Research Article

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Combination therapy for hepatocellular carcinoma: Additive preclinical efficacy of the HDAC inhibitor panobinostat with sorafenib

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Background & Aims: Hepatocellular carcinoma (HCC) is a heterogeneous cancer in which sorafenib is the only approved systemic therapy. Histone deacetylases (HDAC) are commonly dysregulated in cancer and therefore represent promising targets for therapies, however their role in HCC pathogenesis is still unknown. We analyzed the expression of 11 HDACs in human HCCs and assessed the efficacy of the pan-HDAC inhibitor panobinostat alone and in combination with sorafenib in preclinical models of liver cancer.

Methods: Gene expression and copy number changes were analyzed in a cohort of 334 human HCCs, while the effects of panobinostat and sorafenib were evaluated in three liver cancer cell lines and a murine xenograft model.

Results: Aberrant HDAC expression was identified and validated in 91 and 243 HCCs, respectively. Upregulation of *HDAC3* and *HDAC5* mRNAs was significantly correlated with DNA copy number gains. Inhibiting HDACs with panobinostat led to strong anti-tumoral effects *in vitro* and *vivo*, enhanced by the addition of sorafenib. Cell viability and proliferation declined, while apoptosis and autophagy increased. Panobinostat increased histone H3 and HSP90 acetylation, downregulated *BIRC5* (survivin) and upregulated *CDH1*. Combination therapy with panobinostat and sorafenib significantly decreased vessel density, and most significantly decreased tumor volume and increased survival in HCC xenografts.

Conclusions: Aberrant expression of several HDACs and copy number gains of *HDAC3* and *HDAC5* occur in HCC. Treatment with panobinostat combined with sorafenib demonstrated the highest preclinical efficacy in HCC models, providing the rationale for clinical studies with this novel combination.

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Abbreviations: BCLC, Barcelona Clinic Liver Cancer; CI, confidence interval; FACS, fluorescent-activated cell sorting; HCC, hepatocellular carcinoma; HBV, hepatitis B virus; HCV, hepatitis C virus; HDAC, histone deacetylase; HDACi, histone deacetylase inhibitor; HSP90, heat shock protein 90; IACUC, Institutional Animal Care and Use Committee; IHC, immunohistochemistry; PBS, phosphate buffered saline; SC, Spearman's coefficient.

Introduction

Hepatocellular carcinoma (HCC) remains a major health problem worldwide as the third cause of cancer-related mortality and the primary cause of death among cirrhotic patients [1]. Hepatitis B and C, alcohol, and aflatoxin have been identified as major risk factors leading to the development of HCC [2,3]. Resection and transplantation are the only curative treatments available but are greatly hampered by high recurrence rates [3]. Currently, the multi-kinase inhibitor sorafenib is the only FDA-approved treatment for patients with advanced disease, necessitating the development of novel compounds that are effective against this devastating disease [4,5]. A new class of histone deacetylase (HDAC) inhibitors is currently considered to be among the most promising anticancer agents in drug development, most likely due to their potent anti-tumoral effects demonstrated in preclinical studies of hematological malignancies and solid tumors and their promising therapeutic potential in early-phase clinical trials [6]. Vorinostat and romidepsin are HDAC inhibitors (HDACi) that recently received FDA approval for the treatment of cutaneous T cell lymphoma [7].

HDAC inhibitors execute their anti-tumoral activities through hyperacetylation of histone and non-histone proteins, such as the molecular chaperone heat shock protein 90 (HSP90). Histone

Cancer

acetylation leads to decreased affinity for DNA and increased access of transcription factors, while HSP90 acetylation blocks its chaperone function and causes destabilization of client proteins implicated in several important signaling pathways [8]. In addition, HDACi can repress transcriptional activity directly or through destabilization and accelerated decay of mature transcripts. These different mechanisms of action lead to various anti-tumoral effects including the induction of apoptosis, autophagy and differentiation, cell cycle arrest and inhibition of tumor vascularization [9]. Although several key factors in cancer-related signaling pathways have been identified to be regulated by the HDAC family, no exclusive targets have been reported so far. HDACs are known to regulate the expression of some of the pivotal players of hepatocarcinogenesis including the apoptosis inhibitor BIRC5 (survivin), the tumor suppressor gene and Wnt-pathway regulator CDH1 (E-Cadherin), and the cyclin-dependent kinase inhibitor *CDKN1A* (p21) known to be implicated in growth arrest, senescence and apoptosis [10–14].

Panobinostat (LBH589) is a novel pan-HDAC-inhibitor with high efficacy in several preclinical models of cancer [15] and synergistic anti-tumoral activity when administered in combination with chemotherapy or molecular targeted therapies [14,16]. Early clinical trials recently reported promising results with feasible tolerance and side effects, even indicating anti-tumoral activity in patients with lymphoma [17].

Further evidence of the importance of the HDAC machinery in cancer is their observed dysregulation in tumoral tissues and their close correlation with aggressive tumoral behaviour. In cancer, aberrant expression of several of the 11 known classical HDAC family members (HDAC1-11, class I, II, IV) often correlates with disease progression and patient's outcomes [18]. Overexpression of *HDAC1* and *2* has been described in gastric and colorectal cancer [19,20], and HDAC3 has recently been proposed as a potential biomarker for recurrence following liver transplantation in hepatits B (HBV)-associated HCC [21].

