**Experimental in vivo and in vitro treatment with a new histone deacetylase inhibitor belinostat inhibits the growth of pancreatic cancer**

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**Abstract**

**Background:** Treatment options for pancreatic ductal adenocarcinoma (PDAC) are limited. Histone deacetylase inhibitors are a new and promising drug family with strong anticancer activity. The aim of this study was to examine the efficacy of in vitro and in vivo treatment with the novel pan-HDAC inhibitor belinostat on the growth of human PDAC cells.

**Methods:** The proliferation of tumour cell lines (T3M4, AsPC-1 and Panc-1) was determined using an MTT assay. Apoptosis was analysed using flow cytometry. Furthermore, p21Cip1/Waf1 and acetylated histone H4 (acH4) expression were confirmed by immunoblot analysis. The in vivo effect of belinostat was studied in a chimeric mouse model. Antitumoural activity was assessed by immunohistochemistry for Ki-67.

**Results:** Treatment with belinostat resulted in significant in vitro and in vivo growth inhibition of PDAC cells. This was associated with a dose-dependent induction of tumour cell apoptosis. The apoptotic effect of gemcitabine was further enhanced by belinostat. Moreover, treatment with belinostat increased expression of the cell cycle regulator p21Cip1/Waf1 in Panc-1, and of acH4 in all cell lines tested. The reductions in xenograft tumour volumes were associated with inhibition of cell proliferation.

**Conclusion:** Experimental treatment of human PDAC cells with belinostat is effective in vitro and in vivo and may enhance the efficacy of gemcitabine. A consecutive study of belinostat in pancreatic cancer patients alone, and in combination with gemcitabine, could further clarify these effects in the clinical setting.

**Keywords:** Pancreatic cancer, Histone deacetylase inhibitors, Belinostat, Mice

**Background**

Pancreatic ductal adenocarcinoma (PDAC) is a highly aggressive gastrointestinal tumour. It represents the fourth leading cause of cancer related deaths in both males and females in the western world [1]. Its resistance to most forms of therapy such as chemo-, radio-, immuno-, or targeted therapy contributes to the high mortality of this malignancy [2]. Curative resection is the only option to achieve long-term survival, but can be performed in only 15–20% of newly diagnosed pancreatic cancer patients [3].

Despite numerous findings that have contributed to the understanding [4] of PDAC pathogenesis, the reasons for its aggressive biological behaviour remain unclear. Therefore, new drugs for pancreatic adenocarcinoma are urgently needed. One of the most encouraging fields in modern oncological research is the investigation of epigenetics. Histone deacetylase inhibitors (HDACi) are typical representatives of this field of research, and may serve as promising agents with strong anticancer activity. The mechanism of HDACi activity is remodelling of chromatin through a modification of histone molecules, with consecutive initiation of cell differentiation, cell-cycle arrest and induction of apoptosis. Additionally, HDACi might affect neoplastic growth by regulating host immune responses and tumour vasculature [5]. Clinical studies have shown that HDACi can be administered...
safely in humans, and that treatment with these agents seems to be beneficial for some types of cancers [6,7].

A new hydroxamate type HDACi, belinostat (previously called PXD101), has been shown to be a potent anti-tumour agent with sub- to low micromolar IC50 potency in several tumour cell lines. In animal studies, belinostat specifically inhibited tumour growth at nontoxic concentrations [8,9]. Moreover, this compound has recently been evaluated in clinical phase I and II studies in both haematological and solid malignancies [10,11]. However, its efficacy in pancreatic cancer is unknown. Therefore, the aim of this study was to examine the efficacy of the novel pan-HDAC inhibitor belinostat on the growth of human PDAC cells in vitro and in vivo.

Methods

Cell culture and treatment
PDAC cell lines Panc-1 and AsPC-1 were obtained from the American Type Culture Collection (Manassas, VA, USA). T3M4 was a gift from R.S. Metzgar (Duke University, NC, USA). Cells were cultured in RPMI 1640 medium supplemented with 10% foetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin (Invitrogen GmbH, Karlsruhe, Germany) at 37 °C in a humidified 5% CO2 atmosphere. When cells were sub-confluent, medium was changed on both untreated and treated cells. Cells were treated with defined concentrations of a belinostat (Topotarget, København, Denmark) stock dissolved in DMSO. Control cells were grown following a similar protocol in medium supplemented with equivalent volumes of PBS.

