Mucin production determines sensitivity to bortezomib and gemcitabine in pancreatic cancer cells

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Abstract. The prognosis of pancreatic cancer remains disappointing due to a high intrinsic resistance against chemotherapeutic agents. Standard gemcitabine therapies have improved overall survival only marginally and recently, inhibition of the proteasome by the boronic acid derivative bortezomib has been introduced as a novel therapeutic strategy for solid and hematological malignancies including pancreatic cancer. The mucus-producing pancreatic cancer cell line Capan-1 was cultured under standard conditions and treated with different concentrations of gemcitabine or bortezomib. Mucus production was suppressed by siRNA-mediated silencing of apomucin genes. Cell proliferation was determined by 3H-thymidine incorporation and apoptosis was quantified after propidium iodide staining by flow cytometry. Apoptotic cell death was confirmed by TUNEL staining, determination of caspase 3/7 activity, NFκB-activity was determined by EMSA. The unfolded protein response (UPR) was further investigated by PCR, Western blotting and caspase 12 activity assays. Silencing of MUC4 significantly reduced expression of mucins for up to 5 days after transfection. While native cells showed an increased sensitivity to bortezomib treatment, silenced cells were more sensitive to gemcitabine treatment. Bortezomib induces mitochondrial damage in native cells and also activates the UPR by splicing of Xbp-1 and induction of CHOP, which is significantly reduced by silencing of MUC4. Our data suggest that mucinous pancreatic cancers are more sensitive towards proteasome inhibition by bortezomib and that alternative pathways of apoptosis are involved in cell death induction, while tumor cells with a low secretory activity show a better response to gemcitabine.

Introduction

Pancreatic cancer ranks 4th in cancer related deaths in men and women in the US, with an estimated incidence and death rate of approximately 37,680 and 34,390 cases, respectively (1). Overall survival of advanced disease stages remains disappointing due to the failure of currently available chemotherapy regimens (2-4). Standard therapy consists of combinations of gemcitabine, a pyrimidine-analogue, and other cytotoxic or targeted agents but is still limited by a high intrinsic rate of resistance against these therapies (3-5). Recently, inhibition of the ubiquitin-proteasome pathway by the boronic acid dipeptide derivative bortezomib (BTZ) has been introduced as a novel anticancer therapy in hematologic and solid tumors (6). In contrast to other solid tumors, BTZ alone or in combination with gemcitabine could not significantly prolong overall survival but was associated with grade 3/4 toxicities in a phase II trial or in an orthotopic mouse model (7,8). The outcome of these trials was surprising as a clear molecular rationale for using BTZ in pancreatic cancer was established previously by showing that a variety of cell cycle and apoptosis regulators were regulated by proteasomal degradation (6). Among these factors, the transcriptional inhibitor nuclear factor kappa B (NFκB), which is closely linked to pancreatic cancer pathogenesis and resistance, has been shown to be a major target of proteasome inhibition (9-11). NFκB becomes activated by phosphorylation and ubiquitination of its inhibitor inhibitor of NFκB (IκB) by the multi-protein kinase complex inhibitor of IκB kinase (IKK) which finally leads to proteasomal degradation of IκB, release and nuclear translocation of NFκB and target gene activation (12,13). This pathway is activated in most pancreatic cancer cell lines or clinical specimens and is also correlated with poor survival and apoptosis resistance due to overexpression of anti-apoptotic genes (14,15). In preclinical models, inhibition of NFκB has thus been shown to sensitize pancreatic cancer cells to death-receptor or chemotherapy...
induced apoptosis (16-18). Recent studies also indicate a role of the unfolded protein response (UPR) and endoplasmic reticulum (ER)-mediated stress pathways in induction of apoptosis by BTZ, probably due to interference with molecular chaperones like HSP70 and BiP (19,20).

Mucins, like other secreted proteins, are dependent on correct processing by chaperones in the ER (21). We have shown previously that the effect of BTZ in myeloma cells is also dependent on the secretory activity (22). In pancreatic cancer, high expression of mucins has been linked to resistance against chemotherapy, tumor progression and an aggressive phenotype (23-27). We therefore investigated if and to what extent the inhibition of mucin production sensitized pancreatic cancer cells to BTZ treatment and compared these findings with conventional gemcitabine therapy. We found a positive correlation between the expression of mucins and BTZ-induced apoptosis, which was predominantly mediated via the ER stress pathway. In addition, a negative correlation with gemcitabine was also observed.