Herein, we show that several HDAC-family members are aberrantly expressed in HCC with significant correlation of *HDAC3* and *5* upregulation to DNA copy number gains. In addition, we provide evidence that panobinostat has consistent anti-tumoral efficacy in preclinical models of HCC, which is further enhanced when combined with sorafenib. Significantly decreased tumor volume and increased survival *in vivo* in response to this combination establish a rationale for clinical studies with this novel combination.

Materials and methods

Human samples, mRNA expression array and SNP analysis

A total of 230 human samples were obtained from patients with HCC treated with resection or liver transplantation within the HCC Genomic Consortium (Mount Sinai School of Medicine, New York; Hospital Clinic, Barcelona; Istituto Nazionale dei Tumori in Milan) as previously reported [22–25]. After patient's informed consent and Institutional Review Board approvals were obtained, samples were collected and gene expression and SNP array studies were performed as described elsewhere [22]. The training set included 91 fresh frozen HCCs, along with 10 normal, 13 cirrhotic and 18 dysplastic liver samples for gene expression analysis (GSE9843). For SNP array, 101 HCC samples and 101 matching cirrhotic tissues (GSE9829) were analyzed. The validation set included a publicly available dataset of 243 HCCs and paired non-tumoral liver tissue, and 2 pooled, normal liver samples (GSE14520). These patients had predominantly chronic HBV infection [26], while patients within the training set had HCV-related HCC.

Cell lines and in vitro drug treatments

Huh7 (Riken Bioresource Center), Hep3B and HepG2 (ATCC, Manassa, VA) cells were cultured as previously described [22–24]. Panobinostat was provided by Novartis Pharma AG (Basel, Switzerland) following a MTA agreement; sorafenib was purchased from LC Laboratories, Woburn, MA. Both compounds were diluted in DMSO and added 1, 2 and 3 days before cells were processed for further analyses. Final DMSO in all experiments was <0.05%.

Cell viability, proliferation assays, cell cycle analyses, Western blot and real time PCR

MTT, ^{3}H-Thymidine incorporation, and fluorescent-activated cell sorting (FACS) assays were performed as previously reported [24]. Detailed information on Western blot and real time PCR is given in the Supplementary Materials and Methods.

HCC xenograft model and immunohistochemistry

All animal experiments were performed following Mount Sinai School of Medicine Institutional Animal Care and Use Committee (IACUC) approval of protocols. Generation of the experimental model followed previously reported protocols [24]. A detailed description of the xenograft model, the treatment arms and the immunohistochemistry procedures can be found in the Supplementary Materials and Methods.

Statistical analysis

Bars represent the mean + standard error or standard deviation as explained in each figure's legend. Two-tailed *t*-test or *U*-test for continuous variables was used for comparisons between groups and correlations were calculated with the non-parametric Spearman's Coefficient (SC). SNP-array data and gene expression microarray analyses were performed as previously described [22]. Calculations were done by the SPSS package (SPSS 15.0, Chicago, IL).

Results

HDAC alterations in human hepatocellular carcinoma

Aberrant HDAC expression

Gene expression levels of all 11 classic HDACs were analyzed by microarray in a training set of 91 HCC samples, 18 dysplastic nodules, 10 normal and 13 cirrhotic liver samples (Fig. 1A). A subset of HDAC mRNAs (*HDAC1, 2, 4, 5*, and *11*) was significantly upregulated compared to the normal liver, cirrhosis and dysplastic nodules, with the highest expression levels for *HDAC2, 4* and *11* (Fig. 1B). *HDAC3* was significantly upregulated in HCC compared to cirrhosis. By contrast, *HDAC6* and *HDAC7* were significantly downregulated in HCC compared to the normal liver (Supplementary Table 1). To validate these results in an independent set of HCC samples, we analyzed expression levels by independent microarray analysis in 243 paired HCC-cirrhotic samples and two pooled normal liver samples. Significant overexpression of 4 HDACs (1, 2, 4, and 5) and downregulation of *HDAC6* and *HDAC7* were confirmed (Supplementary Fig. 1, and Supplementary Table 2). Data for *HDAC8* and *HDAC10* was not available.

Frequent copy number gains of HDAC3 and HDAC5 are associated with high gene expression levels

We used SNP array technology to evaluate DNA copy number alterations of all 11 HDAC family members. Although no high-level amplifications were detected (cut-off: copy number value 3.4), *HDAC3* and *HDAC5* showed significant DNA gains (Fig. 1C and D). As expected, samples with increased copy numbers in *HDAC3* and *HDAC5* had significantly higher expression of *HDAC3*

