

# The pan-deacetylase inhibitor panobinostat affects angiogenesis in hepatocellular carcinoma models via modulation of CTGF expression

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**Abstract.** Post-translational modifications of chromatin components are significantly involved in the regulation of tumor suppressor gene and oncogene expression. Connective tissue growth factor (CTGF) is an epigenetically regulated growth factor with functions in angiogenesis and cell-matrix interactions and plays a pivotal role in hepatocellular carcinoma (HCC). The pharmacologic inhibition of histone and protein deacetylases represents a new approach to interfere with pathways of apoptosis and angiogenesis. We investigated the effect of the pan-deacetylase inhibitor panobinostat (LBH589) on human HCC cell lines HepG2 (p53wt) and Hep3B (p53null) and in a subcutaneous xenograft model and explored the influence on angiogenesis. Specimens were characterized by quantitative real-time PCR. Protein was separated for western blotting against CTGF, VEGF, VEGF receptor-1 (VEGFR-1/FLT-1), VEGF receptor-2 (VEGFR-2/KDR), MAPK and phospho-MAPK. *In vivo*, HepG2 cells were xenografted to NMRI mice and treated with daily i.p. injections of 10 mg/kg panobinostat. After 1, 7 and 28 days, real-time

PCR was performed. Immunohistochemistry and western blotting were examined after 28 days. An increased significant expression of CTGF was only seen after 24 h treatment with 0.1  $\mu$ M panobinostat in HepG2 cells and Hep3B cells, whereas after 72 h treatment CTGF expression clearly decreased. In the xenografts, treatment with panobinostat showed a minimal CTGF expression after 1 day and 4 weeks, respectively. *In vitro* as well as *in vivo*, VEGF was not affected by panobinostat treatment at any time. In conclusion, panobinostat influences extracellular signaling cascades via CTGF-dependent pathways.

## Introduction

Hepatocellular carcinoma (HCC) is one of the most common cancers worldwide and the third leading cause of cancer-related death (1-4). Major risk factors for HCC development are cirrhosis with underlying chronic viral hepatitis, alcoholic liver disease and non-alcoholic steatohepatitis. Despite the introduction of targeted therapies like the multi-kinase inhibitor sorafenib, treatment with curative intention is still limited with unsatisfactory overall survival results (5-9).

A prerequisite for the growth and spread of malignant solid tumors is the formation of new blood vessels to provide oxygen and nutrient supply to the mass of tumor cells (10). Angiogenesis is a highly complex process involving various growth factors such as vascular endothelial growth factor (VEGF), growth factor receptors like fms-like tyrosine kinase-1 (FLT-1; VEGF receptor-1) or kinase insert domain containing receptor (KDR; VEGF receptor-2) as well as extracellular matrix components like matrix metalloproteinases, inflammatory cells including macrophages and intracellular signaling pathways such as the Hif-1 $\alpha$  cascade (11-13). VEGF is an extracellular signaling molecule stimulating proliferation

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and migration of endothelial (progenitor) cells and regulates the permeability of blood vessels via activating a family of transmembrane tyrosine kinase receptors (14-17). FLT-1 and especially KDR are the central mediators of VEGF signaling in angiogenesis and have been shown to be commonly overexpressed in malignant tumors, including HCC, and their overexpression is associated with limited overall survival of patients (18-22). Although various anti-angiogenic compounds like the multi-kinase inhibitor sorafenib and the monoclonal anti-VEGF antibody bevacizumab have been used for the therapy of HCC, the overall efficacy of anti-angiogenic treatments is still disappointing and these treatments are associated with considerable side effects (23-25).

The role of the surrounding stroma as well as inflammatory cells has recently been recognized as a key feature of tumor-driven angiogenesis (26,27). Connective tissue growth factor (CTGF), also known as CCN2, is a 38-kDa protein and was originally identified as a growth factor secreted by human vascular endothelial cells (28). It is a multifunctional signaling modulator involved in several physiologic and pathologic processes such as fibrosis in kidneys and skin, osteogenesis, angiogenesis and tumor development (29-33). In HCC, CTGF has been shown to contribute to tumor growth in an autocrine loop and its overexpression is negatively correlated with overall survival (34-38). Previously, we have shown that the expression of CTGF can be modulated by the histone deacetylase inhibitor (HDACi) Trichostatin A (TSA) (39).

HDACi are currently evaluated as anticancer agents for various hematologic and solid malignancies (40). Panobinostat (LBH589) represents a novel pan-deacetylase inhibitor that has shown excellent efficacy against HCC growth alone or combined in preclinical models and early clinical trials (41-44). Besides their classical mode of action as transcriptional regulators, HDACi are now considered to interfere with a variety of other cellular pathways like cytosolic protein stabilization and signaling pathways, including angiogenesis-related signaling (45-47).

