Molecular determinants of the antitumor effects of trichostatin A in pancreatic cancer cells

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Abstract

AIM: To gain molecular insights into the action of the histone deacetylase inhibitor (HDACI) trichostatin-A (TSA) in pancreatic cancer (PC) cells.

METHODS: Three PC cell lines, BxPC-3, AsPC-1 and CAPAN-1, were treated with various concentrations of TSA for defined periods of time. DNA synthesis was assessed by measuring the incorporation of 5-bromo-2'-deoxyuridine. Gene expression at the level of mRNA was quantified by real-time polymerase chain reaction. Expression and phosphorylation of proteins was monitored by immunoblotting, applying an infrared imaging technology. To study the role of p38 MAP kinase, the specific enzyme inhibitor SB202190 and an inactive control substance, SB202474, were employed.

RESULTS: TSA most efficiently inhibited BrdU incorporation in BxPC-3 cells, while CAPAN-1 cells displayed the lowest and AsPC-1 cells an intermediate sensitivity. The biological response of the cell lines correlated with the increase of histone H3 acetylation after TSA application. In BxPC-3 cells (which are wild-type for KRAS), TSA strongly inhibited phosphorylation of ERK 1/2 and AKT. In contrast, activities of ERK and AKT in AsPC-1 and CAPAN-1 cells (both expressing oncogenic KRAS) were not or were only modestly affected by TSA treatment. In all three cell lines, but most pronounced in BxPC-3 cells, TSA exposure induced an activation of the MAP kinase p38. Inhibition of p38 by SB202190 slightly but significantly diminished the antiproliferative effect of TSA in BxPC-3 cells. Interestingly, only BxPC-3 cells responded to TSA treatment by a significant increase of the mRNA levels of bax, a pro-apoptotic member of the BCL gene family. Finally, in BxPC-3 and AsPC-1 cells, but not in the cell line CAPAN-1, significantly higher levels of the cell cycle inhibitor protein p21WAF1 were observed after TSA application.

CONCLUSION: The biological effect of TSA in PC cells correlates with the increase of acetyl-H3, p21WAF1, phospho-p38 and bax levels, and the decrease of phospho-ERK 1/2 and phospho-AKT.

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Key words: Pancreatic cancer; Histone deacetylase inhibitor; Trichostatin-A; KRAS; MAP kinases; p21WAF1, AKT

INTRODUCTION

Pancreatic cancer (PC) constitutes the fourth to fifth leading cause of cancer deaths in Western countries. Despite many scientific efforts in recent years, PC still has...
the worst survival rate (< 5%) of all common human tumors\cite{4,5}, and therapeutic progress has been very slow. Important reasons for this dissatisfying situation include the lack of markers for early diagnosis, and the limited efficiency of radio- and chemotherapy.

In the development of ductal adenocarcinoma, the most common form of PC, accumulation of somatic gene mutations in pancreatic progenitor cells plays a key role\cite{6}. For example, oncogenic mutations of the KRAS gene are detectable in approximately 90% of pancreatic adenocarcinomas. Other frequent genetic alterations in PC include loss or inactivation of the anti-oncogenes p53, p16/CDKN2A and DPC4\cite{7}.