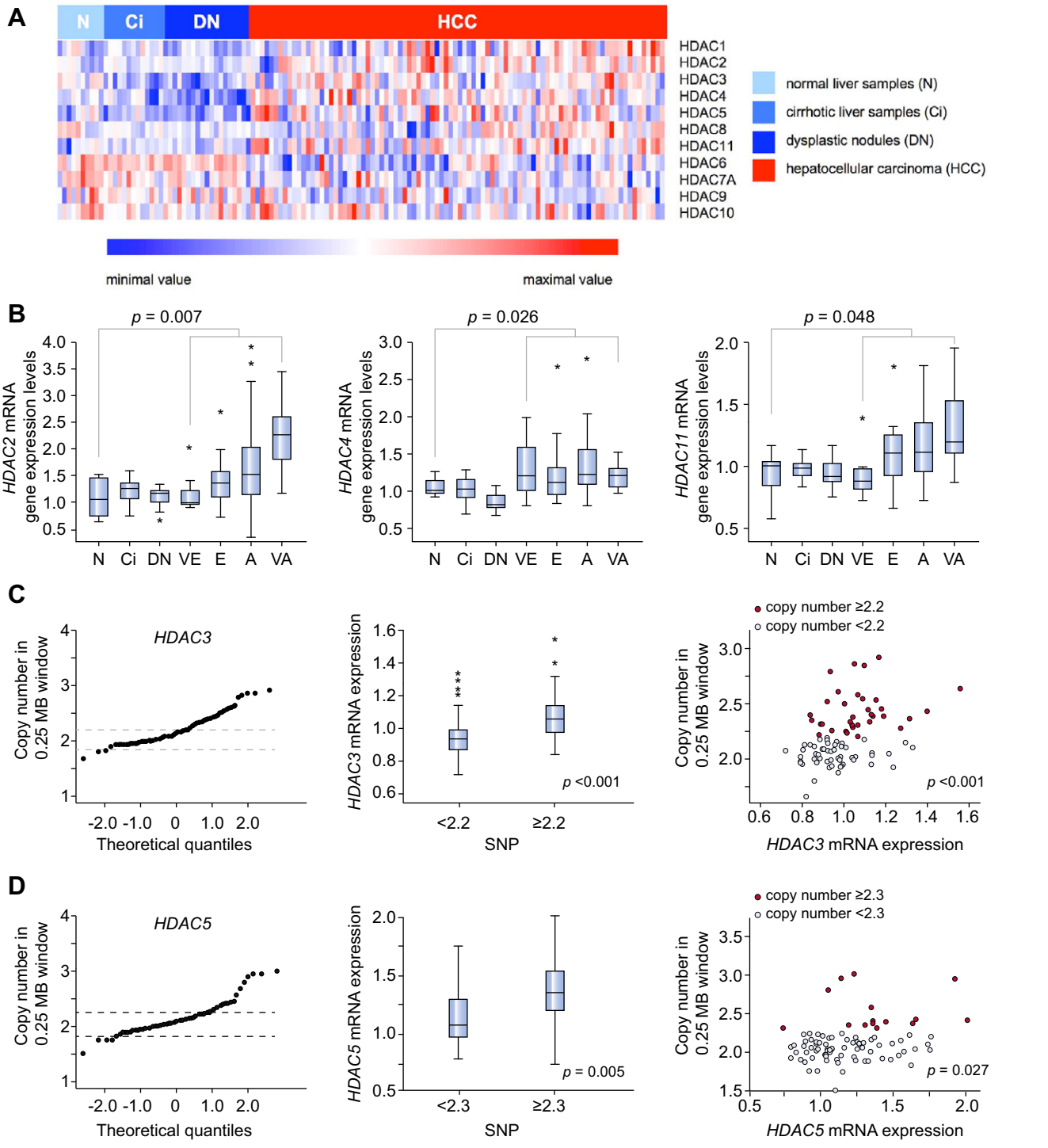


Fig. 1. Aberrant HDAC mRNA expression and copy number changes in human HCC. (A) Heatmap representing expression levels of all HDACs (*HDAC1-11*) in the training set. (B) Boxplots displaying gene expression levels of *HDAC2*, *4* and *11* in the hepatocarcinogenic process (N: normal liver, Ci: cirrhosis, DN: dysplastic nodule, and for HCC VE: very early, E: early, A: advanced and VA: very advanced). (C) *HDAC3* and (D) *HDAC5* DNA copy number levels, expression analyses and their correlation. Qplots show mean SNP array value for the gene's locus for each tumoral sample and dashed lines represent highest/lowest copy number values in paired cirrhotic samples; boxplots show mean expression values in high and low copy number samples. Dotplots display expression and copy number values per sample, red dots = copy number ⩾2.2 and 2.3, respectively; light blue dots = copy number <2.2 and 2.3, respectively). Stars in boxplots represent outlier samples.

and *HDAC5* mRNA levels (p <0.001 and p = 0.005, respectively) (Fig. 1C and D) and showed a significant correlation between amplification and gene expression (p <0.001 and p = 0.0027, respectively) (Fig. 1C and D).