We therefore investigated if and to what extent the pan-deacetylase inhibitor panobinostat mediates its anti-angiogenic properties via regulation of the CTGF signaling pathway in HCC cell lines and in a subcutaneous xenograft model *in vivo*. Our study shows that panobinostat leads to a significant growth delay with prolonged overall survival, mediated by reduced tumor cell proliferation, increased apoptosis and reduced angiogenesis in tumor xenografts (41).

## Materials and methods

**Cell culture.** The human hepatoma cell lines HepG2 (p53wt) and Hep3B (p53null) were obtained from the German collection of microorganisms and cell cultures (DSMZ, Braunschweig, Germany) and maintained under standard conditions as described previously (41). Panobinostat (LBH589) was provided by Novartis Pharma AG (Basel, Switzerland) and was prepared as previously described (41,45). For all *in vitro* experiments, 300,000 cells were seeded to 6-well tissue culture plates (Becton-Dickinson, Heidelberg, Germany) 24 h before treatment. Cells were treated with LBH589 at 0.1 and 0.01  $\mu$ M dissolved in complete growth medium. The medium was not changed during the experiments.

**HepG2 xenograft samples.** Samples from previously established xenografts of HepG2 cells to male athymic nu/nu NMRI mice were used for this study (41).

**Protein isolation and western blotting.** Total protein content was isolated after centrifuging hepatoma cells at 1,000 rpm for 10 min, discarding the liquid phase and adding 50  $\mu$ l of Jie's protein lysis buffer, consisting of 10 mM NaCl<sub>2</sub>, 0.5% NP-40, 20 mM Tris-HCl pH 7.4, 5 mM MgCl<sub>2</sub>, 10  $\mu$ g/ml Prot-I, 1 mM PMSF. After 30 min cooling on ice with intermediate vortexing, the suspension was divided into two portions and stored at -80°C. Samples were subjected to 6-14% SDS-PAGE (Invitrogen, Carlsbad, CA, USA), transferred to a nitrocellulose membrane and blocked for 1 h at room temperature in a TBS or PBS buffer containing 0.1% Tween-20 and 5% low fat milk powder. Membranes were incubated overnight with primary antibodies against CTGF (1:500), VEGF (1:1,000), FLT-1 (1:1,000) (all from Abcam, Cambridge, UK), KDR (1:1,000; Merck Millipore, Darmstadt, Germany), MAPK (1:1,000) and p-MAPK (1:1,000) (both from Cell Signaling Technology, Danvers, MA, USA). Membranes were incubated with a peroxidase coupled secondary antibody (1:2,000, anti-mouse or anti-rabbit IgG; Pierce, Rockford, IL, USA) for 1 h at room temperature (RT). Reactive bands were detected with the ECL chemiluminescence reagent (Amersham Pharmacia Biotech, Freiburg, Germany) and analyzed using GelScan 5 software (BioSciTec, Frankfurt, Germany). Signals were standardized to  $\beta$ -actin (1:5,000; Sigma-Aldrich, Taufkirchen, Germany) content of each sample.

Densitometric quantification of the western blot analyses was done using Bio 1D (Vilber Lourmat, Eberhardzell, Germany) and normalized to untreated controls and  $\beta$ -actin as the loading control.

**Quantitative real-time PCR.** Treated cells and controls were harvested for qPCR after 24-72 h using TRizol<sup>®</sup> (Ambion/Life Technologies, Vienna, Austria) and total RNA extracted using Direct-zol RNA MiniPrep kit (Zymo Research, Irvine, CA, USA). cDNA was synthesized with 0.15 to 0.35  $\mu$ g RNA using the GoScript<sup>™</sup> Reverse Transcription System (Promega, Mannheim, Germany). Finally, real-time PCR was performed with GoTaq<sup>®</sup> qPCR Master Mix (Promega) on a ViiA7 real-time PCR system (Applied Biosystems/Life Technologies) using the following primers (all from Qiagen, Hilden, Germany):  $\beta$ -actin [internal control/reference gene, Qiagen cat. no. QT01680476], VEGF (QT01682072), FLT-1/VEGFR-1 (QT00073640), KDR/VEGFR-2 (QT00069818) and CTGF1 (QT00052899)]. All procedures were performed according to the respective user manuals. Specific amplification was verified using melt curves and gene expression calculated using the  $\Delta\Delta$ Ct method (48). Gene expression levels are presented as mean expression relative to their untreated controls  $\pm$  standard error of the mean (SEM).

