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BASIC RESEARCH

Experimental treatment of pancreatic cancer with two novel histone deacetylase inhibitors

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Abstract

AIM: To investigate *in vitro* and *in vivo* treatment with histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 in pancreatic cancer.

METHODS: Cell-growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied *in vitro* in 8 human pancreatic cancer cell lines using the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. In addition, the anti-tumoral effect of NVP-LBH589 was studied in a chimeric mouse model. Anti-tumoral activity of the drugs was assessed by immunoblotting for $p21^{WAF-1}$, acH4, cell cycle analysis, TUNEL assay, and immunohistochemistry for MIB-1.

RESULTS: *In vitro* treatment with both compounds significantly suppressed the growth of all cancer cell lines and was associated with hyperacetylation of nucleosomal histone H4, increased expression of $p21^{WAF-1}$, cell cycle arrest at G2/M-checkpoint, and increased apoptosis. *In vivo*, NVP-LBH589 alone significantly reduced tumor mass and potentiated the efficacy of gemcitabine. Further analysis of the tumor specimens revealed slightly increased apoptosis and no significant reduction of cell proliferation.

CONCLUSION: Our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human pancreatic cancer, although the precise mechanism of *in vivo* drug action is not yet completely understood. Therefore, further preclinical and clinical studies for the treatment of pancreatic cancer are recommended.

Key words: Histone deacetylase inhibitor; Pancreatic cancer; NVP-LAQ824; NVP-LBH589

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INTRODUCTION

Pancreatic cancer is the fifth to sixth leading cause of cancer death in Europe and the fourth leading cause of cancer death in the USA[1]. The lethality of this malignancy is demonstrated by the fact that the annual incidence is approximately equal to the annual deaths. Unfortunately, carcinoma of the pancreas is increasing in incidence, and its risk factors are poorly understood. Although surgical resection remains the only chance for cure, less than 10% of patients diagnosed with pancreatic cancer are eligible for curative (R0) resection, since up to 90% of patients will present with locally advanced or metastatic disease. In addition, there is a high rate of relapse, even in patients who receive adjuvant therapy[2]. A recent evaluation of the Finnish Cancer Registry, which recorded 4922 pancreatic cancer patients between 1990 and 1996, detected only 89 five year survivors (1.8%)[3]. Metastatic cancer tends to be a rapidly progressing disease, often accompanied by significant weight loss, abdominal pain, nausea, and/or depression. For decades, 5-fluorouracil (5-FU) was the most widely used chemotherapeutic agent in

metastatic pancreatic cancer. Today gemcitabine, a nucleoside analogue that is incorporated into replicating DNA resulting in premature chain termination and apoptosis, is the current standard of care[4]. In a phase Ⅲ approval study 126 patients with metastatic disease who had not received prior chemotherapy were randomized to weekly gemcitabine (n = 63) or weekly bolus 5-FU (n = 63)[5]. Overall survival in patients treated with gemcitabine was significantly improved compared with patients treated with 5-FU; However, there was no convincing gain in median survival time (median survival 5.7 mo *vs* 4.4 mo, P = 0.0025). The primary efficacy measure in this study was clinical benefit response, a composite of patient-oriented parameters including pain, Karnofsky performance status, daily analgesic usage, and body weight. Clinical benefit was experienced in 23.8% of patients treated with gemcitabine compared with only 4.5% of the patients treated with 5-FU (P = 0.022). Fixed-dose-rate (FDR) gemcitabine (1500 mg/m^2 at 10 mg/m^2 per minute) has also been investigated by Tempero *et al* in comparison to 2200 mg/m^2 gemcitabine over 30 min[6]. Although median survival time improved from 5.0 mo in the standard arm to 8.0 mo in the FDR arm (P = 0.013), grade 3 and 4 toxicity increased significantly. Many combination regimens with gemcitabine have been tested in open-label phase Ⅱ or Ⅲ studies with higher response and progression-free survival rates, but no definitive benefit in overall survival, with the only exception being a combination with capecitabine[4,7]. As little progress has been made in the past decade, new strategies should focus on targeting cancer cells at the molecular level. Recently, in a randomized phase Ⅲ placebo-controlled trial, Moore *et al* demonstrated that combining gemcitabine with EGFR inhibitor erlotinib was associated with a modest, but statistically significant survival benefit of 15 d[8]. In contrast, a recent phase Ⅲ trial (SWOG S0205 study) failed to demonstrate a clinically significant advantage of the addition of cetuximab, an anti-EGFR monoclonal antibody, to gemcitabine for overall survival, progression free survival and response[9]. Another approach is targeting VEGF as a key player in tumor growth and resistance to therapy. In a phase Ⅱ trial with 52 patients, a combination of VEGF inhibitor bevacizumab and gemcitabine yielded a 21% response rate and a median survival of 8.8 mo[10]. These data led CALGB to conduct a randomized, double-blind, placebo-controlled, phase Ⅲ trial (CALGB 80303). However, the addition of bevacizumab to gemcitabine did not improve survival[11]. Inhibiting histone deacetylases (HDACs), which regulate interactions between histones and DNA together with histone acetylases (HATs) as counter-players, may be another promising molecular target. Clinical studies published so far have shown that HDAC inhibitors (HDACIs) can be administered safely in humans and that treatment of some cancers with such agents seems to be beneficial[12,13]. NVP-LAQ824 and NVP-LBH589 are new chemical entities belonging to a structurally novel class of cinnamic hydroxamic acid compounds[14-17], which are currently in phase Ⅰ clinical evaluation in advanced refractory solid tumors and hematologic malignancies[18-22]. However, little is known about their potential efficacy in pancreatic cancer. Therefore, the objectives of the current study were to investigate the efficacy of *in vitro* and *in vivo* treatment with the novel pan-HDAC inhibitors NVP-LAQ824 and NVP-LBH589 and to evaluate effects of combination with gemcitabine.