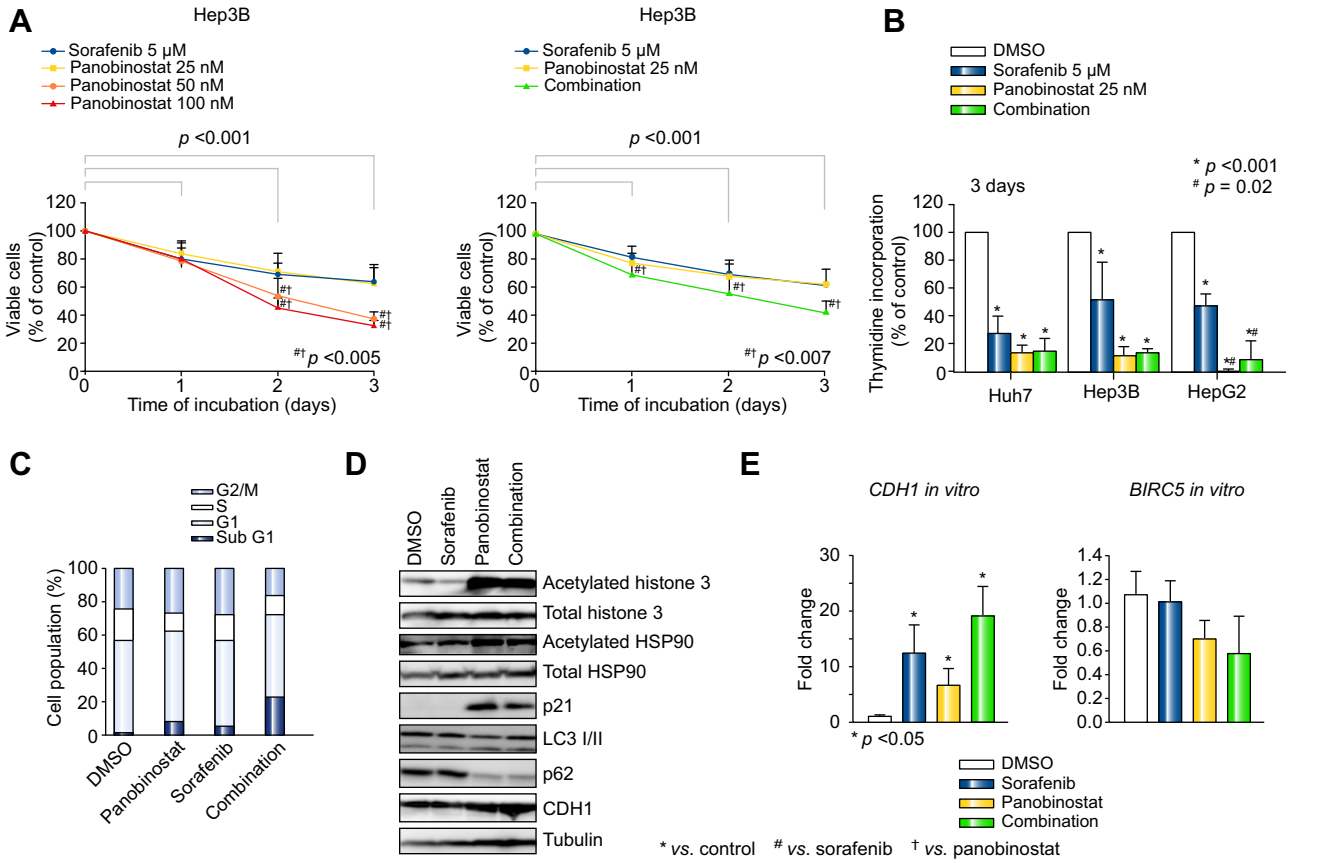


Fig. 2. Panobinostat shows high efficacy in liver cancer cell lines, enhanced by sorafenib. (A) Cell viability studies after treatment with panobinostat, sorafenib and their combination for 1, 2, and 3 days in Hep3B cells. (B) Proliferation studies after 3 days of treatment in Huh7, Hep3B and HepG2 cells. (C) Cell cycle analysis after 3 days of treatment in Huh7 cells. (D) Western blots of acetylated/total histone H3, acetylated/total HSP90, p21, LC3, p62 and CDH1 in Huh7 cells. (E) Expression levels of *CDH1* and *BIRC5* in Huh7 cells after treatment for 1 day. Mean values + standard deviation are shown.

Expression of CDH1 and BIRC5 mRNA correlates with gene expression of multiple HDACs in human HCC

We next analyzed the mRNA expression of 2 previously described HDAC target-genes, *CDH1* and *BIRC5*, in our cohort of samples. *CDH1* was significantly downregulated in human HCC samples of our training set when compared to the normal liver ($p = 0.001$), the cirrhotic liver ($p < 0.001$) and to dysplastic nodules ($p < 0.001$) (Supplementary Fig. 2A). *BIRC5* mRNA was significantly upregulated in human HCC samples compared to the normal liver, cirrhotic and dysplastic nodule samples ($p < 0.001$) (Supplementary Fig. 3A). mRNA levels of both genes were correlated to gene expression of multiple HDACs, most significantly *CDH1* to *HDAC4* and *5* (Supplementary Fig. 2B) and *BIRC5* to *HDAC1*, *2*, and *11* (Supplementary Fig. 3B).

