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# Antifibrogenic effects of histone deacetylase inhibitors on pancreatic stellate cells

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ABSTRACT

Pancreatic stellate cells (PSCs) are essentially involved in pancreatic fibrogenesis and considered as a target for antifibrotic therapies. Here, we have analyzed the effects of three histone deacetylase inhibitors (HDACIs), sodium butyrate, sodium valproate (VPA) and trichostatin A (TSA), on profibrogenic activities of PSC and elucidated molecular targets of HDACI action. Therefore, cultured PSCs were exposed to HDACI. Cell proliferation and viability were assessed by 5-bromo-2′-deoxyuridine (BrdU) incorporation and trypan blue staining assays. Exhibition of the myofibroblastic PSC phenotype was monitored by immunofluorescence analysis of α-smooth muscle actin (α-SMA) expression. [$^3$H]-proline incorporation into acetic acid-soluble proteins was measured to quantify collagen synthesis. Levels of mRNA were determined by quantitative reverse transcriptase real-time PCR. Protein expression, phosphorylation and acetylation were analyzed by immunoblotting, and gel shift assays were performed to study DNA binding of nuclear proteins. HDACI enhanced histone H3 acetylation in a dose-dependent manner. In the same dose range, they strongly inhibited cell proliferation, α-SMA expression and collagen synthesis. A significantly increased rate of cell death was observed in response to TSA at 1 μM. While all three HDACI inhibited mRNA expression of endothelin-1, only VPA significantly reduced expression of transforming growth factor-β1. Both mediators exert autocrine profibrogenic effects on PSC. Furthermore, HDACI-treated PSC displayed a diminished DNA binding of AP-1, a key transcription factor in profibrogenic signaling. Together, the results suggest that HDACI exert antifibrogenic effects on PSC. Interruption of AP-1 signaling and autocrine loops enhancing PSC activation might be key mechanisms of HDACI action.



## 1. Introduction

In chronic pancreatitis, accumulation of extracellular matrix (ECM) proteins frequently leads to the development of pancreatic fibrosis, resulting in an exocrine and endocrine insufficiency of the gland [1,2]. Fibrosis is also a hallmark of pancreatic cancer and likely to play an active role in disease progression (reviewed in [3]). Despite significant efforts in recent years, specific therapies to retard or even reverse pancreatic fibrosis have not been established yet.

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*Abbreviations:* HDACI, histone deacetylase inhibitor; PSC, pancreatic stellate cell; VPA, sodium valproate; TSA, trichostatin A; BrdU, 5-bromo-2′-deoxyuridine; α-SMA, α-smooth muscle actin; ECM, extracellular matrix; HSC, hepatic stellate cell; PDGF, platelet-derived growth factor; TGF, transforming growth factor; ET, endothelin; CTGF, connective tissue growth factor; IFN, interferon; ECL, enhanced chemoluminescence; IMDM, Iscove's modified Dulbecco's medium; HPRT, hypoxanthine-guanine phosphoribosyl transferase; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; EMSA, electrophoretic mobility shift assay; S.E.M., standard error of the mean; AP-1, activator protein-1; ab, antibody.

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Pancreatic stellate cells (PSCs) are considered as the main source of ECM proteins in the diseased organ [3]. In response to profibrogenic mediators, PSC undergo phenotypic changes termed activation: the cells proliferate, synthesize and secrete increased amounts of ECM proteins (e.g., collagen types 1 and 3) and stain strongly positive for the myofibroblastic marker protein α-smooth muscle actin (α-SMA), which is organized in networks of so-called stress fibers [4–9]. Furthermore, in the course of activation PSC loose most of their characteristic vitamin A-containing fat droplets [4]. Together, these phenotypic changes closely resemble the process of hepatic stellate cell (HSC) activation in the fibrotic liver, suggesting common mechanisms in the development of fibrosis in both organs. Typical mediators of PSC activation include, in addition to ethanol metabolites, various cytokines [5,8,9]. Thus, platelet-derived growth factor (PDGF) exerts strong mitogenic effects on PSC, while transforming growth factor (TGF)-β stimulates matrix protein synthesis [10,11]. PSC activation is maintained and further enhanced through autocrine loops that have been suggested for mediators such as endothelin (ET)-1 [12,13], connective tissue growth factor (CTGF) [14,15] and members of the TGF-β family (TGF-β1; Activin A) [16,17]. Extracellular antagonists of PSC activation are less well characterized. In a recent study, we found that interferons (IFNs), particularly IFN-γ, exert antiproliferative effects on PSC and inhibit collagen synthesis in vitro [18], but the in vivo effects of these cytokines remain to be analyzed.