While the essential contribution of somatic gene mutations is well established, recent studies have implicated epigenetic alterations in pancreatic carcinogenesis as well\cite{8}. Thus, several genes with tumor suppressor properties, such as p16/CDKN2A (if not genetically inactivated)\cite{9}, p57\cite{10}/CDKN1C\cite{11} and BNIP3\cite{12}, were shown to frequently undergo epigenetic promoter silencing by aberrant methylation of CpG islands. Hypomethylation of CpG's, in contrast, represents a key principle of epigenetic regulation. Histone acetylation is associated with a repressed chromatin state, whereas inhibition is associated with an activated chromatin state. Histone acetylation and further post-translational modifications are tightly controlled by two classes of enzymes, histone acetyltransferases and histone deacetylases (HDACI)\cite{13,14}. Inhibitors of histone deacetylases (HDACI) display anti-cancer activities and are, therefore, of growing clinical interest\cite{15,16}. With respect to PC, recent experimental studies have suggested that HDACI may efficiently inhibit growth and induce apoptosis even of drug resistant cell lines\cite{17-19,20,21}. Furthermore, a synergistic action with classic cytostatic drugs, such as gemcitabine, has been demonstrated\cite{22-24}. We have recently shown that HDACI also exert antifibrotic efficiency by inhibiting functions of pancreatic stellate cells\cite{25}. Since fibrosis is considered a progression factor of PC, this “side effect” might enhance antitumor activities of these drugs.

The molecular targets of HDACI in PC are incompletely characterized. Although expression of cell-cycle inhibitors (e.g. p21\cite{26}/CDKN1A\cite{27,28}) and modulation of pro-apoptotic pathways\cite{29,30,31,32,33} have been implicated in HDACI action, the molecular determinants of HDACI efficacy or ineffectiveness are currently unclear. Here, we have chosen three human PC cell lines that differ in their biological sensitivity to the HDACI trichostatin A (TSA) for a comparison of TSA effects at the molecular level. We have focussed on pathways that are considered to be important both for PC cell growth/survival as well as HDACI action. By this approach, we have identified molecular correlates of TSA effectiveness in PC cells.

MATERIALS AND METHODS

Materials

Media and supplements for cell culture were obtained from Biochrom (Berlin, Germany), the 5-bromo-2'-deoxyuridine (BrdU) labelling and detection enzyme-linked immunosorbent assay kit from Roche Diagnostics (Mannheim, Germany), and SB202190 and SB202474 from Merck Chemicals/Calbiochem (Darmstadt, Germany). TRI reagents and all chemicals used for reverse transcription and Taqman™ real-time polymerase chain reaction (PCR) were from Applied Biosystems (Foster City, CA, USA). PVDF membrane was supplied by Millipore (Schwalbach, Germany), anti-p21\cite{26}® immunoglobulin (mouse monoclonal) by Becton Dickinson Biosciences Pharmingen (Heidelberg, Germany), the other primary antibodies (all raised in rabbits) by New England BioLabs (Frankfurt, Germany), and Odyssey® blocking buffer, stripping buffer and secondary antibodies for immunoblotting by LI-COR (Bad Homburg, Germany). Tissue culture dishes (Corning plasticware), standard laboratory chemicals and TSA were from Sigma-Aldrich (St. Louis, MO, USA). The HDACI was dissolved in ethanol and stored at -20°C as a stock solution (3.3 × 10⁻³ mol/L).

Cell culture

All cells used in this study represent human pancreatic carcinoma cell lines. CAPAN-1 cells were grown in IMDM supplemented with 17% foetal calf serum (FCS) and 10 mL/L non-essential amino acids (dilution of a 100 × stock solution). BxPC-3 cells were cultured in RPMI with 10% FCS. The cell culture medium for AsPC-1 cells was DMEM containing 10% FCS. All culture media were supplemented with 10³ U/L penicillin and 100 mg/L streptomycin. The cell lines were grown at 37°C in a 5% CO₂ humidified atmosphere. When reaching subconfluency, the cells were harvested by trypsinization, and recultured according to the experimental requirements.

Quantification of DNA synthesis

DNA synthesis was assessed by measuring incorporation of BrdU into newly synthesized molecules. Therefore, PC cells growing in 96-well plates were pretreated with drugs as indicated, 24 h after drug application, BrdU labelling was initiated by adding labelling solution at a final concentration of 1 × 10⁻³ mol/L (in the culture medium). After an overnight incubation, labelling was stopped, and BrdU uptake was measured according to the manufacturer’s instructions.