Materials and methods

Cell culture. The mucus-producing human ductal pancreatic cancer cell line Capan-1 was obtained from the Deutsche Sammlung für Mikroorganismen und Zellkulturen (DSMZ, Braunschweig, Germany) and cultured under standard conditions in RPMI-1640 medium with 20% (v/v) fetal bovine serum and 1% penicillin/streptomycin (all from Biochrom, Braunschweig, Germany) and bortezomib (Velcade; Janssen-Cilag, Neuss, Germany) was added after 16 h. Cells were transferred to glass fibre sheets (Cell Harvester, Inotech, Dottikon, Switzerland) after an additional 8 h (total treatment time is 24 h). Thymidine incorporation was determined with a β-imager plate and a BAS reader 5148 (Fuji, Düsseldorf, Germany). Analysis was performed with AIDA software (Raytest, Berlin, Germany).

Isolation of total RNA, reverse transcription and PCR analysis. Total RNA was isolated using RNApure (PeqLab, Erlangen, Germany) according to the manufacturer's protocol. Isolated RNA was resolved in DEPC water (Sigma, Munich, Germany) and concentration was determined photometrically at 260 nm in a Genios plate reader (Tecan, Crailsheim, Germany). RNA (1 µg) was used for reverse transcription using Superscript II reverse transcriptase (Invitrogen, Karlsruhe, Germany), oligo-dT-primer (Biomol, Berlin, Germany) and random hexamer primer (Promega, Heidelberg, Germany). cDNA was stored at -20°C until use.

Quantitative real-time PCR was performed on the Light-Cycler system (Roche Diagnostics, Mannheim, Germany) using the DNA Master Kit SYBR green I (Roche Diagnostics) as described previously (29) and QuantiTect Primer assays (Qiagen) for GAPDH (QT01192646) as an internal standard, Bcl-2 (QT00025011), Bax (QT00031192), CHOP (QT00082278), MUC1 (QT01667239), MUC2 (QT01004675), MUC3 [QT01529976(A); QT01679692(B)] and MUC4 (QT00045479).

Regular PCR was performed for determining Xbp-1 splicing using the MasterMix Kit (Fermentas, St. Leon Rot, Germany) on a DNA Engine (PTC-200) Peltier Thermal Cycler (Bio-Rad, Munich, Germany). PCR conditions were 90°C for 10 min and 35 cycles with 57°C for 20 sec, 70°C for 20 sec and 90°C for 30 sec. Specific primers for Xbp-1 forward (CCTTGTGGTTGAGAACCAGG) and reverse (TCTCTGTCTCAGAGGGGATC) were obtained from MWG Biotech AG (Ebersberg, Germany). PCR products were stained with ethidium bromide (Sigma) and subjected to gel electrophoresis on a 1.7% (w/v) agarose/TAE gel. Specific primers for Bcl-2 (QT01667239) and GAPDH (QT01192646) were 90°C for 10 min and 35 cycles with 57°C for 20 sec, 70°C for 30 sec and 90°C for 10 sec. Specific primers for Bcl-2 forward (CCTTGTGGTTGAGAACCAGG) and reverse (TCTCTGTCTCAGAGGGGATC) were obtained from MWG Biotech AG (Ebersberg, Germany). PCR products were stained with ethidium bromide (Sigma) and subjected to gel electrophoresis on a 1.7% (w/v) agarose/TAE gel (Sigma). Bands were visualised under UV light. For these experiments, Capan-1 cells treated for 6 h with 5 µg/ml of the glycosylation inhibitor tunicamycin (Sigma) were used as a positive control.

Flow cytometry of cell cycle, cell death and mitochondrial transmembrane potential. Treated cells and supernatants were collected and processed as described by staining with hypotonic propidium iodide solution (28). The mitochondrial transmembrane potential $\Delta W_m$ was determined by staining of $10^5$ treated cells with 3,3‘-dihexyloxacarbocyanine iodide (DiOC$_6$, Sigma) for 20 min at 37°C (30). All flow cytometric analyses were performed on an Epics Profile Analyzer (Coulter, Miami, FL, USA).

Caspase activity assays. Cells ($5 \times 10^5$) were treated with bortezomib or gemcitabine as indicated for 16 h. Activity of caspase 3/7 and 8 was determined with Caspase-Glo assays (Promega, Madison, WI, USA) according to the manufacturer's instructions. Luminescence signals were determined on a Genios plate reader (Tecan). The Caspase-12 Fluorimetric Assay Kit (BioCat, Heidelberg, Germany) was used for fluorimetric quantification.