**Immunohistochemistry (IHC).** Antigen retrieval was performed by heat induced epitope retrieval in pH 9.0 antigen retrieval buffer (Dako, Glostrup, Denmark) at 95°C for 60 min. Endogenous peroxidase blocking was carried out for 10 min with peroxidase blocking reagent (Dako). Subsequently, primary antibody against CTGF (1:50; Abcam) was applied for 30 min at

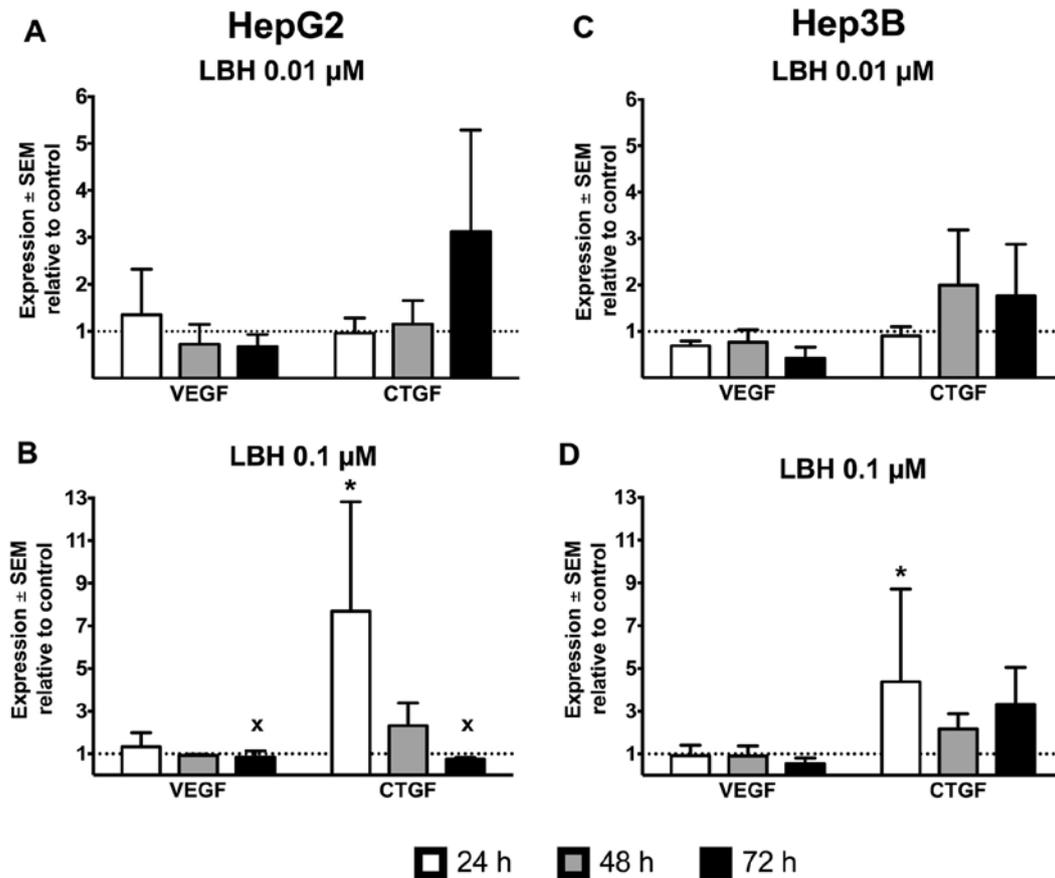


Figure 1. Effect of panobinostat (LBH589) on gene expression of connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF) and fms-like tyrosine kinase-1 (FLT-1) *in vitro* in HepG2 and Hep3B cells. (A) Quantitative real-time RT-PCR analysis after 24, 48 and 72 h treatment with 0.01  $\mu$ M panobinostat (A) and 0.1  $\mu$ M panobinostat (B) in HepG2 cells, with 0.01  $\mu$ M panobinostat (C) and 0.1  $\mu$ M panobinostat (D) in Hep3B cells. Gene expression was normalized to  $\beta$ -actin and expressed relative to untreated controls (set at 1.0). Asterisks indicate statistically significant differences in gene expression vs. the untreated controls ( $p < 0.01$ ). The symbol (x) indicates that very few viable cells remained after 72 h of treatment in these cells.

RT and detected using the EnVision Detection system (Dako). Visualisation was performed using diaminobenzidine (DAB) as the chromogen substrate (Roche Molecular Biochemicals, Mannheim, Germany), all according to the manufacturers' instructions. Slides were counterstained with hematoxylin.