Effect of panobinostat alone or in combination with sorafenib in liver cancer cell lines

Panobinostat affects cell viability and proliferation alone and in combination with sorafenib in culture

On the basis of the data obtained in human HCC analyses, we investigated the effects of panobinostat (Novartis Pharma AG, Basel, Switzerland) in experimental models of HCC. Panobinostat significantly decreased cell viability compared to control in a dose and time dependent manner at 25, 50 and 100 nM concentrations in Hep3B and HepG2 cells at 1, 2 and 3 days after treatment (12.1–77.7% decline, $p < 0.05$) (Fig. 2A and Supplementary Fig. 4A, respectively). In Huh7 cells, cell viability significantly declined by 47.7% and 23%, respectively, 2 and 3 days after the administration of 50 and 100 nM concentrations compared to control ($p < 0.001$) (Supplementary Fig. 4B). Interestingly, panobinostat 50 and 100 nM significantly decreased cell viability by 15.7–41.8% compared to sorafenib alone ($p < 0.004$) 3 days after treatment in all 3 cell lines (Fig. 2A, Supplementary Fig. 4A and B). Combination therapy enhanced this effect on cell viability compared to single treatment in Hep3B and HepG2 cells at all time points analyzed (Fig. 2A, Supplementary Fig. 4C).

Twenty-five nM panobinostat also significantly decreased proliferation by up to 88.9% in Huh7 and Hep3B cells and completely blocked proliferation in HepG2 cells, 2 and 3 days after administration (Fig. 2B, Supplementary Fig. 5). It is noteworthy that panobinostat led to greater decrease of proliferation than sorafenib treatment alone after 2 and 3 days of treatment (Fig. 2B, Supplementary Fig. 5). Nevertheless, combination treatment did not further enhance the effect on proliferation (Fig. 2B, Supplementary Fig. 5).

Panobinostat induces apoptosis, acetylation of histone H3 and HSP90, and promotes autophagy in culture

We then analyzed the effects of panobinostat and the combination of panobinostat with sorafenib on the cell cycle by FACS

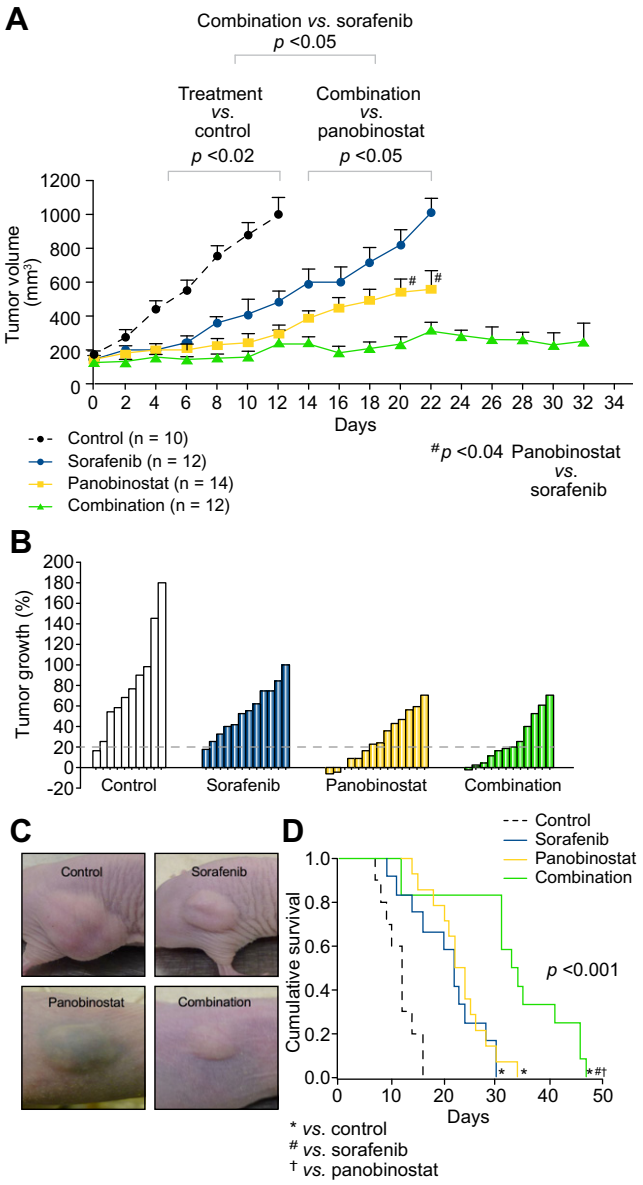


Fig. 3. Combination of panobinostat and sorafenib mostly decreases tumor volume and improves survival in HCC xenografts. (A) Tumor volume of HCC xenografts treated with panobinostat, sorafenib and the combination of both. Plotted values represent mean tumor volume until half of the mice for each treatment arm have been sacrificed. Mean values + standard error are shown. (B) Waterfall plot showing % tumor growth from randomization to day 12 of treatment. Bars below 20% show stable disease or response. (C) Representative pictures of HCC xenografts of each group at the day of euthanization. (D) Kaplan–Meyer survival analyses of mice in different treatment arms.

analysis. Panobinostat alone increased the percentage of necrotic/apoptotic cells in sub-G1 to 8.5% compared to 1% in the control group. Combination with sorafenib significantly enhanced this effect to 23% in the combination group *vs.* sorafenib ($p = 0.002$) and control ($p = 0.003$), suggesting apoptosis as the main cause of cell death (Fig. 2C).