3H-thymidine incorporation assay. Capan-1 cells ($10^5$) were seeded in 96-well round bottom plates (Nunc) and were treated with indicated concentrations of bortezomib or gemcitabine. 3H-thymidine ($10 \mu$Ci) (Amersham Pharmacia Biotech, Freiburg, Germany) was added after 16 h. Cells were transferred to glass fibre sheets (Cell Harvester, Inotech, Dottikon, Switzerland) after an additional 8 h (total treatment time is 24 h).
of caspase 12 activity according to the manufacturer's protocol and as described previously (30). Fluorescence signals were measured on a Genios plate reader (Tecan) with excitation at 400 nm and emission at 505 nm.

TUNEL assay. Cells (10⁶) were seeded in chamber slides (Nunc) and were treated as indicated for 24 h. Supernatant was discharged and slides were air-dried at 4°C. TUNEL staining was performed using the In situ cell death detection Kit (Roche) according to the manufacturer's instructions. Final staining was done with 3,3′-diaminobenzidine (DAB) substrate (Roche). Slides were analyzed by light microscopy.

Protein isolation and Western blotting. Cells (1.5x10⁶) were collected and lysed by adding 2X sample buffer as described (29). Protein concentration was determined by the BCA Protein Assay Reagent (Thermo Fisher Scientific, Bonn, Germany) and measured at 595 nm in a Genios plate reader (Tecan). Extinction was normalized to a standard curve calculated with dilution series of bovine serum albumin (Sigma).

Samples were incubated at 70°C with 10% reducing agent and 25% LDS sample buffer (both from Invitrogen) and subjected to electrophoresis on precast 14% bis-tris-polyacrylamide NuPAGE gels (Invitrogen) at 125 mA and 200 V for 50 min. Blotting was performed with transfer buffer (Invitrogen) containing 10% (v/v) methanol (Sigma) in a cooled blot chamber at 90 mA and 90 V for 60 min on nictrocellulose membranes (Whatman Biometra, Göttingen, Germany). Membranes were blocked with PBS containing 0.1% Tween-20 (Sigma) and 3.5% low fat milk for 12 h at 4°C and incubated with the primary antibodies given in Table I for 1 h at room temperature. After washing 3x for 10 min with the blocking buffer, appropriate secondary antibodies (Table I) were incubated for 1 h at room temperature. Reactive bands were visualised with ECL chemiluminescence reagent (Amersham Pharmacia Biotech). Results were densitometrically evaluated by AIDA™ (Raytest, Berlin, Germany).

Electrophoretic mobility shift analyses (EMSA). Electrophoretic mobility shift assays (EMSA) were done as described previously (31). Briefly, nuclear extracts were prepared from 5x10⁶ cells treated as indicated. Nuclear extracts (10 μM) were added to the probe mix. Probes were end labeled with 32P-dGTP (Hartmann Analytik, Braunschweig, Germany). The reaction mixtures were incubated at room temperature for 15 min and run on a 4% non-denaturing polyacrylamide gel. For specificity control, nuclear extracts were incubated for 15 min at room temperature with unlabeled double-stranded oligonucleotides or a respective mutant variant at 100-fold molar excess. Gels were dried on Whatman paper and exposed to X-ray film for 48 h at -70°C. Alternatively, gels were analyzed using phosphoimaging instrumentation (Fujiifilm FLA-3000, Agilent Technologies, Palo Alto, CA).

Statistical analysis. Statistical evaluation was done with Microsoft Excel 2003™ (Microsoft Corp., Seattle, WA) and SPSS v. 15.0™ (SPSS Inc., Chicago, IL) software packages. The significance of all of the data was proven using the t-test, and P<0.05 was considered significant.

### Results

**siRNA-mediated silencing of mucin production.** Capan-1 pancreatic carcinoma cells with a high mucin production were transfected with siRNA against various mucin genes. Transfection efficacy was determined by quantitative real-time RT-PCR after 24-120 h. All used siRNAs significantly reduced the expression of the respective mucin mRNA in a time-dependent manner, reaching a maximum suppression after 96 h (Fig. 1A). The knockdown of mucin genes was followed by a delayed reduction of the mucin concentration in cell culture supernatant (Fig. 1B). Here, silencing of MUC4 showed the most distinctive reduction of mucin secretion. We therefore used Capan-1 cells transfected with siRNA against MUC4, named sCapan-1, for all further experiments.

There were no differences in viability or proliferation between native Capan-1 or sCapan-1 cells as determined by 3H-thymidine incorporation (data not shown). Phase contrast microscopy revealed a thinner mucin margin surrounding sCapan-1 cells and time for trypsinisation was reduced compared to native cells (2 vs. 4 min at 37°C, 0.01% trypsin/EDTA).

