Proteomic identification of aldo-keto reductase AKR1B10 induction after treatment of colorectal cancer cells with the proteasome inhibitor bortezomib


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Abstract
Targeting the ubiquitin-proteasome pathway with the proteasome inhibitor bortezomib has emerged as a promising approach for the treatment of several malignancies. The cellular and molecular effects of this agent on colorectal cancer cells are poorly characterized. This study investigated the antiproliferative effect of bortezomib on colorectal cancer cell lines (Caco-2 and HRT-18). In order to define the proteins potentially involved in the mechanisms of action, proteome profiling was applied to detect the proteins altered by bortezomib. The in vitro efficacy of bortezomib as a single agent in colorectal cancer cells was confirmed. Proteome profiling with two-dimensional PAGE followed by mass spectrometry revealed the up-regulation of the major inducible isoform of heat shock protein 70 (hsp72) and lactate dehydrogenase B in both cell lines, as well as the induction of aldo-keto reductase family 1 member B10 (AKR1B10) in HRT-18 cells. Both AKR1B10 and hsp72 exert cell-protective functions. This study shows for the first time a bortezomib-induced up-regulation of AKR1B10. Small interfering RNA-mediated inhibition of this enzyme with known intracellular detoxification function sensitized HRT-18 cells to therapy with the proteasome inhibitor. To further characterize the relevance of AKR1B10 for colorectal tumors, immunohistochemical expression was shown in 23.2% of 125 tumor specimens. These findings indicate that AKR1B10 might be a target for combination therapy with bortezomib.

Introduction
Colorectal cancer is a leading cause of cancer death throughout the world (1). Although chemotherapy has improved the outcome of patients with metastatic disease, new therapies with novel mechanisms of action are warranted. Major molecular pathways involved in the pathogenesis of colorectal carcinoma such as β-catenin, Smad4, p53, and nuclear factor κB (NF-κB) downstream of Ras are regulated by the ubiquitin-proteasome system providing a rationale for proteasome inhibition (2). The 26S proteasome, a large multicatalytic protease complex, degrades several regulatory proteins and inhibitors after their ligation with the protein ubiquitin and mediates various cellular functions such as transcription, stress response, cell cycle regulation, apoptosis, ribosome biogenesis, cellular differentiation, DNA repair, and oncogenesis. Its involvement in malignancy and the finding that it regulates the key antiapoptotic transcription factor NF-κB has formed the basis for the exploration of proteasome inhibition as an antineoplastic strategy (3).

Bortezomib (Velcade, formerly known as PS-341) is a novel dipeptide boronic acid that reversibly inhibits the chymotryptic activity of the proteasome (4). Preclinical studies have shown that this compound decreases proliferation, induces G2-M arrest and apoptosis, enhances the activity of chemotherapy or radiation, and reverses chemoresistance in a variety of hematologic and solid malignancy models. Bortezomib is already approved for the treatment of patients with relapsed or refractory multiple myeloma and has shown activity in solid tumors (5). In colorectal cancer, preclinical testing with bortezomib has shown synergy with irinotecan or ionizing radiation and resulted in significant tumor regression in xenograft models (6–8). However, the results of clinical efficacy are very limited and biomarkers related to response or resistance are lacking (9).

Recently, proteomic technologies evolved as a useful strategy to track biological responses to therapy (10–12). With this method, hundreds to thousands of proteins are displayed at the same time and dynamic changes in response to drug therapy can be detected. To define proteins...
that are up-regulated or down-regulated after therapy with bortezomib, we created proteome profiles of the colorectal cancer cell lines, HRT-18 and Caco-2, using two-dimensional PAGE (13). The highly differentiated Caco-2 cell line (doubling time, 80 hours) and the poorly differentiated HRT-18 cell line (doubling time, 20 hours) are characterized by high epidermal growth factor receptor expression and a mutation status with mutant APC/wild-type p53/wild-type KRAS/wild-type BRAF for Caco-2 and mutant AP/C/mutant p53/mutant KRAS/wild-type BRAF for HRT-18 (11, 14–16). Differential analysis before and after therapy revealed three proteins specifically up-regulated. Our data suggest that proteome-based technologies can reveal proteins influenced by proteasome inhibition and help to further elucidate the complex mode of action.

**Materials and Methods**

**Cell Cultures**

The colorectal cancer cell lines Caco-2 and HRT-18 were purchased from American Type Culture Collection. Caco-2 was grown in MEM with Earle’s salts (PAA Laboratories) supplemented with 2 mmol/L of l-glutamine, 1.5 g/L of sodium bicarbonate, 0.1 mmol/L of nonessential amino acids, 50 units/mL of penicillin, 50 μg/mL of streptomycin, and 10% FCS (v/v; Sigma-Aldrich). HRT-18 cells were maintained in RPMI 1640 (PAA Laboratories) containing 2 mmol/L of l-glutamine, 50 units/mL of penicillin, 50 μg/mL of streptomycin, and 10% FCS (v/v). Cultures were incubated in a 5% CO2-humidified atmosphere.