In order to confirm the previously described mechanism of action of panobinostat, we analyzed the acetylation status of the 2 known HDAC targets, histone H3 and the molecular chaperone HSP90. Panobinostat alone and in combination with sorafenib led to a significant hyperacetylation of histone H3 and HSP90 in Huh7 cells 1 day after treatment (Fig. 2D).

We also observed a significant induction of the known HDAC target p21 at the protein level in the panobinostat treated cells 1 day after administration (Fig. 2D, Supplementary Fig. 6).

We finally aimed at assessing autophagy activation in panobinostat and sorafenib-treated Huh7 cells by analyzing the protein expression of LC3 and p62, two well-known autophagy markers [27]. Panobinostat administration, but not treatment with sorafenib, led to a significant decrease of p62 along with increased LC3 II levels, indicating that panobinostat might induce autophagy in Huh7 cells (Fig. 2D, Supplementary Fig. 6).

Panobinostat changes gene expression levels of CDH1 and BIRC5 in vitro

We further analyzed mRNA levels of *CDH1* in Huh7 cells after treatment with sorafenib, panobinostat and their combination. Although both compounds increased *CDH1* gene expression, only the combination therapy led to a statistically significant rise ($p = 0.02$, Fig. 2E). Western blot analysis showed upregulation of CDH1 protein expression after panobinostat therapy for 1 day following treatment (Fig. 2E, Supplementary Fig. 6). The expression levels of *BIRC5* mRNA were slightly decreased after treatment with panobinostat, but not with sorafenib, in Huh7 cells (Fig. 2E).

Effect of panobinostat alone or in combination with sorafenib in HCC xenografts

Panobinostat and sorafenib additively reduce tumor volume and increase survival in vivo

Treatment with panobinostat as single agent, and as expected with sorafenib, led to a significant delay in tumor growth compared to control mice ($p < 0.02$, day 4–12) (Fig. 3A). After 12 days of treatment, the median tumor volume was 987.3 mm^3 for control, 288.2 mm^3 for panobinostat and 464.9 mm^3 for sorafenib-treated mice. Tumor volume was further reduced to 225.7 mm^3 in the group that received combination therapy ($p < 0.05$, Fig. 3A). After 20 days of treatment, this additive effect was still evident with a median tumor volume of 227 mm^3 in the combination group, 531 mm^3 in the panobinostat group and 806 mm^3 in mice receiving sorafenib (Fig. 3A, $p = 0.04$ for panobinostat *vs.* sorafenib, $p = 0.0002$ for combination *vs.* sorafenib, and $p = 0.005$ for combination *vs.* panobinostat). To assess the response rate for each group, we analyzed the tumor growth rate less than 20% at day 12 (Fig. 3B). This effective delay in tumor growth was evident for 7/12 (58.3%) mice of the combination group, compared with 6/14 (42.9%) in the panobinostat, 1/10 (10%) in the sorafenib and 1/12 (8.3%) in the control group. This low growth rate was maintained in 6/12 (50%) panobinostat-treated mice until the study end points were reached (data not shown). As an example, Fig. 3C shows xenograft tumors 5 min before euthanization: while in the panobinostat group 2, intratumoral hematomas occurred, not interfering with continuation of the therapy, no adverse events could be observed in the other treatment arms. Nevertheless, panobinostat treatment at 15 mg/kg per day induced weight loss of up to 20% of the body weight in 5/14 (35.7%) animals, and this toxicity led us to use a dose of 7.5 mg/kg in the combination arm. Overall survival increased in the single treatment groups (median 22 days for sorafenib, 23 days for panobinostat) compared to control (median survival 12 days, $p < 0.001$), while combination treatment further