Immunoblotting

Cells were harvested by medium aspiration and addition of boiling lysis buffer\cite{34} directly to the cell monolayer. Cellular proteins received from equal numbers of cells were separated by SDS-polyacrylamide gel electrophoresis (10%-12% gels, depending on the experimental requirements) and transferred onto PVDF membrane by semi-dry blotting. After blotting, the filters were blocked and processed by incubation with the indicated primary antibody as previously described\cite{35}. The secondary antibody was IRDye® 800CW conjugated goat anti-mouse IgG and goat anti-rabbit IgG, respectively. All immunoblots were scanned at a wavelength of 800 nm, using an Odyssey®
Infrared Imaging System. Signal intensities were quantified by means of the Odyssey® software version 3.0. Prior to reprobing with additional primary antibodies, the blots were treated with stripping buffer according to the instructions of the manufacturer.

Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

The pancreatic cancer cell lines were challenged with TSA as indicated. Afterwards, total RNA was isolated with TRI reagent according to the manufacturer’s instructions. The RNA was reverse transcribed into cDNA by means of TaqMan™ Reverse Transcription Reagents and random hexamer priming. Relative quantification of target cDNA levels by real-time PCR was performed in an ABI Prism 7000 sequence detection system (Applied Biosystems) using TaqMan™ Universal PCR Master Mix and the following Assay-on-Demand™ human gene-specific fluorescently labelled TaqMan™ MGB probes: Hs00180269_m1 (bax), Hs00355782_m1 (p21Waf1), and Hs99999905_m1 [glyceraldehyde-3-phosphate dehydrogenase (GAPDH), used as house-keeping control gene]. Following the guidelines of the manufacturer, PCR was performed under the following conditions: 95℃ for 10 min, 50 cycles of 15 s at 95℃, 1 min at 60℃. The reactions were run at least in duplicate, and repeated 6 times with independent samples. Relative expression of each mRNA compared with GAPDH was calculated according to the equation △Ct = Ct_EXP - Ct_GAPDH, The relative amount of target mRNA in control cells and samples treated with drugs as indicated was expressed as 2^(-ΔΔCt), where △ΔCt = △Ct_SAMPLE - △Ct_CONTROL.

Statistical analysis

Results are expressed as mean ± SE for the indicated number of separate cultures per experimental protocol. Statistical significance was analyzed using the indicated statistical test. P < 0.05 was considered to be statistically significant.

RESULTS

TSA enhances histone acetylation in PC cell lines

In initial experiments, we compared the effects of TSA on the acetylation of histone H3 in the three different pancreatic cancer cell lines used in this study (Figure 1). In all cell lines, a dose-dependent increase of H3 acetylation was observed, suggesting an inhibition of histone deacetylase activity. The effect of TSA was stronger in BxPC-3 cells than in the other two cell lines, and AsPC-1 cells were somewhat more sensitive to TSA treatment than CAPAN-1 cells. The functional consequences of TSA action were investigated in subsequent experiments.

TSA inhibits DNA synthesis of pancreatic cancer cells

TSA significantly inhibited the incorporation of BrdU into newly synthesized DNA in all cell lines tested, but with remarkably different efficiency (Figure 2): While BxPC-3 cells showed a significant response at a TSA concentration of 10 μmol/L, AsPC-1 and CAPAN-1 cells were less sensitive to TSA treatment with 10 μmol/L BrdU incorporation.
The phosphatidylinositol (PI) 3-kinase pathway revealed that its phosphatase PTEN, the best-characterized negative regulator, the PI 3-kinase/phosphatidylinositol 3-phosphate 3-phosphatase PTEN, was expressed and phosphorylated in all cell lines (Figure 3A, panel 1 and 2, and Figure 3B). Furthermore, only in BxPC-3 cells TSA at 10 × 10⁻⁷ mol/L almost completely blocked phosphorylation of AKT (Figure 3A, panel 3 and 4, and Figure 3C), which acts downstream of the phosphatidylinositol (PI) 3-kinase [23]. A further evaluation of the PI 3-kinase/AKT pathway revealed that its best-characterized negative regulator, the PI 3-kinase phosphatase PTEN [23], was expressed and phosphorylated in all three cell lines in a TSA-independent manner (Figure 3A, panel 5 and 6; quantification data not shown). These data suggest that PTEN was not involved in the mediation of TSA effects on AKT.