**Drug Sensitivity Assays**

Bortezomib resolved in 0.9% NaCl was obtained from the institutional pharmacy. Drug sensitivity testing was done using WST-1 assay and direct cell count for Caco-2 and HRT-18 cells, at different times (24, 48, 72, and 96 h) and doses (range, 0.01–0.1 μmol/L) of bortezomib. These concentrations are within those achieved clinically in the plasma of patients treated with bortezomib (17). To screen for IC50 values, an indirect measure of cell viability and proliferation was done by determination of the metabolic activity of cells with a colorimetric assay based on cleavage of the tetrazolium salt WST-1 by mitochondrial dehydrogenases (cell proliferation reagent WST1; Roche Diagnostics). For this assay, cells were seeded in 96-well plates (5,000 cells/well) and allowed to adhere for 24 h. On day 2, the medium was removed and replaced with medium containing bortezomib at the doses and times mentioned above.

Direct cell count and viability of bortezomib-treated cells was assessed 48 h after exposure to bortezomib (0.01–0.08 μmol/L) using hemocytometer and trypan blue dye exclusion test (Sigma-Aldrich) according to the protocol of the manufacturer in a six-well plate format (150,000 cells/well).

To determine the percentage of cells that are actively undergoing apoptosis 48 h after incubation with bortezomib (0.02–0.05 μmol/L), Annexin V FITC and propidium iodide–stained cells were subjected to flow cytometry (Becton Dickinson) according to the recommendations of the manufacturer (Annexin V FITC Apoptosis Detection Kit; BD PharMingen). Corresponding cell culture assays were prepared in a six-well plate format (150,000 cells/well).

**Two-dimensional PAGE**

HRT-18 and Caco-2 cells (5 × 10⁶) were seeded in T75 flasks and analyzed with two-dimensional PAGE 6, 12, 24, and 48 h after treatment with 0.03 μmol/L of bortezomib. Controls were taken at times 0 and 24 h. For sample preparation, cells were scraped, harvested, and lysed as previously described (11).

For first-dimension isoelectric focusing, 100 μg of protein per sample diluted to 450 μL with rehydration buffer (7 mol/L urea, 2 mol/L thiourea, 1% ASB-14, 0.5% IPG buffer, and 60 mmol/L DTT) were loaded on immobilized 24 cm (pH 3–10) NL gradient strips (Amersham Biosciences). Active rehydration (50 V) was carried out at 20°C for 12 h. Isoelectric focusing was done at 250 V for 30 min, 500 V for 1 h, 2,000 V for 1 h, and finally, at 8,000 V until 55,000 V/h were reached in total.

For the second dimension, samples were separated on 12.5% polyacrylamide gels with the Ettan DALT twelve System following the standard procedures recommended by the manufacturer (Amersham Biosciences). After electrophoresis, gels were silver-stained, scanned with ImageScanner, and analyzed with ImageMaster 2D Platinum software (Amersham Biosciences). To obtain a standard gel for each individual cell line, spot detection and matching were done using gels from three runs. These standard gels were then matched to yield information about differentially expressed proteins. We selected spots overexpressed or underexpressed by more than 4-fold to exclude artifacts caused by gel-to-gel variation observed with silver staining, and then checked spots by eye in order to avoid possible software errors (13, 18).

Protein analysis was done as previously published (11). Three protein digests were separated using capillary high-performance liquid chromatography connected online to an LCQ ion trap instrument (ThermoFinnigan) equipped with a nanospray interface. Tandem mass spectra were searched against a human database using SEQUEST (LCQ BioWorks; ThermoFinnigan).

**Verification of hsp72 and AKR1B10 Expression Profile by Western Blot**

HRT-18 and Caco-2 cells were lysed and processed as described previously (11). For each lane, 40 μg of protein was separated by 12% SDS-PAGE and electroblotted onto nitrocellulose membranes (Schleicher & Schuell), incubated for 1 h at room temperature in blocking buffer containing rabbit anti-hsp70 (hsp72) polyclonal antibody (Stressgen Biotechnologies), or AKR1B10 polyclonal antibody (A01; Abnova Corporation). Immunodetection was done with an enhanced chemiluminescence system (Amersham Biosciences) after incubation with horseradish peroxidase–conjugated antioimmun mouse or antirabbit secondary antibody (Amersham Biosciences).