**Statistical analysis and scientific graphing.** Significant differences in qPCR gene expression were calculated using the t-test for paired samples and corrected for multiple comparisons using the Holm-Sidak post-hoc test.  $P < 0.05$  was regarded as statistically significant. Statistics and scientific graphing were performed using Prism 6 (GraphPad Software Inc., La Jolla, CA, USA).

## Results

***In vitro* experiments.** Treatment of the p53-wild-type HepG2 cell line with 0.1  $\mu$ M panobinostat induced a strong increase of CTGF mRNA expression to 7.7-fold after only 24 h and values dropped to 2.3-fold after 48 h. At 72 h, treatment with panobinostat led to a drastic reduction of cells numbers, thus very few viable cells were left for analysis, even after repeated experiments, and these cells expressed CTGF 0.8-fold compared to the untreated controls (Fig. 1A and B). VEGF was induced 1.3-fold after 24 h, and levels after 48 and 72 h dropped to 0.9-

and 0.8-fold, respectively. At 0.01  $\mu$ M, a less dramatic change in gene expression was observed: VEGF was upregulated to 1.3-fold at 24 h, and dropped (in a similar fashion as in 0.1  $\mu$ M treated cells) to 0.7- and 0.6-fold at 48 and 72 h. At this lower concentration, CTGF was gradually induced 1.0-, 1.2- and 3.1-fold at 24, 48 and 72 h, respectively. The gradual time-dependent CTGF induction at 0.01  $\mu$ M in the HepG2 cells was paralleled by a strong induction at 24 h at 0.1  $\mu$ M followed by a drop of CTGF in the remaining viable cells at 48 and 72 h at this higher concentration.

The clear time- and dose-dependent change in gene expression seen in the p53-wild-type HepG2 cells was not observable in the p53-deficient Hep3B cells. VEGF expression remained at 0.9-fold of untreated controls at 24 and 48 h and dropped to 0.5-fold after 72 h incubation with 0.1  $\mu$ M panobinostat. At the lower concentration of 0.01  $\mu$ M LBH, VEGF ranged between 0.7- and 0.4-fold during the treatment period. However, CTGF was induced 4.4-, 2.2- and 3.3-fold after 24, 48 and 72 h incubation with 0.1  $\mu$ M panobinostat (and to only 0.9-, 2.0- and 1.8-fold under 0.01  $\mu$ M panobinostat) (Fig. 1C and D). FLT-1 was not expressed *in vitro* at any time-point in the HepG2 and Hep3B cells.

In western blot analysis, we could not detect VEGF expression in HepG2 cells and neither cell line expressed FLT-1 on protein level (Fig. 2). VEGF in Hep3B cells was induced at