Finally, we studied how TSA treatment affected phosphorylation of the MAP kinase p38, which like ERK plays a profound role in tumorigenesis [24]. Here, in all three cell lines a dose-dependent enhancement of phosphorylation was observed (Figure 3A, panel 7 and 8, and Figure 3D). The effect was most pronounced in BxPC-3, followed by AsPC-1 and CAPAN-1 cells.

**Effects of p38 MAP kinase inhibition on DNA synthesis of PC cells**

To study the biological consequences of p38 MAP kinase activation by TSA, the specific inhibitor SB202190 and an inactive control substance, SB202474, were employed. In the absence of TSA, incubation of BxPC-3 cells with the drugs did not significantly affect BrdU incorporation (Figure 3A). However, at any concentration tested, BrdU incorporation was significantly stronger inhibited in BxPC-3 cells than in the other two cell lines. Action of HDACI has previously been linked to the suppression of cell proliferation and induction of apoptosis [13-15]. Therefore, we chose two of the best-studied candidate genes, p21/waf1 [25], and BAX [26], to compare the effects of TSA on gene expression in the three PC cell lines. Various HDACI target genes identified so far have been linked to the induction of cell cycle arrest or apoptosis. We chose two of the best-studied candidate genes, BAX and p21/waf1 (CDKN1A, Cip1), to compare the effects of TSA on gene expression in the three PC cell lines. BAX, a pro-apoptotic member of the BCL gene family [25], and the cell-cycle inhibitor gene p21/waf1 were found to be ex-
Emonds E et al. Effects of trichostatin A

A

|            | BxPC-3 | AsPC-1 | CAPAN-1 |
|------------|--------|--------|---------|
| TSA (× 10⁻⁷ mol/L) | 0 | 1 | 4 | 10 | 0 | 1 | 4 | 10 | 0 | 1 | 4 | 10 |
| P-ERK 1/2 | ![Image](image1.png) | ![Image](image2.png) | ![Image](image3.png) |
| ERK 1/2   | ![Image](image4.png) | ![Image](image5.png) | ![Image](image6.png) |
| P-AKT     | ![Image](image7.png) | ![Image](image8.png) | ![Image](image9.png) |
| AKT       | ![Image](image10.png) | ![Image](image11.png) | ![Image](image12.png) |
| P-PTEN    | ![Image](image13.png) | ![Image](image14.png) | ![Image](image15.png) |
| PTEN      | ![Image](image16.png) | ![Image](image17.png) | ![Image](image18.png) |
| P-p38     | ![Image](image19.png) | ![Image](image20.png) | ![Image](image21.png) |
| p38       | ![Image](image22.png) | ![Image](image23.png) | ![Image](image24.png) |
| Actin     | ![Image](image25.png) | ![Image](image26.png) | ![Image](image27.png) |

B

![Graph](image28.png)

C

![Graph](image29.png)
vs BxPC-3, AsPC-1 and CAPAN-1 cells were treated with TSA at concentrations up to $10 \times 10^{-7}$ mol/L for 24 h. A: Expression and phosphorylation of the indicated proteins was analyzed by immunoblotting. Phospho-proteins were detected first, followed by a reprobing of the blots with anti-protein specific antibodies. Actin was used as a housekeeping control protein; B-D: Fluorescence signal intensities of phospho (P) proteins and total proteins were quantified using Odyssey® software version 3.0. Subsequently, the ratios phospho-ERK/ERK protein (B), phospho-AKT/AKT protein (C) and phospho-p38/p38 protein (D) were determined. A ratio of 100% corresponds to control cells cultured without TSA. Data of 8 independent experiments were used to calculate mean values and SE; $^a$P < 0.05 vs control cultures, $^b$P < 0.05 vs BxPC-3 cells (Wilcoxon's rank sum test).