MTT assay for cell proliferation
A 3-(4,5-dimethylthiazol-2-yl)-2.5-diphenyltetrazolium-bromide (MTT) (Sigma-Aldrich Chemie, Steinheim, Germany) assay was used to assess cell proliferation reflected by metabolic activity of the cells. The cells were seeded in 96-well plates at a density of 5000 cells/well in 250 μl of complete medium. After the cells became adherent (in 24 h), they were exposed to belinostat (25nM, 50nM, 100nM, 300nM, 500nM, 800nM, or 1000nM) for 48 h. Cells exposed to PBS served as controls. After the indicated times, 10 μl of MTT (5 mg/ml, dissolved in PBS, pH 7.4) were added to each well and incubated for 4 h. After incubation, the culture medium was aspirated and the plates were dried by inversion for about 15 min. Subsequently, the formazan products were solubilised with acidic isopropanol (100 μl for each well) and the optical density was measured at λ = 570 nm with a Multiscan EX plate reader (Thermo Electron). All assays were performed in triplicate. Proliferation in the control group was set as 100%.

Immunoblotting
After 24 h treatment with Belinostat (100 nM for T3M4 and ASPC or 300 nM for Panc-1), cells were washed twice with ice cold PBS before lysis with SDS lysis buffer (2% w/w SDS, 1 mM DTT, 10% v/v Glycerol, 62.5 mM Tris–HCl pH6.8, 0.05% w/v Bromphenolblue). Protein concentration was determined by BCA protein assay (Pierce Chemical Co., Rockford, IL, USA). Cell lysates (30 μg protein/lane) were separated on a 15% SDS/polyacrylamide gel and electroblotted on PVDF membrane (Whatman, Maidstone, UK). Membranes were then incubated in blocking solution (5% milk in 20 mM Tris–HCl, 150 mM NaCl, 0.1% Tween-20), followed by overnight incubation at 4 °C with anti acH4, CHIP-Grade antibody (Upstate Biotechnology, Billerica, USA) at a dilution of 1:75,000, or rabbit polyclonal anti- p21Cip1/Waf1 (Abcam, Cambridge, UK) at a 1:200 dilution. The membranes were then washed in TTBS and incubated with secondary antibodies: POD conjugated donkey anti-rabbit (Promega, Mannheim, Germany) at 1:150,000 dilution (blot for acH4) or POD conjugated goat anti-rabbit IgG (GE Healthcare Limited, Buckinghamshire, UK) at 1:2000 dilution (blot for p21Cip1/Waf1).

Antibody detection was performed using an enhanced chemiluminescence reaction (Amersham Bioscience). Equal lane loading was confirmed using anti-actin (Clone AC-15) antibody (Sigma-Aldrich Chemie). The actin signal obtained after incubation with anti-actin antibody on the same membrane was used as an internal control, in addition to loading all lanes with the same amount (30 μg) of protein. All assays were performed in triplicate. For the semiquantitative analysis of the immunoblots, densitometry using the ImageJ program was carried out, and the signal intensity of acH4 or p21Cip1/Waf1 expression was normalised to its corresponding signal intensity of actin.

Induction and analysis of cell death by flow cytometry
Belinostat was diluted in phosphate-buffered saline (PBS) to a final concentration ranging from 100 to 1000 nM, according to the concentrations indicated in each experiment. Gemcitabine (Synchem OHG, Felsberg, Altenburg, Germany) was applied at a final concentration of 0.01 mM in PBS. All cells were treated and cultured under the same conditions (37 °C with 5% CO2 in RPMI 1640), and exposed to the drugs 24 h before the experiments.