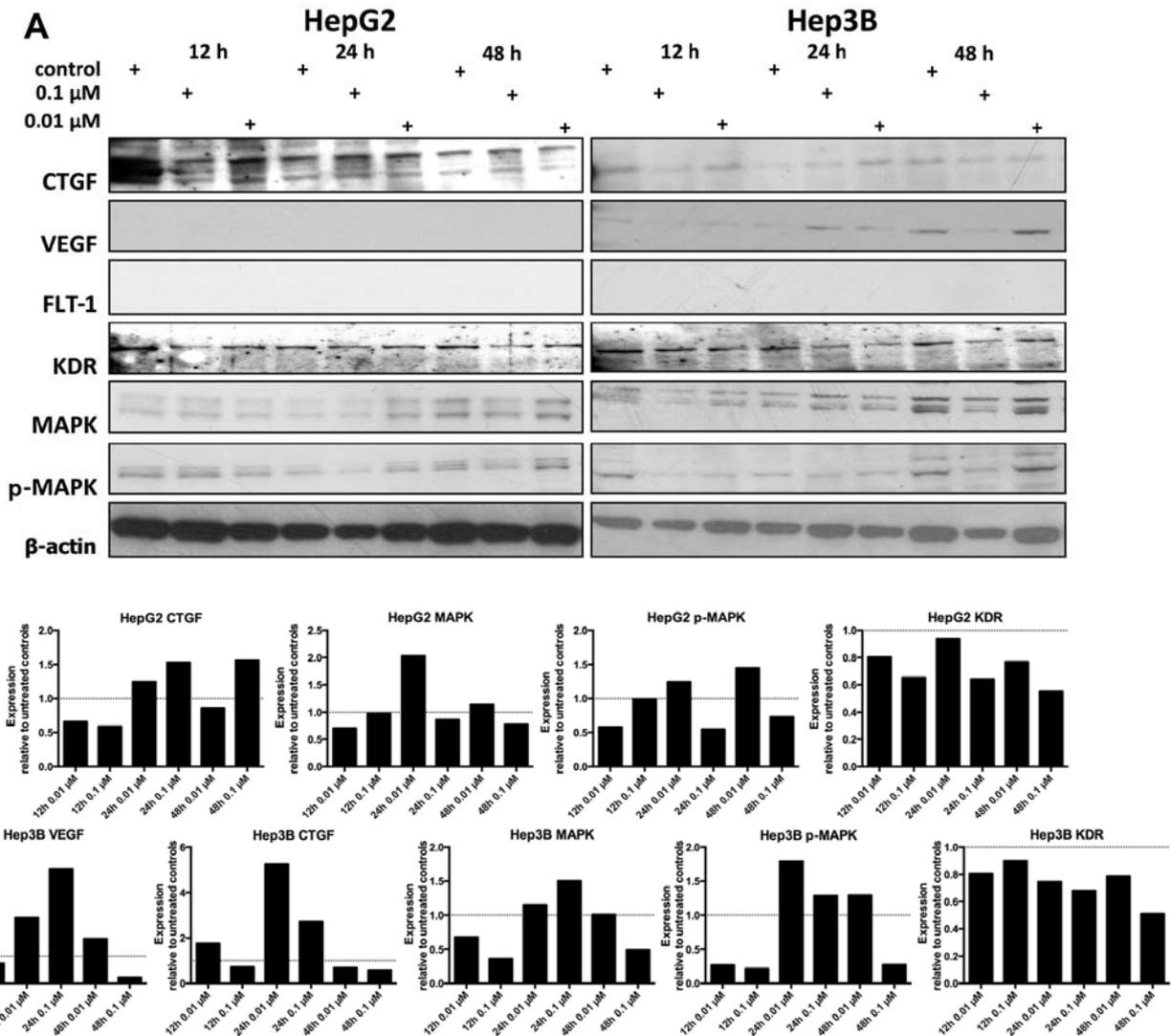


Figure 2. Western blot analysis of expression of connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), fms-like tyrosine kinase-1 (FLT-1), kinase insert domain containing receptor (KDR), MAPK, p-MAPK *in vitro* and densitometric quantification. HepG2 and Hep3B cells were incubated with 0.1 and 0.01 μM panobinostat for 12, 24 and 48 h. Western blot results show representative examples for expression of CTGF, VEGF, FLT-1, KDR, MAPK and p-MAPK as well as β-actin, which served as loading control (A). Protein expression analyzed using western blot analysis was quantified by densitometry (B). Shown are expression values for CTGF, MAPK, p-MAPK, KDR and VEGF after treatment of HepG2 and Hep3B cell lines with 0.01 and 0.1 μM of panobinostat for 12-48 h.

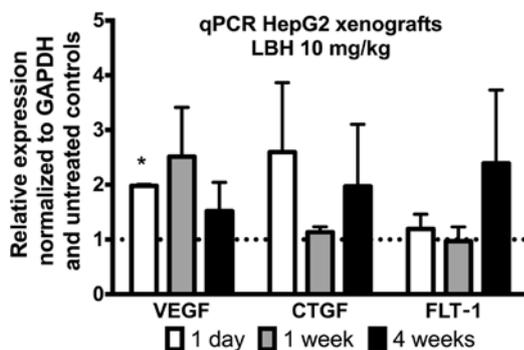


Figure 3. Effect of panobinostat (LBH589) on gene expression of connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF) and fms-like tyrosine kinase-1 (FLT-1) *in vivo*. HepG2 xenograft specimens were analyzed for mRNA expression of CTGF, VEGF and FLT-1 after 1, 7 and 28 days of daily i.p. injections of 10 mg/kg panobinostat. Results were normalized to β-actin content of each sample and represent mean ± SD of 8 independent samples per group and are expressed relative to expression levels of untreated control animals set at 1.0 for each time-point.

24 h by panobinostat 2.4- and 4.2-fold at concentrations of 0.01 and 0.1 μM, respectively, as calculated by densitometry. Longer incubation (48 h) reduced VEGF expression to 1.6- and 0.2-fold at 0.01 and 0.1 μM. CTGF was induced most effectively in HepG2 cells by 0.1 μM panobinostat at either 24 or 48 h to levels of 1.5- and 1.6-fold, respectively. In Hep3B cells, 24 h incubation induced CTGF most prominently, but the lower dose of 0.01 μM was more effective than 0.1 μM and the CTGF levels were 5.3- and 2.7-fold elevated in this setting. In both cell lines, elevated levels of MAPK were accompanied by elevated levels of p-MAPK and vice versa. The two highest levels of MAPK in HepG2 (2.0-fold at 24 h 0.01 μM and 1.1-fold at 48 h 0.01 μM) were accompanied by the two highest levels of p-MAPK (1.2-fold at 24 h 0.01 μM and 1.4-fold at 48 h 0.01 μM), resulting in a MAPK/p-MAPK ratio of 1.67 and 0.79 at 24 and 48 h for 0.01 μM. At all other time-points and concentrations, MAPK and p-MAPK were suppressed compared to the untreated controls. The lowest