**NF-κB Western Blot Analysis**

The predominant form of NF-κB exists as a heterodimeric complex of 50 kDa and 65 kDa protein subunits. In its
inactive form, NF-κB is sequestered in the cytoplasm. Activated NF-κB is free to translocate to the nucleus and induces the expression of proinflammatory and antiapoptotic genes. Isolation of nuclear and cytoplasmic fractions was done according to the procedure described by Wang et al. (19). Western blotting for NF-κB was done as described above using p65-specific antibodies (Cell Signaling Technology, Inc.). For a positive control, tumor necrosis factor-α, known to induce NF-κB, was applied (10 ng/mL, 2 h). Other samples were treated with bortezomib (0.03 μmol/L), and translocation of p65 was assessed 3 and 6 h after treatment.

**AKR1B10 Silencing by Small Interfering RNA Transfection**

Two specific small interfering RNAs (siRNA) targeted to 3’-untranslational siRNA1, 5’CGAGAUCGAGGGCUGU- GUU(5’; according to Yan et al. (20)) and to encoding siRNA2, exon 2, 5’GUGCCUAUGCUAUCAGAA(5’) regions of AKR1B10 were chemically synthesized (Ambion). For negative control, the AllStars Negative Control siRNA and for a FITC-conjugated transfection control, the Negative Control siRNA AF488 was used (Qiagen). For siRNA delivery, 5,000 HRT-18 cells in complete RPMI medium were seeded in 96-well plates on top of the siRNA-HiPerFect Reagent transfection complexes for reverse transfection, according to the instructions of the manufacturer (Qiagen). Transfected cells were incubated under their normal growth conditions (37°C, 5% CO2). Gene silencing was monitored after 24 to 96 h of incubation with real-time reverse transcription PCR (RT-PCR) analysis. The effects of combination therapy with bortezomib were studied with drug sensitivity assays (see above) and cell cycle analysis. Morphologic changes were characterized by phase contrast microscopy of cultured cells (Olympus IX70 inverted tissue culture microscope, Olympus SC35 camera).

**RT-PCR Analysis**

cDNA synthesis was carried out directly with cell lysates (5 μL) prepared from cultured HRT-18 cells plated in 96-well plates at a density of 5 × 10^5 cells per well (control, negative siRNA, siRNA1, and siRNA2) using the FastLane Cell Multiplex kit (Qiagen). Subsequently, RT-PCR assays were done on an ABI Prism 7000 Sequence Detection System using the Quantitect Probe RT-PCR kit (Qiagen). For AKR1B10 expression determination, we used the FAM-labeled expression assay (Applied Biosystems) spanning exons 3 and 4. The control housekeeping gene was human β-actin and measured with a VIC-labeled expression assay (Applied Biosystems). Both were analyzed in the same setup. The variables for PCR amplification were 95°C for 10 min for activation and 40 cycles at 95°C for 15 s, and 60°C for 1 min. Relative expression of mRNA was determined from the point at which each amplification curve crossed the threshold (Ct) line. AKR1B10 expression was normalized to Ct values of the housekeeping gene β-actin using the comparative threshold cycle method $2^{-\Delta\Delta CT}$ and expressed as a percentage of expression in cells transfected with negative siRNA (21).

**Cell Cycle Analysis**

HRT-18 cells (300,000) were seeded on six-well plates, transfected with siRNA1 (30 nmol/L), siRNA2 (10 nmol/L), or negative siRNA control (30 nmol/L) and incubated for 24 h to trigger AKR1B10 silencing. Subsequently, cells were treated with 0.03 μmol/L of bortezomib. After 24 h of treatment, cells were harvested, washed twice with PBS, stained in 500 μL of DNA staining solution (50 μg/mL propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100), incubated in the dark for 30 min and kept at 4°C until FACS analysis. Nuclear DNA content was determined by analysis of propidium iodide-stained nuclei, reflecting the fraction of cells at each point in the cell cycle ($G_0$-$G_1$, S, and $G_2$-$M$, FACS caliber; Becton Dickinson). Results were represented as DNA histograms using CellQuest software (Becton Dickinson).

**AKR1B10 Enzymatic Activity Assay**

The carbonyl reduction activity of AKR1B10 was monitored spectrophotometrically by measuring the decrease in the absorbance of the cofactor NADPH at 340 nm. Preparation of the recombinant enzyme and assay conditions were done as previously described by coauthor Martin and colleagues (22). Bortezomib was added in concentrations up to 1 mmol/L.