The viability of PDAC cells after exposure to belinostat and/or gemcitabine was analysed using annexin V/propidium iodide (PI)-staining (Annexin V-FITC Apoptosis Detection Kit I, BD Biosciences, Heidelberg, Germany). This method allows discrimination between early (annexin V+/PI-) and late (annexin V+/PI+) apoptotic, as well as necrotic (annexin V-/PI+) and viable (annexin...
V-/PI-) cells. The staining was performed according to the manufacturer’s instructions. Briefly, the cells were suspended in a buffer solution (10 mM HEPES/NaOH at pH 7.4, 140 mM NaCl, 2.5 mM CaCl₂) at a final concentration of 1 x 10⁶ cells/ml. After addition of Annexin V-FITC, the cells were incubated for 15 min at room temperature without light exposure. Flow cytometry was performed using the FACS LSR II system (BD Biosciences). A total of 10,000 ungated events were acquired for each sample, and data were analysed with the BD FACS Diva® with the CSD module (BD Biosciences). In order to determine late apoptotic cells, PI was added to the samples. Flow cytometry was performed immediately thereafter.

**In vivo tumourigenicity study**
A total of 300,000 T3M4 cells in 200 μl RPMI 1640 medium were injected subcutaneously behind the anterior forelimb of four-week-old athymic mice through a 26-gauge needle. The injection sites were examined daily for the appearance of tumours. Treatment was started on the seventh day after tumour inoculation. Mice were divided into groups receiving belinostat (0.1 mg/g, 5 x weekly), gemcitabine (0.15 mg/g, 2 x weekly), or a combination of both (Belinostat at 0.1 mg/g, 5 x weekly, plus gemcitabine at 0.15 mg/g, 2 x weekly) i.p., whereas the control group (sham) received only PBS (20 μl/g, 5 x weekly). Treatment was continued for 28 consecutive days, and tumours were measured twice weekly with Vernier callipers. Tumour volumes were calculated using the formula: V = (L x W²)/2, with L representing the length and W the width of the tumour [12].

The same treatment was performed after tumour inoculation by direct injection in the pancreatic tail via laparotomy. In this case, the tumours were compared at the end of 28- days of treatment.

After completion of treatment, the animals were sacrificed, and tumours were excised, fixed, and embedded in paraffin. The numbers of mice in each treatment cohort was 6. All experiments on animals were approved in accordance with German law on the care and use of laboratory animals.

**Immunohistochemistry**
Paraffin-embedded tissue sections (2–3 mm thick) were deparaffinised in xylene and rehydrated in progressively decreasing concentrations of ethanol. Thereafter, slides were placed in washing buffer. Antigen retrieval was carried out by microwaving the tissue sections in 10 mM citrate buffer for 10 min. Sections were then incubated first with normal goat serum (DAKO Corporation, Carpinteria, CA, USA) for 45 min to block non-specific-binding sites, and then with a mouse monoclonal Ki-67

![Figure 1 Proliferation of PDAC cells (MTT assay). Treatment with belinostat reduces the proliferation of pancreatic carcinoma cell lines in vitro in a dose-dependent manner. T3M4, AsPC-1 and Panc-1 cells were cultivated with increasing concentrations of belinostat. After 48 h, cell viability was determined. Data are presented as mean ± SEM. * p < 0.05 vs. control.](image-url)
antibody, diluted 1:5 (DAKO Corporation). Incubation was performed for 18 h at 4 °C. Slides were then rinsed in washing buffer and incubated with a biotinylated secondary goat anti-mouse antibody (DAKO Corporation) for 45 min at room temperature. The slides were then washed in washing buffer, and each section was exposed to 100 μl DAB-chromogen substrate mixture (DAKO Corporation), then counterstained with Mayer’s haematoxylin. The sections were washed again, dehydrated in increasing concentrations of ethanol, and mounted with xylene-based mounting medium. Every staining was controlled with a negative control. For semi-quantitative analysis, slides were scored in a blinded manner by two observers. Using 400-fold image magnification, the positive and negative stained cells were counted at three independent units area. Next, the percentage of the positive stained cells was calculated.

Statistical analysis
Results are expressed as mean ± standard error of the mean (SEM). Analysis of variance (ANOVA) was used to show an overall difference between groups, the Student t test for pairwise comparison of normal distributed parameters, and the Mann–Whitney U test for parameters without normal distribution. Significance was defined as p < 0.05. Graphical presentations were performed using GraphPad Prism version 4.02 for Windows (GraphPad Software).