**DISCUSSION**

The molecular targets of HDACi in PC cells have been studied by several groups in recent years. The results suggest that inhibitors of cell-cycle progression, such as p21$^{\text{Waf1}}$/CDKN1A, and regulators of cell survival/death are involved in the mediation of antiproliferative and pro-apoptotic effects of HDACi$^{[13-15,17]}$. The molecular basis of variations in the biological efficiency of HDACi in different PC cells, however, has not been systematically studied so far. It is also largely unknown how precisely the increase of histone acetylation is linked to the biological and molecular effects of HDACi described above. To address these questions, we chose an experimental model system of three PC cell lines that differ significantly in their biological responsiveness to the HDACi trichostatin A. BxPC-3 cells, which displayed the strongest increase of histone H3 acetylation and decrease of DNA synthesis, were the only wild-type KRAS$^{[16]}$ cells in this study. Interestingly, only BxPC-3 cells also showed a significant inhibition of ERK 1/2 and AKT phosphorylation in response to TSA treatment. Both ERK 1/2 (through Raf-1 and MEK) and AKT (through PI 3-kinase) are downstream of KRAS$^{[16]}$. We therefore hypothesize that oncogenic KRAS$^{[16]}$ mutations may reduce, but not abolish, TSA efficiency by preventing an inhibition of AKT and ERK 1/2 signalling.

Although BxPC-3 cells were most sensitive to TSA treatment, DNA synthesis of CAPAN-1 and AsPC-1 cells could also be reduced by the drug. In CAPAN-1 cells, however, a significant effect was achieved at the high TSA concentration of $1 \times 10^{-6}$ mol/L. Searching for further molecular explanations of these phenomena, we found that CAPAN-1 cells showed the weakest increase of acetyl-histone H3, p21$^{\text{Waf1}}$ and phospho-p38 levels. Furthermore, an unexpected decrease of bax mRNA expression in all cell lines (Figure 5A and B). In TSA-treated BxPC-3 cells, a significant increase of bax mRNA levels was observed, whereas AsPC-1 cells displayed a non-significant tendency towards higher bax mRNA levels, only. Surprisingly, CAPAN-1 cells responded to TSA application by a dose-dependent, significant decrease of bax mRNA expression (Figure 5A).

Treatment of BxPC-3 and AsPC-1 cells with TSA caused a strong increase of p21$^{\text{Waf1}}$ mRNA expression, while in CAPAN-1 cells this effect was less pronounced (Figure 5B). Similar results were obtained at the level of p21$^{\text{Waf1}}$ protein expression (Figure 5C). Here, in CAPAN-1 cells the TSA effect did not reach statistical significance.
ssion was observed in these cells.

Taken together, our data suggest that the biological efficiency of TSA in the different PC cell lines correlates with the increase of histone H3 acetylation, p21<sup>Waf1</sup> expression and MAP kinase phosphorylation, and the decrease of ERK 1/2 and AKT phosphorylation. Using a specific inhibitor of p38, we also obtained evidence for a direct involvement of this kinase in the mediation of the antiproliferative effect of TSA in the cell line BxPC-3.

Along these lines, the efficiency of TSA in PC cells might be restricted by antagonistic effects of other factors, such as oncogenic KRAS, on intracellular pathways that are critical for HDACI action.