**Patient Samples**

This study was comprised of 125 human consecutive colorectal cancer patients, who underwent surgery in the years 1996 to 1998 according to the recommendations of the German Society of Surgery. Complete clinical records and follow-up information were available in all cases. The minimum follow-up period for patients still alive was 60 months. Surgical material was fixed in formalin and routinely processed. The tumors were classified according to the Tumor-Node-Metastasis System (6th edition) and histopathologic diagnosis was made according to the WHO classification of tumors of the rectum and colon (23). Patients with International Union Against Cancer stages III and IV colorectal carcinoma received standardized chemotherapy in the adjuvant setting. According to the recommendations of the German Surgical Oncology Working Group (CAO), patients with stages I and II colorectal carcinoma were not advised to undergo any adjuvant treatment.

**Tissue Microarray Construction**

Vital tumor areas were selected and marked on the respective H&E-stained slides. The most representative tumor area was carefully selected and marked on each H&E slide. In the case of tumor heterogeneity, areas with the lowest degree of differentiation were selected. The tissue microarrays were constructed using a manual tissue-array instrument (Beecher Instruments). Three 0.6-mm-thick tissue cores were taken from each colorectal carcinoma specimen. One section from each tissue microarray was stained with H&E. Each block contained normal colon tissue as controls.

**AKR1B10, Cyclooxygenase-2, and Phosphorylated AKT Immunohistochemistry**

To confirm the specificity of the AKR1B10 antibody (monoclonal rabbit anti-human AKR1B10 antibody, clone 1A6; Abnova Corporation), and to obtain positive controls, cell pellets of the colorectal cell lines Caco-2 and HRT-18 were made. Caco-2 cells and HRT-18 cells (5 × 10^6) were seeded in T75 flasks, untreated control cells and bortezomib-treated HRT-18 cells (0.03 μmol/L) were removed from the flasks by
trypsinization and pelleted in 1× PBS 48 h after incubation. The pellets were fixed with 4% formaldehyde, placed in filter paper and in a cassette, and routinely processed for paraffin embedding. Immunohistochemistry was done on 5-μm-thick paraffin sections of cell pellets, or tissue microarray and antigen retrieval was carried out with 0.01 mol/L of citrate buffer (pH 6.1) for 20 min in a hot water bath (95°C). The primary antibody was incubated for 30 min at 1:300 dilution. Detection was achieved with the Zyto-Chem-Plus HRP kit (Zytomed). Replacement of the primary antibodies with mouse immunoglobulin served as negative control. Cyclooxygenase-2 (COX-2) and phosphorylated Akt immunohistochemistry were done as previously described (24, 25).

Ki67 Immunohistochemistry and Terminal Deoxyribonucleotide Transferase–Mediated dUTP Nick End Labeling

Ki67 immunohistochemistry was done as reported recently (24). The growth fraction was defined as the percentage of Ki67-positive randomly chosen nuclei per 600 tumor cells. In situ DNA fragmentation was established using the terminal deoxyribonucleotide transferase–mediated dUTP nick end labeling technique in paraffin-embedded sections. We used the ApopTag Plus Peroxidase In situ Apoptosis Detection kit (Intergen Company) according to the recommendations of the manufacturer.

Statistical Analysis

For statistical evaluation of cell culture experiments, mean values and SD were calculated from three independent experiments and compared with Student’s independent samples t test using SPSS 15.0 software (SPSS, Inc.). Significance was defined as P < 0.05. For patient studies, we analyzed a possible relation between AKR1B10 expression with clinicopathologic variables, the Ki-67 labeling index, apoptotic rate, COX-2, and pAKT expression, as recently reported by coauthor Schmitz and colleagues for this patient collective (24). All data were statistically analyzed using the SPSS 15.0 software (SPSS). Relationships between ordinal variables were investigated using a two-tailed analysis (or Fisher’s exact test if the number of patients was small). The relationship between categorical data and numerical data was determined using the Kruskal-Wallis test or the Mann-Whitney test, depending on the number of groups. For survival analysis, only patients who died from colorectal cancer were included in statistical analysis. Disease-specific overall survival curves were estimated using the Kaplan-Meier method, and any differences in the survival curves were compared using the log-rank test. Cox regression analysis was done for multivariate survival analysis. Ninety-five percent confidence intervals were used throughout.