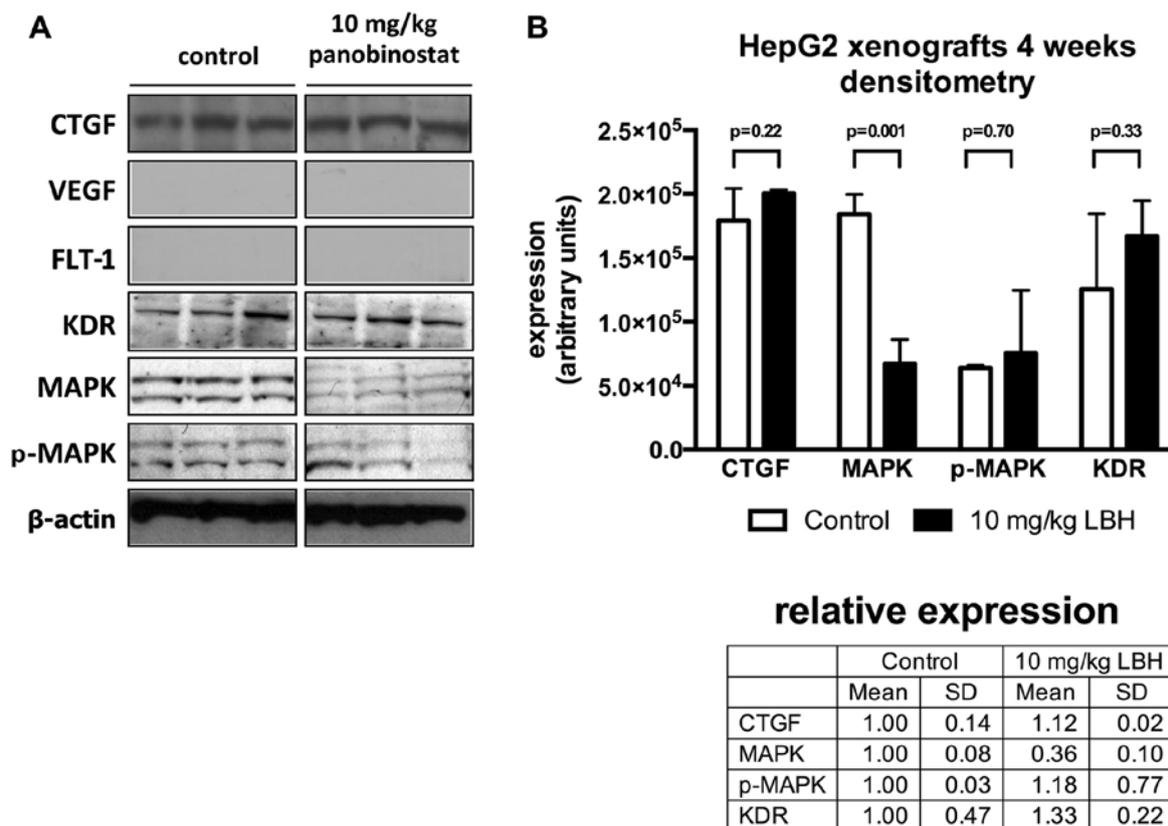


Figure 4. Western blot analysis of expression of connective tissue growth factor (CTGF), vascular endothelial growth factor (VEGF), fms-like tyrosine kinase-1 (FLT-1), kinase insert domain containing receptor (KDR), MAPK, p-MAPK and densitometric analysis of protein expression *in vivo*. Western blot analysis of representative HepG2 xenograft specimens from untreated controls or animals receiving 10 mg/kg panobinostat. Tumor samples were obtained at the end of the treatment period and subjected to semiquantitative western blotting against CTGF, VEGF, FLT-1, KDR, MAPK and p-MAPK.  $\beta$ -actin served as a loading control (A). Protein expression was quantified by densitometry. Shown are expression values for CTGF, MAPK, p-MAPK, KDR and after treatment of HepG2 xenograft specimens treated with 10 mg/kg panobinostat for 4 weeks (B).

expression in HepG2 was thus observed at 12 h 0.01  $\mu$ M for both MAPK (0.7-fold) and p-MAPK (0.6-fold) (ratio MAPK/p-MAPK, 1.17) and in Hep3B at 12 h 0.1  $\mu$ M also for both MAPK (0.4-fold) and p-MAPK (0.2-fold) (ratio 2). KDR levels were generally suppressed at all time-points and concentrations, with the lowest level of 0.55-fold in HepG2 and 0.51-fold in Hep3B (vs. untreated controls) at 48 h 0.1  $\mu$ M in both cell lines.