**Effects of gemcitabine and bortezomib on sCapan-1.** As high levels of mucins have been associated with resistance to the pyrimidine analogue 5-fluorouracil (23), we investigated the effect of gemcitabine, the current standard pyrimidine analogue for pancreatic cancer, on native and sCapan-1 cells. 3H-thymidine incorporation showed a complete suppression of cell growth in native Capan-1 cells at gemcitabine concentrations of 2 μM or higher. Interestingly, sCapan-1 cells showed an increased sensitivity and growth suppression was already observed at 0.1 μM gemcitabine (Fig. 2A). In parallel, apoptosis induction as determined by flow cytometry of sub-G1-events after propidium iodide staining showed a higher sensitivity to 2 μM gemcitabine in sCapan-1 and reduced rates of apoptosis in native cells (Fig. 2B).

In contrast, native Capan-1 cells showed an enhanced sensitivity towards treatment with the proteasome inhibitor BTZ. Growth rate, as determined by 3H-thymidine incorporation,
was significantly suppressed (>90%) in Capan-1 cells at 0.5 µM, while this level of growth reduction was achieved in sCapan-1 cells only at 2 µM after 24 h (Fig. 2C). Paralleling these results, sCapan-1 cells also showed a delayed and diminished apoptosis response to BTZ compared to the parental cell line as shown by quantification of sub-diploid events after propidium iodide staining (Fig. 2D). Treatment with 1 µM BTZ lead to a maximum of ~40% apoptosis in native cells, while silenced cells reached only 25% after 36 h of incubation. Apoptotic cell death was confirmed by TUNEL staining (Fig. 2E). These findings indicate that mucin secretion influences differential pathways of chemotherapy response to either classical pyrimidine analogues or to novel targeted therapies like BTZ.

**Bortezomib induces apoptosis via mitochondrial pathways.** To address the underlying pathways of apoptosis induction, we examined the expression of the mitochondrial membrane proteins bax and bcl-2 in native and silenced Capan-1 cells after treatment with 1 µM BTZ by quantitative RT-PCR. Pro-apoptotic bax was induced 6-fold in Capan-1, while the expression was reduced in sCapan-1 cells. Also anti-apoptotic bcl-2 was downregulated in both cell lines, a stronger suppression was observed in native cells compared to sCapan-1, shifting the overall bax/bcl-2 ratio to 130.02 and 0.48, respectively (Fig. 3A and B). These results were confirmed on the protein level by Western blot analysis (Fig. 3C). The functional relevance of the pro-apoptotic bax/bcl-2 ratio was further confirmed by showing an increased breakdown of the mitochondrial transmembrane potential ∆Ψm by DiOC6 staining in native Capan-1 cells after BTZ treatment compared to sCapan-1 (Fig. 3D) which was significantly correlated to the apoptosis values determined by propidium iodide staining (R² = 0.87 for Capan-1 and R² = 0.74 for sCapan-1). Furthermore, activity of the executioner caspases 3/7 and their activator caspase 8 was significantly higher in treated Capan-1 cells compared to sCapan-1 (Fig. 3D and E). Co-incubation with the pan-caspase inhibitor zVAD reduced this activation to control level in both cell lines.

**Apoptotic response is independent of NFκB pathway inhibition.** Inhibition of the transcription factor nuclear factor kappa B (NFκB) is considered as the main antitumor target of BTZ (9). We analyzed the activation of NFκB by an enzymatic mobility shift assay (EMSA, Fig. 4A). DNA binding of NFκB
was high in both untreated cell lines. NFκB activation was suppressed in both cell lines after treatment with 0.5 µM BTZ for 20 h. Yet, the DNA binding activity of NFκB is not necessarily paired with an increased transcription (32), which could also be influenced by other factors like RNAi related pathways. We therefore analyzed the mRNA level of IκB (nuclear
factor of kappa light polypeptide gene enhancer in B-cells (IKB), an inhibitor of NFkB function (33). Although NFkB activation induces IKB transcription in an inhibitory feedback loop, we found a significant decrease in IKB mRNA after BTZ treatment in both cell lines (Fig. 4B). The similar sensitivity towards NFkB inhibition in both cell lines is discrepant to the observed apoptosis induction in Capan-1 and sCapan-1 cells after BTZ treatment, indicating that additional pathways of cell death induction are involved.

Secretory activity sensitizes endoplasmic reticulum-mediated apoptosis by bortezomib. As protein and mucus secretion is linked to integrity of the endoplasmic reticulum (ER), we investigated if BTZ disturbs ER processes after silencing of
MUC4 and activates the unfolded protein response (UPR) which could provide further insight into the observed differences in apoptosis induction between native and silenced Capan-1 cells.