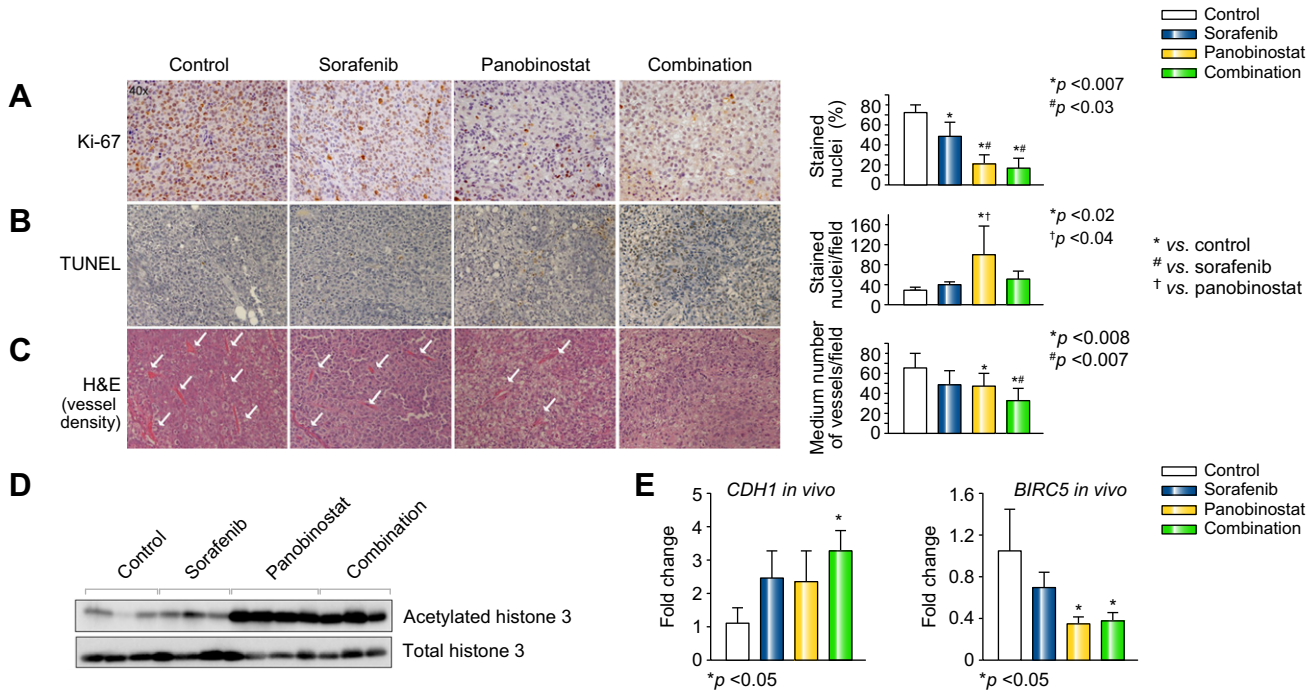


Fig. 4. Drug-induced effects on proliferation, apoptosis, angiogenesis and target-gene expression in HCC xenografts. (A) Ki-67 immunostaining for proliferation analysis, (B) TUNEL immunostaining for apoptosis analysis and (C) hematoxylin and eosin (H&E) staining for vessel density assessment in HCC xenografts. Original magnification for all pictures is 40×. (D) Western blot results for acetylated and total histone H3 in HCC xenografts. (E) Expression levels of *CDH1* and *BIRC5 in vivo*. Mean values + standard deviation are shown.

enhanced the median survival to 34 days in comparison to control and single treatments (p <0.001, Fig. 3D).

Panobinostat induces apoptosis, decreases proliferation and reduces vessel density in HCC xenografts

To further assess proliferation and apoptosis *in vivo*, Ki-67 and TUNEL immunohistochemistry was performed. We observed a significant decrease in the proliferation index (stained nuclei per field) from 72.1 ± 7% in control animals to 48.1 ± 13.8% in sorafenib treated mice, 20.5 ± 9.3% in the panobinostat and 17 ± 8.9% in the combination group (p <0.007) (Fig. 4A). Panobinostat and combination therapy led to a significantly lower proliferation index than sorafenib treatment alone (p <0.03) (Fig. 4A), but no synergism could be detected. TUNEL staining revealed a significant increase of apoptotic cells in the panobinostat-treated mice compared to the control and sorafenib groups (p <0.04). Although combination treatment increased apoptosis compared to sorafenib, the difference was not statistically significant (Fig. 4B). Finally, we analyzed neoangiogenesis *in vivo* by assessing the vessel density in each tumor. Panobinostat treatment alone led to a significant decrease of vessel density compared to control animals, which could be enhanced by combination with sorafenib leading to a significantly lower vessel density compared to control and sorafenib treated mice (p <0.007) (Fig. 4C). We also confirmed the activity of panobinostat in HCC xenografts with a significant increase of acetylated histone H3 in all analyzed tumors (Fig. 4D).

Panobinostat suppresses BIRC5 and induces CDH1 gene expression in combination with sorafenib in vivo

Finally, we analyzed the gene expression of *CDH1* and *BIRC5* in HCC xenografts collected from mice treated with panobinostat, sorafenib and their combination. Although sorafenib and panobinostat alone increased *CDH1* expression levels, only the combination led to a statistically significant increase of *CDH1* mRNA *in vivo* (p = 0.02) (Fig. 4E). *BIRC5* gene expression levels significantly declined after treatment with panobinostat alone (p = 0.01) and after treatment with combination (p = 0.04). Although sorafenib led to a mild decrease of *BIRC5* levels, no synergistic effect could be observed (Fig. 4E).

Discussion

After sorafenib approval in advanced HCC, the scientific community aimed at further enhancing the survival benefit with combination therapies and/or novel drugs [28,29]. Combination therapies are currently tested in one phase III study (sorafenib with erlotinib) and in several phase II studies [5]. Nonetheless, none of them is testing the efficacy of an HDAC inhibitor in this difficult-to-treat cancer, a family of drugs already approved in other malignancies [6].

This study provides compelling data supporting the role of HDACs as novel targets for the treatment of HCC. We report aberrant expression of most HDACs and identify *HDAC3* and *HDAC5* overexpression to be associated with copy number gains in human HCC. In addition, we show that treatment with the HDAC inhibitor panobinostat is highly efficient in preclinical models of HCC, boosted by the combination with sorafenib.