Results
Effects of belinostat in vitro
Antiproliferative effect of belinostat on pancreatic cancer cells
Belinostat caused a significant dose-dependent decrease in cell proliferation in all cell lines tested. The ED50

Figure 2 Apoptosis and necrosis in PDAC cell lines after treatment with belinostat and (or) gemcitabine. The percentage of dying tumour cells after treatment with belinostat, gemcitabine or belinostat + gemcitabine, stratified into early apoptotic, late apoptotic and necrotic fractions in the different PDAC cell lines. Belinostat increased apoptosis in a dose-dependent fashion in T3M4, AsPC-1 and Panc-1 cell lines. Moreover, the combination of belinostat and gemcitabine significantly enhanced apoptosis in T3M4 and Panc-1. Data are presented as mean ± SEM. * p < 0.05 vs. control.
concentrations for belinostat were ~100nM for T3M4, ~200nM for AsPC-1 and ~600 nM for Panc-1 (Figure 1).

Apoptosis induction in pancreatic cancer cells by belinostat treatment
As shown in Figure 2, treatment with belinostat induced dose dependent apoptosis in all cell lines tested. The differences compared to control were significant at concentrations of 500 nM or more in all cell lines tested.

Belinostat increases gemcitabine-mediated apoptosis in pancreatic tumour cells
When concomitant use of both drugs (500 nM belinostat and 0.01 mM gemcitabine) was tested in T3M4, AsPC-1 and Panc-1 cells, the combined treatment significantly enhanced the proapoptotic activity compared to gemcitabine treatment alone in Panc-1 (~3 fold) and T3M4 (~1.5 fold) cells (Figure 2).

Inhibition of histone deacetylation after belinostat treatment
In Western Blot analysis with an anti-ac-histone H4 antibody, treatment with belinostat significantly increased acetylation of histone 4 in all cell lines tested (Figure 3A).

Belinostat induces expression of p21Cip1/Waf1
In addition, belinostat was effective in increasing the level of p21Cip1/Waf1, which is related to HDACi-induced growth arrest in pancreatic carcinoma cells. Figure 3B demonstrates the increased expression of p21Cip1/Waf1 after belinostat treatment in Panc-1 cells.
Inhibition of *in vivo* tumour growth by belinostat

Tumours in the belinostat treatment group showed significantly reduced growth in both subcutaneous and intrapancreatic tumours compared with the control group, in *in vivo* experiments (Figures 4A and B). The combination of belinostat and gemcitabine therapy showed no additional growth inhibition.

Routine hematoxylin/eosin histological examination showed no morphological differences between the tumours in the treatment and control groups. However, analysis of their proliferation rates (Figure 5) using an anti-Ki-67 antibody, showed a significantly lower number of proliferating cells per unit area in the belinostat group (47.8 ± 5.7 cells per high-power field) compared with the control group (79.1 ± 2.2).

**Discussion**

PDAC remains a therapeutic challenge with a poor overall prognosis. Only surgery with adjuvant chemotherapy can achieve a long-term perspective in patients with localized...
tumours [13-15]. Adjuvant postoperative chemotherapy based on 5-FU or gemcitabine has been shown to improve the survival of these patients [16]. However, even under optimal treatment conditions, the 5-year survival rate doesn’t exceed 25% [13]. Additionally, palliative treatment in advanced tumour stages is associated with a poor prognosis and a median survival of about 6 months. To improve this situation, investigation of new therapeutic agents for PDAC treatment is essential.