Results

Sensitivity of Caco-2 and HRT-18 Cells to Bortezomib

The colorectal cancer cell lines Caco-2 and HRT-18 were exposed for 24 to 96 hours to various concentrations of bortezomib (0.01–0.1 μmol/L), revealing a dose-dependent and time-dependent response. The IC50 values obtained by WST-1 colorimetric assay ranged from 0.03 to 0.04 μmol/L in Caco-2, and from 0.04 to 0.06 μmol/L in HRT-18 cells (Fig. 1A). For proteome studies, we intended to define a bortezomib concentration with antiproliferative but low apoptotic effect. Analysis of cell viability with trypan blue dye exclusion method 48 hours after therapy showed a dose-dependent increase of dead cells, reaching LC50 earlier in Caco-2 (~0.05 μmol/L) than in HRT-18 cells (~0.08 μmol/L; Fig. 1B). At this time point, Annexin V FITC/propidium iodide FACS analysis revealed apoptosis induction with a dose-dependent increase of Annexin V FITC–positive cells (Fig. 1C). Based on these results, proteome studies were done with a bortezomib concentration of 0.03 μmol/L.

Confirmation of Bortezomib-Induced hsp72 and AKR1B10 Expression

In both colorectal cancer cells, treatment with 0.03 μmol/L of bortezomib increased the level of hsp72 as detected by two-dimensional PAGE (Fig. 3A). Western blot analysis confirmed the up-regulation of the stress-inducible form of hsp72 (hsp70). This effect appeared after 6 hours of treatment, increased progressively with time, and declined after 24 hours (Fig. 3B). According to two-dimensional PAGE profiles, up-regulation of AKR1B10 expression occurred in HRT-18 cells after treatment with bortezomib (0.03 μmol/L). This cell line constitutively expressed AKR1B10 (Fig. 3A). Western blot analysis verified an increase in the amount of this enzyme after 6 hours to maximum levels obtained 48 hours after treatment. In the Caco-2 cell line, AKR1B10 was not expressed (Fig. 3B).

Involvement of NF-κB in Bortezomib-Induced AKR1B10 Expression

Because of an association observed between NF-κB activation and up-regulation of aldose reductase, another member of the aldo-keto reductase family, we examined a
potential involvement of NF-κB induction in up-regulation of AKR1B10 (26). HRT-18 cells were separated into cytosolic and nuclear fractions and translocation of NF-κB (p65) protein was analyzed by Western blot analysis using p65-specific antibodies. The untreated sample as well as bortezomib-treated samples (0.03 μmol/L) showed only the presence of inactive, cytosolic NF-κB without translocation to the nucleus. In the positive control, after treatment with the known NF-κB-inducer tumor necrosis factor-α (10 ng/mL, 2 hours), translocation of p65 to the nucleus occurred (Fig. 4).

Influence of Anti-AKR1B10 siRNA on the Sensitivity of HRT-18 Cells to Bortezomib

siRNA Transfection and Silencing Effects. Two siRNAs, targeted to the 3′ untranslated (siRNA1) and encoding...
(siRNA2) regions of AKR1B10 mRNA were used to confirm the specificity of the AKR1B10 knockdown. Transfection with a nontargeting fluorescent control siRNA indicated that 86.5% (±3.5%) of HRT-18 cells had been transfected (ratio of transfection reagent to culture medium, 1:266.7). As monitored by RT-PCR analysis, both siRNA1 and siRNA2 induced specific AKR1B10 down-regulation in HRT-18 cells as compared to transfection with a nontargeting siRNA-negative control and to cells incubated with a transfection reagent without siRNA (Fig. 5A). Expression of AKR1B10 was normalized to the housekeeping gene β-actin using the $2^{-\Delta\Delta CT}$ comparative method, and data were expressed relative to cells transfected with negative control siRNA. In the presence of 30 nmol/L of siRNA1 or 10 nmol/L of siRNA2, the relative expression of AKR1B10 was reduced and maximum silencing occurred 72 hours after siRNA delivery (74.7 ± 9.3% for siRNA1 and 78.2 ± 4.5% for siRNA2).

**Effects of Combined Treatment**

After successful transfection, we investigated whether the silencing of AKR1B10 mRNA affected susceptibility to bortezomib in the HRT-18 cell line. In pilot experiments, siRNA-transfected and siRNA-untransfected cells were exposed to bortezomib (0.03 μmol/L; the concentration used for proteome and Western blot studies), and effects have been studied with WST-1 assay, phase-contrast light microscopy, and cell cycle analysis. Drug treatment was undertaken 24 hours after siRNA transfection, so that maximum silencing of AKR1B10 occurred 48 hours after drug delivery. After therapy with 0.03 μmol/L of bortezomib, metabolic activity of specifically transfected cells was reduced to 23.45 ± 4.37% (siRNA1) or 32.67 ± 11.11% (siRNA2) after 48 hours of incubation, and to 40.85 ± 3.84% (siRNA1) or 17.64 ± 8.71% (siRNA2) after 72 hours of incubation, as compared with control cells (incubated only with transfection reagent; Fig. 5B). Phase-contrast light microscopy...
images of these cells showed profound morphologic changes 72 hours after therapy. Whereas drug-naïve cells reached confluency, bortezomib-treated cells showed reduced density and an increase in cell size. Cells with combined treatment presented cell islets with large single cells (Fig. 5C). An exemplary analysis to study effects at the 96-hour time point displayed a decrease of viable cells (trypan blue negative) of 65.5% with combined therapy (siRNA1/bortezomib, 0.03 μmol/L) compared with monotherapy (bortezomib, 0.03 μmol/L + transfection reagent; mean value of viable cells ± SD siRNA1/bortezomib, 455,000 ± 45,000; bortezomib, 0.03 μmol/L + transfection reagent, 1,096,666.7 ± 73,181.6; \( P = 0.005 \)).