**HepG2 xenografted nude mice.** Treatment of nude mice bearing HepG2 xenografts with daily i.p. injections of 10 mg/kg panobinostat resulted in an increase of VEGF on mRNA level 2.0-, 2.5- and 1.5-fold after 1 day, 1 week and 4 weeks, respectively (Fig. 3). CTGF was upregulated 2.6-, 1.1- and 2.0-fold while FLT-1 reached an expression of 1.2-, 1.0- and 2.4-fold in treated vs. the untreated mice. After 4 weeks of treatment of the HepG2 xenografts in the nude mice, the western blot analysis revealed a relative expression change of CTGF (1.1-fold, vs. untreated xenografts), p-MAPK (1.2-fold) and KDR (1.3-fold) and a downregulation of MAPK to 0.4-fold ( $p=0.001$ ) of untreated controls (Fig. 4).

In immunohistochemical staining of the explanted xenografts after 4 weeks (Fig. 5), the 10 mg/kg panobinostat treated samples showed an expression of CTGF [low (Fig. 5A), moderate (Fig. 5B)] in contrast to 2.5 mg panobinostat treated samples (Fig. 5C) and untreated controls (Fig. 5D).

## Discussion

In the present study we investigated the anti-angiogenic properties of the pan-deacetylase inhibitor panobinostat via regulation of the CTGF signaling pathway in HCC cell lines and a subcutaneous xenograft model *in vivo*.

Panobinostat (LBH589) plays an important role in anti-angiogenesis (45). Recently, Di Fazio *et al* showed a panobinostat-mediated significant growth delay of a subcutaneous HCC xenograft model with prolonged overall survival, mediated by reduced tumor cell proliferation, increased apoptosis and especially reduced angiogenesis (41). As key message of this study, panobinostat induces alternative apoptotic pathways dependent on the p53 status. Furthermore, it was shown macroscopically that the microvascular density and tumor size of panobinostat treated HCC xenografts were significantly decreased.

Our results show that panobinostat induces a context-dependent differential expression of CTGF *in vitro* and *in vivo* in a subcutaneous xenograft model after daily i.p. injections of 10 mg/kg panobinostat. The previously shown tumor growth inhibition (41) was associated with the inhibition of the MAPK signaling pathway and with inhibition of tumor vascularization. For this study, *in vivo* samples from those previous experiments (41) were used. Interestingly, the protein levels of CTGF in the western blot analyses were downregulated, while the