In recent years, epigenetic mechanisms have emerged as major determinants of gene expression and implicated in the regulation of complex differentiation and developmental processes, both under physiological and pathological conditions. In addition to promoter methylation, histone modification, particularly histone acteylation, is considered as a key principle of epigenetic regulation [19,20]. Histone deacetylation, which is associated with a repressed chromatin state, is tightly controlled by two classes of enzymes, histone acetyltransferases and histone deacetylases [19–21]. Inhibitors of histone deacetylases (HDACI) display anti-cancer activities and are, therefore, of growing clinical interest [22]. The role of epigenetic regulatory mechanisms in the control of PSC functions has not been studied so far. Recent studies, however, suggest that HDACI exert antifibrotic effects on hepatic stellate cells [23,24] and lung fibroblasts [25]. The underlying molecular mechanisms are still poorly understood. Here, we have characterized the effects of three structurally diverse HDACI, sodium butyrate, sodium valproate (VPA) and trichostatin A (TSA), on key functions of activated PSC. Furthermore, we have studied target genes of HDACI action in PSC and linked changes in histone acetylation to the modulation of profibrogenic signal transduction pathways. Therefore, an established in vitro model was applied, using PSC that were isolated from the pancreas of healthy rats and activated by sustained culture [26].

## 2. Materials and methods

### 2.1. Materials

The enhanced chemoluminescence (ECL) Plus kit, horseradish-peroxidase-labelled antibodies and [$^3$H]-proline were purchased from GE Healthcare (Freiburg, Germany), Taqman™ reagents from Applied Biosystems (Foster City, CA, USA) and recombinant rat PDGF-BB from R&D Systems (Minneapolis, MN, USA). Iscove's modified Dulbecco's medium (IMDM) and all supplements for cell culture were delivered by Biochrom (Berlin, Germany). Ascorbat, β-aminoproprionitrile, the α-SMA antibody, TSA, sodium butyrate, VPA, ET-1 and standard laboratory chemicals were from Sigma–Aldrich (St. Louis, MO, USA). Nycodenz was obtained from Nycomed (Oslo, Norway), TRIzol from Invitrogen (Carlsbad, California, USA), Alexa Fluor goat anti-mouse IgG from MoBiTec (Göttingen, Germany) and all further antibodies from Santa Cruz Biotechnologies (Santa Cruz, CA, USA).

### 2.2. Cell culture

Stellate cells were isolated from the pancreas of male LEW.1W inbred rats by collagenase digestion of the organ followed by Nycodenz density gradient centrifugation as previously published [26,27]. PSCs collected from the top of the gradient were washed and resuspended in IMDM supplemented with 10% fetal calf serum, 1% non-essential amino acids (dilution of a 100× stock solution), 100 U/ml penicillin and 100 μg/ml streptomycin at 37 °C in a 5% $CO_2$ humidified atmosphere. All experiments were performed with cells growing in primary culture, or, depending on the experimental settings, with cells of the first passage. If replating of the cells was required, PSCs were harvested by trypsinization on day 7 after isolation and recultured at equal seeding densities. Trypan blue staining was performed to distinguish live from dead cells and to determine absolute cell counts.

### 2.3. Quantification of DNA synthesis

To quantify DNA synthesis, incorporation of 5-bromo-2′-deoxyuridine (BrdU) was measured using the BrdU labelling and detection enzyme-linked immunosorbent assay kit (Roche Diagnostics, Mannheim, Germany). Therefore, cells were plated in 96-well plates in complete culture medium supplemented with HDACI as indicated. After 24 h, BrdU labelling was initiated by adding labelling solution at a final concentration of 10 μM. Another 24 h later, labelling was stopped, and BrdU uptake was measured according to the manufacturer's instructions.

### 2.4. Quantitative reverse transcriptase-PCR using real-time TaqMan™ technology

Total RNA from PSC pretreated with HDACI for 24 h as indicated was isolated with TRIzol reagent according to the manufacturer's instructions. Next, 1 μg of 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™ rat gene-specific fluorescently labelled TaqMan™ MGB probes: 00561129_m1 (ET-1), Rn00572010_m1 (TGF-β1), and Rn01527838_g1 (hypoxanthine-guanine phosphoribosyl transferase [HPRT]; house-keeping gene control).