Cell cycle analysis revealed an increase in cells in G2-M phase 24 hours after therapy with 0.03 μmol/L of bortezomib in transfected and untransfected HRT-18 cells (Fig. 5D). At this time point, the amount of dead cells detectable by means of sub-G1 assay accounted for <5%.

To extend these observations, dose-response curves revealed a significantly lower IC_{50} with the presence of anti-AKR1B10 siRNA [IC_{50} with siRNA1 and siRNA2, 0.015 μmol/L; \( P = 0.02 \) (siRNA1), \( P = 0.01 \) (siRNA2); Fig. 5E]. Both siRNAs sensitized HRT-18 cells to low doses of bortezomib (0.01–0.03 μmol/L) with...
significant earlier and stronger decrease of viable cells. Annexin V/propidium iodide FACS analysis confirmed the induction of apoptosis, as shown for monotherapy with bortezomib (Fig. 1C).

AKR1B10 Enzymatic Activity Assay
Spectrophotometric measurements of NAPDH oxidation with recombinant AKR1B10 and bortezomib revealed a depletion of the cofactor at high doses of the drug. The

Figure 5. Effects of AKR1B10 silencing on bortezomib-treated HRT-18 cells. Cells were transfected with specific siRNAs directed against AKR1B10 (siRNA1, 30 nmol/L; siRNA2, 10 nmol/L) or a nontargeting siRNA (neg siRNA, 30 nmol/L) and cultured 24 h before being exposed to the indicated concentrations of bortezomib. A, time-dependent AKR1B10 expression in HRT-18 cells transfected with siRNA1 or siRNA2. RT-PCR results are normalized to expression of the housekeeping gene β-actin using the formula $2^{-\Delta \Delta CT}$, and data are presented as the ratio of cell numbers transfected with negative control siRNA. B, cell viability based on metabolic activity of HRT-18 cells under various experimental conditions determined by means of WST-1 assay 48 and 72 h after therapy with bortezomib (0.03 μmol/L). Columns, means expressed as a percentage of control cells exposed to transfection reagent only (TFR). C, morphologic changes 72 h after 0.03 μmol/L of bortezomib treatment of untransfected and transfected HRT-18 cells visualized by phase contrast imaging (original magnification, ×10). D, cell cycle distribution 24 h after 0.03 μmol/L of bortezomib treatment of untransfected and transfected HRT-18 cells. E, dose-response curves of siRNA transfected (siRNA1 and siRNA2) and untransfected HRT-18 cells (+TFR) 48 h after treatment with bortezomib (0.01–0.03 μmol/L) obtained with trypan blue dye exclusion test. The percentage of viable cells (excluding trypan blue) is expressed relative to control cells (untransfected + TFR, siRNA1 and siRNA2 transfected) without bortezomib treatment. For bortezomib 0.01 and 0.02 μmol/L corresponding data of the AKR1B10-negative cell line Caco-2 were added to the graphic. A and E, points, mean of three experiments; bars, SD; *, P < 0.05; **, P ≤ 0.001 as compared with corresponding controls (Student’s t test).
reaction rate in the presence of 0.167 mmol/L of bortezomib was 2.6 nmol/(min·mg) and 7.85 nmol/(min·mg) with 1 mmol/L of bortezomib.

**AKR1B10, COX-2, and Phosphorylated AKT Immunohistochemical Analysis in Colorectal Cancer**

Immunohistochemical analysis of *in vitro* cell pellets confirmed the specificity of AKR1B10 staining with constitutive expression and bortezomib-related up-regulation in HRT-18 cells and absent expression in Caco-2 cells (Fig. 6A–C). Most specimens lacked AKR1B10 immunostaining. Although normal mucosa revealed a consistent positive immunostaining of the apical mucosal epithelial cells, cells in the basal crypt remained negative. Tumor cells showed a combined nuclear and cytoplasmic immunostaining. If any of the tumor cells showed specific AKR1B10 immunostaining regardless of staining intensity, the case was classified as positive. In total, AKR1B10 immunohistochemistry of the tissue microarray containing 125 tumor samples from patients with colorectal cancer revealed positive immunostaining in 29 tissue samples (23.2%; for representative example, see Fig. 6D). The amount of AKR1B10-positive cells ranged from 1% to 45% (mean, 9.81 ± 11.26%).