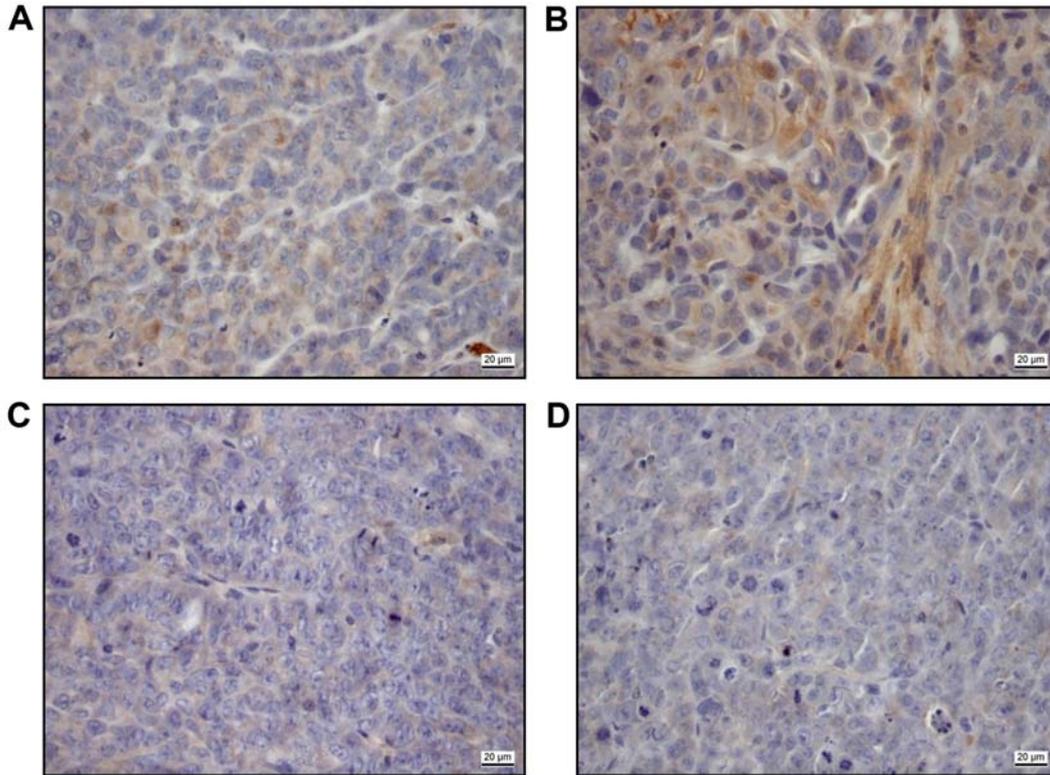


Figure 5. Immunohistochemical analysis of connective tissue growth factor (CTGF) expression (magnification, x400). HepG2 xenografts were treated with daily i.p. injections of 10 mg/kg panobinostat for 28 days. Panobinostat treated samples (10 mg/kg) showed an expression of CTGF [low (A), moderate (B)] in contrast to 2.5 mg panobinostat treated samples (C) and untreated controls (D) (inset control tissue of liver cirrhosis).

mRNA expression of CTGF in HepG2 and Hep3B cells after 24 and 48 h were mainly upregulated. An increased expression of CTGF was also seen in the immunohistochemical analysis of panobinostat treated xenograft samples. Because CTGF can be both secreted and membrane bound, western blot analysis of the cell lysate may not reflect the total protein amount produced when compared to mRNA expression. While in mesangial cells it is bound to the cell surface (49), it has been shown that in hepatocellular carcinoma cells, a fraction of the produced protein remains cell-bound (38). This may explain why our mRNA and protein expression data do not necessarily correlate. One other reason may be that the CTGF mRNA pool does not necessarily translate into a 1:1 expression on the protein level. A further reason could be the fact that in the *in vivo* xenograft model, the CTGF expression is especially triggered by endothelial cells. This assumption is supported by findings of Komorowsky *et al* (39): treatment of cultured endothelial cells with different HDAC inhibitors upregulated CTGF mRNA and protein. Their data indicate that the effect of HDAC inhibitors on CTGF expression is largely cell dependent in non-tumor cells.

In this study VEGF and FLT-1 (VEGFR-1) are not expressed and thus do not seem to play a role in treatment with panobinostat, especially in the xenograft model. VEGF and the VEGFR system are known to be main regulators of angiogenesis. In this study there is absolutely no expression of VEGF and FLT-1, especially in the controls of the xenograft samples. It could be speculated that an alternative angiogenesis pathway is activated in this xenograft model involving

the xenograft environment (50), especially because KDR (VEGFR-2) is expressed and induced in the xenografts after treatment.

Panobinostat is a pan-deacetylase inhibitor and, like many other compounds targeting histone deacetylases, a promising drug against different malignant tumors (51-55). Especially solid malignant tumors including prostate (56,57), breast (58,59) and lung cancers (60,61) are supposed to be angiogenesis-dependent as shown for hepatocellular carcinoma (62-64). Tumor progression of HCC is associated with angiogenesis and the increase in microvascular density is associated with a poor prognosis. The inhibition of tumor angiogenesis and its complete comprehension is the aim when developing a successful anti-angiogenic tumor therapy. Angiogenesis is the formation and growth of new blood vessels from the already existing vasculature. It is necessary for various inflammatory, ischemic, infectious and immune disorders and also for malignant processes (65,66).

Malignant cells are able to secrete pro-angiogenic factors including VEGF, which induces tumor blood vessel formation (67). The *in vivo* angiogenic activity of secreted VEGF may be regulated by extracellular inhibitors, because it is also produced in avascular tissues such as the cartilage. CTGF can inhibit VEGF-induced angiogenesis via complex formation of VEGF with CTGF through a protein-to-protein interaction (68,69). On the other hand, CTGF expresses mitogenic activity in endothelial cells to promote angiogenesis, although the potency of angiogenic activity of CTGF is not well-evaluated (70,71).

In conclusion, panobinostat induces differential expression of CTGF in a context-dependent manner. In this setting, the anti-angiogenesis is obviously not mediated via the classical VEGF-driven cascade.

Other pathways are supposed to be involved in the CTGF mediated angiogenesis and further experiments are necessary to explore the role of CTGF.

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