Recently, dysregulation of several HDACs has been reported for gastric, prostate, breast and liver cancer [19,30]. These aberrant expression profiles are believed to contribute to carcinogenesis by disturbing the balance of histone acetylation and non-acetylation needed for normal cell growth and proliferation. In accordance

with this data, we detected and confirmed several significantly dysregulated HDACs in two large datasets of human HCC.

Up to now, only very limited data about transcriptional regulation of HDACs has been available. Mutation, methylation and copy number analyses of different HDAC isoforms need to be performed in order to confirm our observed gains in *HDAC3* and *5* and to detect additional mechanisms of regulation. HDACs themselves act as transcriptional regulators, either through inhibiting transcription by histone deacetylation or through activating expression by functioning as transcriptional co-activators [31]. It has previously been shown that *CDH1* is silenced by a repressor complex containing HDAC1 and 2 in pancreatic cancer cells, and that it can be re-expressed after treatment with the HDACi trichostatin A or *HDAC2* specific siRNA knockdown [11]. In accordance with this report from *in vitro* data, we observed highly significant correlations of several *HDACs* with *CDH1* gene expression in human HCC. The other reported HDAC target *BIRC5* is frequently downregulated by HDAC inhibition in many cancer models including HCC [10,32]. Nonetheless, no human data had shown correlation between HDACs and *BIRC5* gene expression. Even though a vast number of putative HDAC target genes have been reported, we chose *CDH1* and *BIRC5* as two pivotal players with recently reported contributions to the development of HCC as part of a molecular signature discriminating dysplastic nodules from early HCC [25,33].

HDACis are currently among the most promising anti-tumoral drugs in both preclinical and clinical development. Preliminary results suggest high anti-tumoral efficacy also in solid tumors, particularly when administered in combination with chemotherapy, radiotherapy or molecular targeted agents [18]. The pan-HDACi panobinostat demonstrated high anti-tumoral activity in preclinical models of solid tumors and seemed to be well tolerated after oral administration in clinical studies [34]. We observed that combining panobinostat with sorafenib strongly potentiated individual treatment efficacy *in vitro* and *in vivo*. Similar effects have been reported with lapatinib, a dual EGFR/HER2 inhibitor, in colon cancer cells [14], irradiation in non-small lung cancer cells [35] and doxorubicin in multiple myeloma cells [16].

Although combination treatment led to several significant additive effects *in vitro* (e.g. cell viability, apoptosis, *CDH1* expression), the underlying mechanism could not be elucidated. Recent reports showed that sorafenib increases *CDH1* levels through the Raf-MAPK-pathway and that HDACs directly suppress *CDH1* expression and this might partially explain the observed additive effect on *CDH1* expression in our study [36]. Assumed additive effects on cell viability still require further investigation, since our hypothesis of panobinostat further altering the RAS/MAPK- and PI3K/Akt/mTOR pathway along with sorafenib was not confirmed (data not shown).

The fact that panobinostat alone led to 85–100% decrease in proliferation depending on the cell line analyzed might be related to the status of p53 activity in different cell lines and also to the panobinostat-specific inhibition of *BIRC5* [37]. This might result in the observed p21 up regulation together with a possible direct p21 induction through inhibition of a HDAC co-factor. Other authors have thoroughly explored the mechanisms of induction of apoptosis by HDACi both by p53-dependent and p53-independent pathways [38]. Panobinostat, but not sorafenib, is also able to induce autophagy in liver cancer cells, a mechanism already observed in lymphoma cells [37]. Nonetheless, we were unable to confirm that this occurs through alteration of the Akt/mTOR pathway [39]. Autophagy activation might indeed be a separate mechanism by which panobinostat modulates cell viability in HCC, but this definitely requires further investigation.

Translated into our xenograft model, the combination of panobinostat with sorafenib led to the highest decrease of tumor volume and the most significantly improved survival rates in treated mice along with a significant decrease of vessel density in HCC xenografts. While sorafenib targets neo-angiogenesis through the VEGF pathway [4], it has been demonstrated that panobinostat reduces angiogenesis by inhibition of endothelial cell formation [40]. In this setting, the additive effect of both compounds reported in this study compellingly warrants further investigation.

Concerns over the toxicity of amplified drug toxicity in combination treatment trials have been the current bottleneck to translating positive preclinical experiments into clinical trials in HCC. This has been the case with some combinations of molecular targeted therapies in HCC and recent reports suggest that combining sorafenib and everolimus appears difficult to manage [41]. Thus, the combination of sorafenib and panobinostat should be explored in the setting of phase I-II studies, particularly assessing a potential synergistic anti-angiogenic toxicity.

In conclusion, dysregulation of the HDAC-family might play a significant role in hepatocarcinogenesis. While copy number changes lead to aberrant HDAC gene expression in HCC, additional mechanisms need to be explored. Inhibition of all classical HDACs certainly leads to high anti-tumoral efficacy in preclinical models of HCC. Combining panobinostat with the standard of care sorafenib greatly enhanced its anti-tumoral activity, supporting the rationale for clinical studies with this combination.

Conflict of interest

Prof. Llovet has a Consultancy Agreement and received research support from Bayer Pharmaceutical. The other authors have no conflict of interest to declare.

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jhep.2012.01.009.

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