34.17	42.67	28.33
Growth signaling/Cell cycle			
insulin-like growth factor 1 receptor	10.37	7.23	2.44
insulin-like growth factor 2 receptor	5.64	5.45	1.5

SW1736 cells were treated with 100 nM bortezomib for indicated time periods. Values represent the ratio of transcriptional changes in bortezomib-treated relative to untreated cells measured by gene chip analysis.

Supplemental Table 3: Transcriptional Profile of SW1736 cells

	12h	24h	48h
Apoptosis-related			
Bcl2-like1	37.61	67.72	31.39
death-associated protein kinase3	10.25	6.48	3.05
TNFR SF6 – Apoptosis antigen	8.96	1.44	1.98
Bcl-x beta (Bcl-2-like)	8.52	14.75	6.66
Fas-Apo-1	7.71	4.13	2.67
Tumor necrosis factor superfamily 7 (TNFSF7)	26.33	2.07	1.6
Tumor necrosis factor superfamily 9 (TNFSF9)	10.13	2.65	0.12
Tumor necrosis factor receptor member 6	7.71	4.13	2.67
Tumor necrosis factor superfamily 10 (TNFSF10)	5.29	1.21	1.42
Tumor necrosis factor superfamily 5 (TNFSF5)	5.28	1.32	1.22
Tumor necrosis factor superfamily 11B (TNFSF11B)	5.08	2.49	1.03
Thyroid-related			
paired box gene (Pax8)	540.96	0.49	0.85
Pax8 transcript variant 8B	96.4	14.2	22.2
thyroid transcription factor (TTF) 2	39.92	0.83	1.33
Pax8 transcript variant 8E	28.96	0.07	0.23
Pax8A isoform	17.92	0.29	0.24
Pax8C isoform	8.15	1.27	0.57

SW1736 cells were treated with or 10 nM bortezomib for indicated time periods. Values represent the ratio of transcriptional changes in bortezomib-treated relative to untreated cells measured by gene chip analysis.