ER stress and UPR are usually initiated by activation of the endonuclease activity of IRE1, which excises a 26-bp fragment from XBP-1 mRNA. In our hands, splicing of XBP-1 occurred in native Capan-1 cells but not in sCapan-1 cells after treatment with 1 µM BTZ (Fig. 5A).

Western blot analysis showed a strong induction of CHOP expression after treatment with 1 µM BTZ in Capan-1, but only a minor response in sCapan-1 cells. This was paralleled by a delayed induction of caspase 12 expression, the initiator caspase of the ER stress pathway, after 24 h in BTZ-sensitive Capan-1 cells (Fig. 5B). Interestingly, we could not detect significant changes in protein or mRNA levels of ER stress related chaperones like BiP, which may be due to high levels of BiP already in untreated control cells (data not shown). These results highlight the capability of BTZ to induce additional apoptotic pathways in pancreatic cancer cells beyond the previously described NFκB signalling.

Discussion

Advanced pancreatic cancer is characterized by a high intrinsic resistance to conventional chemotherapies and novel effective and tolerable treatment regimens are therefore urgently needed (3). Recent data suggest that production of mucin molecules may be directly linked to the inhibition of apoptosis in pancreatic cancer cells (34). Mucins are a family of high molecular weight proteins and are often aberrantly expressed in cancers (35-37). In addition to altering surface properties of tumor cells, several mucin molecules also have intracellular domains that are directly connected to oncogenic signalling pathways (38,39).
MUC4 has been reported to be expressed in pancreatic adenocarcinomas but not in adjacent normal tissue (40,41) and was proposed to be a novel tumor marker for this disease as the prognosis of patients with high MUC4 expression was significantly reduced (42). The intracellular domain of MUC4 contains an amino acid sequence that acts as a direct stimulating ligand for the tyrosine kinase receptor ErbB2 (43,44). ErbB2 itself has been shown to be significantly overexpressed in human pancreatic ductal adenocarcinomas (45,46) and to regulate inflammatory and cell viability pathways, e.g., by NFκB (47). MUC4 has thus been demonstrated to stimulate proliferation pathways and to inhibit apoptosis induced by chemotherapeutic drugs in different cancer cell lines (27,34,48).

We have previously established RNA interference based strategies to inhibit oncogene function in pancreatic cancer models (28,49) and others have shown that the knockdown of MUC4 interferes with the above described tumorigenic pathways (24,26,27). We have shown here that the siRNA-mediated knockdown of different mucin genes leads to a significant and sustained suppression of both mucin mRNA and protein and interferes with sensitivity to gemcitabine and BTZ. In our hands, silencing of MUC4 increases the sensitivity towards treatment with the pyrimidine analogue gemcitabine, confirming the observation that the inhibition of mucin production by benzyl-α-GalNAc restores sensitivity of Capan-1 cells to 5-fluorouracil (23).

The proteasome inhibitor BTZ has been shown to possess a strong anti-tumor activity against pancreatic cancer cells in vitro (8,50). Surprisingly, BTZ did not prove to be effective in in vivo models or early clinical trials of pancreatic cancer (7,8,51). In our study, BTZ inhibited proliferation and induced apoptosis in both native and silenced Capan-1 cells with a significantly stronger effect in MUC4-expressing native Capan-1 cells. These results are in line with our previous findings that a high immunoglobulin production sensitizes myeloma cells to BTZ treatment (22). In native Capan-1 cells, BTZ induces mitochondrial apoptosis as shown by a shift of the bax/bcl-2 ratio and breakdown of ΔΨm, but is also capable of activating novel alternative pathways of cell death. As described in the myeloma model (22), we also found a strong activation of the unfolded protein response (UPR) and endoplasmic reticulum stress (ER) stress pathways especially in native Capan-1 cells as demonstrated by splicing of Xbp-1 and increased expression of CHOP. The misfolding of proteins activates the UPR by enhancing chaperone activity and by ubiquitinating misfolded proteins for proteasomal degradation. These pathways have been described for BTZ treated pancreatic cancer cells (19,20) and our results support the hypothesis that the secretory activity, i.e., the degree of mucin production, regulates the apoptotic response by ER stress. This hypothesis is further supported by the notion that pancreatic epithelial cells have a high protein synthesis capacity and therefore possess an extensive ER which sensitizes these cells to UPR (52-54).

Although inhibition of NFκB signalling is currently conceived as the main effect of BTZ on apoptosis and cell growth (9) we did not detect significant differences between the two investigated cell lines in either NFκB or IkB signalling that could explain the observed differences in BTZ-induced apoptosis. It was previously described that BTZ does not influ-