Considering established crosstalks between growth-regulatory pathways, we hypothesize that the diverse molecular effects of TSA observed in this study are, at least in part, causally related. Specifically, we suggest direct links between modulation of MAP kinase (ERK, p38) signalling by the drug and induction of p21<sup>Waf1</sup> expression, since activation of p38 and inhibition of ERK phosphorylation have previously been shown to enhance p21<sup>Waf1</sup> promoter activation in other contexts<sup>[27-29]</sup>. Furthermore, AKT has been implicated in functional inactivation of p21<sup>Waf1</sup> through the phosphorylation of the protein, resulting in its cytoplasmic localization<sup>[30]</sup>. While cellular localization of p21<sup>Waf1</sup> was not investigated in this study, we observed an inverse correlation between p21<sup>Waf1</sup> expression and AKT activity.

A detailed knowledge of the molecular mechanisms...
of TSA action is essential to understand what determines the HDACI sensitivity and resistance of tumors. Our data encourage further studies in the field of pancreatic cancer, where novel therapeutic approaches are particularly needed.

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COMMENTS

Background
Pancreatic cancer has the worst prognosis of all common human tumors, indicating an urgent need for novel therapeutic approaches. Histone deacetylase inhibitors, such as trichostatin-A (TSA) or the clinically available drug SAHA, display anti-cancer activities in vitro and in vivo. They are therefore under experimental investigation for the treatment of different human malignancies. Histone deacetylase inhibitor (HDACI) act as epigenetic regulators by increasing histone acetylation, which is associated with a repressed chromatin state.

Research frontiers
While the immediate targets of HDACI action are clear, the precise molecular links between histone protein acetylation and suppression of cancer cell growth remain to be established. In most types of cancer cells, inhibitors of cell cycle progression and regulators of cell survival/apoptosis are important mediators of HDACI effects. However, a systematic overview of the underlying molecular mechanisms is missing. Thus, it is currently unknown which genes and proteins critically determine the efficacy or ineffectiveness of HDACI in pancreatic cancer (PC) cells.

Innovations and breakthroughs
The results of this study provide molecular insights into HDACI action in pancreatic cancer cells. The data suggest that the biological efficiency of TSA in different PC cell lines, indicated by the inhibition of DNA synthesis, correlates with the increase of histone H3 acetylation, but also both with genetic alterations and specific effects at the level of intracellular signal transfer as well as gene expression. In detail, expression of wild-type KRAS, a strong increase of bax, p21WAF1 and phospho-p38 levels, and a diminished phosphorylation of ERK 1/2 and AKT were found to be associated with a high biological efficiency of the drug. The authors hypothesize that PC cells differ in their biological sensitivity to TSA, since not all tumor cells exhibit the full range of molecular targets required for an effective HDACI action.

Applications
The results of this study open an avenue for the further investigation of the molecular determinants of HDACI action in PC cells. An improved understanding of HDACI effects at the molecular level may ultimately lead to the establishment of markers predicting the HDACI sensitivity or resistance of tumors.

Terminology
Pancreatic cancer is a malignancy of the pancreas with poor prognosis that typically displays the histological features of a ductal adenocarcinoma. HDACI, including trichostatin A, are substances that increase histone acetylation by inhibiting the activity of histone deacetylases. MAP kinases (such as ERK 1/2 and p38), Pten and AKT are intracellular proteins that are involved in the intracellular transfer of receptor-derived signals regulating e.g. cell growth and survival. p21WAF1 belongs to a family of proteins that prevent cells from entering the S-phase of the cell cycle, while bax is an inducer of apoptotic cell death.

Peer review
It is an interesting and well written manuscript, and a scientifically sound modest extension of what is known regarding histone deacetylase inhibitors and cancer.

Recent advances
Several recent advances in the field of HDACI and their potential in the treatment of different human malignancies have been published, including

- The first clinical trial with a histone deacetylase inhibitor (TSA) in patients with pancreatic cancer
- The identification of novel targets for HDACI action
- The development of novel HDACI with improved selectivity and reduced toxicity

These advances encourage further studies in the field of pancreatic cancer and provide new opportunities for therapeutic approaches.
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