The family of HDACi represents a novel approach in oncological research. In defined - predominantly haematological - tumour entities, HDACi have already passed the stage of experimental research and been investigated clinically [17]. Regarding PDAC, promising results have been shown using SAHA, TSA, butyrate and some other histone deacetylase inhibitors in experimental studies [6,18-20]. Belinostat is a novel member of the family with a distinct pan-HDAC inhibitory effect. It has been shown to be strongly effective in experimental settings of ovarian, bladder and colon cancer, as well as haematological tumour entities [9,21-24]. Consecutive clinical trials have proven an anti-tumour effect of belinostat as a monotherapy in T-cell lymphomas and thymomas. In addition, belinostat has demonstrated beneficial effects in combination with other anti-cancer drugs for the treatment of ovarian and bladder cancer, CUP, multiple myeloma and acute myeloid leukaemia. Despite these findings, no data are available concerning belinostat in the context of PDAC treatment. Consequently, in the present study, the efficacy of belinostat for PDAC treatment was investigated in experimental in vitro and in vivo settings for the first time.

Comparable to the results of previous studies in bladder [8], colorectal [25] or hepatocellular carcinoma [21], we found a strong dose dependent antiproliferative activity of belinostat in three pancreatic cancer cell lines (T3M4, Panc-1 and AsPC-1) with an IC50 concentration in the nanomolar range, similar to other tumour entities [8,25].

This antiproliferative effect can be explained by a strong proapoptotic activity in pancreatic cancer cells, demonstrated by annexinV/propidium iodide staining. This is in line with other studies on AML [26] and hepatocellular carcinoma cells [21], underlining that apoptosis induction is an important mechanism of the anti-tumourous effect of HDACi, and particularly belinostat. As apoptosis induction is an important mechanism of anti-cancer chemotherapy [27], we tested the
influence of concomitant use of belinostat and gemcitabine. As described in studies with other HDACi like trichostatin A [6] and 4-phenylbutyrate [18], the combination of gemcitabine and belinostat strongly enhanced the proapoptotic effects of each substance alone. This may be due to the expression of proapoptotic proteins like Caspase-8 and Bid, and activation of the gemcitabine-mediated JNK-pathway [18].

Increase in histone H4 acetylation has been shown to be helpful in monitoring belinostat activity [28]. Consequently, we examined belinostat-dependent expression of acH4 in PDAC cells. Acetylation of H4 was increased in all cell lines tested, confirming the inhibitory effect of belinostat on HDAC activity in pancreatic cancer cells.

Cyclin-dependent kinase inhibitor p21Cip1/Waf1 is a key protein participating in cell cycle regulation. Previous studies have shown that HDACi activates expression of p21Cip1/Waf1 through enhanced histone acetylation around the p21Cip1/Waf1 promoter [29]. We performed western blot analysis with treated and control Panc-1 cells to clarify the effect of belinostat on p21Cip1/Waf1 expression. Belinostat induced an upregulation of p21Cip1/Waf1, as has been described for other HDAC inhibitors in pancreatic cancer [18,30]. Increased expression of p21Cip1/Waf1 in these studies was associated with normalization of the cell cycle and induction of apoptosis.

Regarding the effect of belinostat in vivo, we observed that belinostat was an effective growth inhibitor of T3M4 pancreatic cancer cells in a nude mouse model. Mice treated with belinostat showed xenograft growth inhibition for more than 28 days after tumour inoculation, without any signs of toxicity. The reduction in the tumour volume was associated with decreased cell proliferation, as shown by Ki-67 immunohistochemistry. Similar observations in in vivo tumour models were shown in previous studies, e.g. in human ovarian cancer s.c. xenografts; the efficacy of the treatment with belinostat was further enhanced when a combination therapy with carboplatin was added [24]. Plumb et al. [22] described a significant dose-dependent growth delay of human colon tumour xenografts in mice after belinostat treatment, without signs of toxicity.

In contrast to our in vitro observations, we could not find an additional effect of combined therapy with belinostat and gemcitabine in vivo. A possible explanation for this discrepancy is the relatively high dosage of gemcitabine administered in the in vivo study. This might have covered a possible additional belinostat effect.

Conclusion

In summary, this preclinical study using in vitro and in vivo pancreatic cancer models shows that belinostat is effective as a monotherapy of pancreatic cancer, primarily by inhibition of proliferation and induction of apoptosis. In vitro results revealed that belinostat can be successfully combined with gemcitabine to potentiate induction of apoptosis in the tumour cells. These findings should be confirmed in the clinical setting in PDAC patients.
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