Regarding COX-2 expression, a total of 123 tumors were analyzed; the two others showed loss of discs. Tumor cells exhibited a specific cytoplasmic staining pattern. Classification was done according to the amount of positively stained tumor cells: negative (0–10%) and positive (>10%). In all, 93 tumors were classified as positive and 30 as negative.

Phosphorylated Akt immunostaining was available for 124 cases. Disc loss occurred in one case. Immunohistochemistry of colorectal tumor tissue revealed a specific cytoplasmic staining, whereas normal mucosal tissue exhibited no staining. According to their cytoplasmic staining intensity, tumors were classified as negative, moderate, or strong. In all, 26 tumors were classified as negative, 71 as moderate, and 27 as strong.

**Correlation between Pathologic Data and Immunodetectable AKR1B10**

To clarify which factors correlate with AKR1B10 immunostaining, we carried out a statistical analysis that examined clinicopathologic variables and the expression of molecules reported previously in this patient cohort (24). AKR1B10 expression was not correlated with tumor-node-metastasis stage of tumors, amount of Ki-67–positive cells, or apoptotic rate (data not shown). Overall survival did not significantly differ between AKR1B10-positive and AKR1B10-negative tumors as assessed by univariate Kaplan-Meier survival analysis. With regard to previously characterized molecular markers in this patient cohort (24), AKR1B10 expression was significantly associated with the expression of COX-2 ($P = 0.022$) and pAKT ($P = 0.045$).

**Discussion**

The proteasome inhibitor bortezomib exhibits antiproliferative, proapoptotic, and antiangiogenic activities in several cancer models (6). Inhibition of NF-κB, stabilization of p21, p27, p53, Bid and Bax, inhibition of caveolin-1 activation, activation of c-Jun-NH2-kinase, as well as endoplasmic reticulum stress response are the molecular effects of this proteasome inhibition, but the mechanisms involved in sensitivity or resistance to this agent remain poorly understood. Although bortezomib showed significant activity in colorectal cell lines and xenograft models, it was inactive as a single agent in a phase II trial in metastatic colorectal cancer (7, 9). To define proteins with altered expression following therapy, we investigated colorectal cancer cell lines using a two-dimensional gel-based proteomic approach. Analysis of the proteome profiles before and after treatment revealed three
proteins with a significant increase in expression levels after treatment with bortezomib. All these proteins have previously been shown to be involved in malignant growth. Whereas the lactate dehydrogenase subunit LDHB is primarily a key mediator of glycolysis, hsp72 and AKR1B10, are directly involved in cytoprotective mechanisms and their dynamic expression was confirmed by Western blot (27, 28). Hsp are stress-inducible proteins with antiapoptotic properties that act as molecular chaperones for nascent proteins and assist in protecting and repairing proteins whose conformation is altered by stress, such as heat or anticancer drugs. The major stress-inducible isoform of the hsp70 family, hsp72, is expressed in human tumors, correlates with poor prognosis, and contributes to resistance to cancer therapy (29–31). Proteasome inhibitors seem to up-regulate hsp synthesis by increasing the amount of misfolded proteins as shown in breast tumor, hepatoma HepG2, myeloma, and B-lymphoma cells (32–36). Similar to these reports, in this study, hsp72 up-regulation occurred very early after the exposure of the two colorectal cancer cell lines, Caco-2 and HRT-18, to bortezomib. In contrast to induction of hsp72 after proteasome inhibition, up-regulation of AKR1B10, a member of the aldo-keto reductase (AKR) superfamily has not been previously shown. The 13 human AKRs are NAD (P)H-dependent oxidoreductases implicated in intracellular detoxification, cell carcinogenesis, and resistance to cancer therapeutics (27). They reduce aldehydes and ketones to yield primary and secondary alcohols and are regulated in response to osmotic, electrophilic, and oxidative stress. In addition to endogenous substrates, AKRs are phase I drug-metabolizing enzymes for a variety of carbonyl-containing drugs (37). AKR1B10 involvement has been described for resistance to cyclophosphamide, etacrynic acid, Adriamycin, daunorubicin, and mitomycin (38–40). Special functions of this isoform include the conversion of retinals into retinols, resulting in the reduction of intracellular retinoid acid, a signaling molecule regulating cell proliferation and differentiation (41). Furthermore, this enzyme is a regulator of fatty acid biosynthesis, an essential cell membrane component in cancer cells (42). Recently, the involvement of AKR1B10 has even been shown for non-small cell lung carcinoma, esophageal carcinogenesis, hepatocellular carcinoma, and uterine cancer in smokers (18, 43–45). Because of our observation of AKR1B10 induction following therapy with bortezomib and its reported biological functions, this protein was chosen for further functional studies.

In contrast to AKR1B10-negative Caco-2 cells, the faster growing cell line HRT-18, with less sensitivity to low doses of bortezomib, showed strong AKR1B10 up-regulation after therapy. This induction was found to be independent of NF-kB activation, contrasting NF-kB dependence observed for the enzyme, aldose reductase, the most investigated member of the AKR family (26). Constitutive expression of AKR1B10 has already been reported for the same cell line (HRT-18) and knockdown of AKR1B10 expression resulted in growth inhibition and cell susceptibility to reactive carbonyls (20). We investigated the effect of AKR1B10 silencing on treatment with bortezomib using two different siRNAs, targeting encoding and 3′untranslational regions of the AKR1B10 gene. Consistent with the findings of Yan et al. (20), AKR1B10 silencing per se caused a time-dependent but mild growth inhibition. Combined treatment of siRNA and bortezomib sensitized HRT-18 cells to low doses of bortezomib with significant earlier and stronger reduction of viable cells. G2-M arrest followed by induction of apoptosis was shown to be the underlying antiproliferative mechanism, as expected for treatment with bortezomib (3). There are two major possible explanations why additional inhibition of AKR1B10 increased sensitivity to bortezomib. (a) Bortezomib is known to cause the generation of intracellular reactive oxygen species (46, 47). Consequently, production of endogenous reactive carbonyls that lead to cellular damage is expected. Inhibition of AKR1B10 may prevent a cytoprotective response to drug-induced oxidative injury (37, 48). (b) Furthermore, AKR1B10 could be directly involved in the detoxification of bortezomib, by carbonyl reduction of the carbonyl moiety present in this agent [N-(2,3-pyrazine)carbonyl-t-phenylalanine-t-leucine boronic acid; ref. 49]. Silencing of this oxidoreductase would prolong the duration of drug exposure and thereby increase cell damage. Using a NADPH-regenerating system, we could show that bortezomib is indeed a substrate of AKR1B10. The reaction rate observed is comparable to values reported for AKR1B10 and daunorubicin (22). However, the drug doses necessary to recognize in vitro enzyme activity are more than a thousand-fold higher than the growth-inhibitory concentrations observed in tumor cells. The detoxification of endogenous reactive carbonyls may be more relevant than a direct interaction with bortezomib (48). Nevertheless, the substrate relevance of bortezomib metabolites remains to be elucidated.

The additional detection of AKR1B10 up-regulation in the WiDr colorectal cancer cell line (Fig. 3C) excludes that this is a unique HRT-18 cell type–specific phenomenon. To rule out the possibility that this protein is present exclusively in cultured cells, the in vitro relevance of this protein was determined by immunohistochemical analysis of patient samples. AKR1B10 expression was found in ~25% of colorectal carcinomas. This finding is comparable to the frequencies observed for adenocarcinoma of the lung (29.2%), cervical cancer (20.0%), and endometrial cancer (15.8%; refs. 43, 45). A higher percentage of expression has been described in squamous cell carcinoma of the lung (84.4%); moreover, the expression was highly correlated with smoking (43). Our finding that AKR1B10 expression is associated with COX-2 and pAKT expression cannot be directly explained. All three molecules have proproliferative properties. COX-2 transcription is suppressed by retinoids, suggesting a possible link to AKR1B10 via inhibition of retinoid acid synthesis (41, 50). Despite reported in vitro evidence for enhanced cell growth in the presence of AKR1B10, we found no association with tumor growth fraction or survival of patients (20). However, a possible association with response to bortezomib remains to be discussed, and patient samples from ongoing clinical studies would be helpful in clarifying this issue.
In summary, proteomic screening of colorectal cancer cell lines before and after treatment with bortezomib allows the identification of proteins that interfere with the complex network of proteasome inhibition. Further functional investigation of these proteins might provide a deeper understanding of sensitivity to bortezomib. Especially, the aldo-keto reductase AKR1B10 may act in a cytoprotective manner in response to bortezomib-mediated proteasome inhibition, thus providing an interesting target for therapeutic interventions.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

References


Proteomic identification of aldo-keto reductase AKR1B10 induction after treatment of colorectal cancer cells with the proteasome inhibitor bortezomib


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