MATERIALS AND METHODS

Materials

Eight human pancreatic cancer cell lines (Hs766T, As-PC-1, CFPAC-1, Capan-2, Panc-1, MiaPaca-2, HPAF-2 and L3.6pl) were examined[23-27]. All cell lines were cultured in a 37℃ incubator with 50-100 mL/L CO_2 in appropriate media. The HDACIs NVP-LAQ824 and NVP-LBH589 were provided by Novartis (Basel, Switzerland) and dissolved in dimethyl sulfoxide (DMSO) (10 mmol/L stock). Hoechst dye, sodium butyrate and monoclonal (mc) β-actin antibody were purchased from Sigma (Sigma-Aldrich Chemie GmbH Munich, Germany), mc p21$^{WAF-1/Cip-1}$ from Cell Signaling (Cell Signaling Technology, Beverly, USA), mc acH4 antibody from Upstate (Upstate Biotechnology, Lake Placid, USA), mc MIB-1 antibody from Dako (Glostrub, Denmark), and gemcitabine (diluted in D5W and 50 mL/L DMSO) from our hospital pharmacy. Six to eight-wk-old female athymic NMRI nude mice were supplied by Taconic (Taconic Europe, Ry, Denmark) and held under pathogen-free conditions. Humane care was administered, and study protocols complied with the institutional guidelines.

Inhibition of cell growth

Cytotoxic effects of both drugs were determined by the 3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma-Aldrich Chemie GmbH Munich, Germany) assay. 1-5 × 10^3 cells were seeded in triplicate in 96-well plates (100 μL/well) and allowed to attach overnight. The medium was then replaced with media (100 μL) containing the designated drug or vehicle control (50 mL/L DMSO in D5W) followed by an incubation for 3 or 6 d. For the 6 d experiment, medium was changed after 3 d. Three hours before the end of the incubation period, 10 μL of PBS containing MTT (5 g/L) was added to each well. Following this, the medium was removed. The precipitate was then resuspended in 100 μL of lysis buffer (DMSO, 100 g/L SDS). Absorbance was measured on a plate reader at 590 nm using a reference wavelength of 630 nm. Each experiment was performed in triplicate.

Immunoblotting

Cell culture monolayers were washed twice with ice-cold PBS and lysed with RIPA-buffer containing Tris-HCl (50 mmol/L, pH 7.4), NP-40 (10 g/L), sodium-desoxycholate (2.5 g/L), NaCl (150 mmol/L), EDTA (1 mmol/L), sodium-orthovanadate (1 mmol/L), and

one tablet of complete mini-EDTA-free protease inhibitor cocktail (Boehringer, Mannheim, Germany, in 10 mL buffer). Histones for anti-acH4 immunoblotting were isolated by acid extraction [cells were lysed in ice-cold lysis buffer (HEPES 10 mmol/L; pH 7.9), $MgCl_2$ (1.5 mmol/L), KCl (10 mmol/L), DTT (0.5 mmol/L), PMSF (1.5 mmol/L), and additional protease inhibitor]. One molar HCl was added to a final concentration of 0.2 mol/L, followed by an incubation on ice for 30 min and centrifugation at 13000 r/min for 10 min. The supernatant was retained and dialysed against 200 mL of 0.2 mol/L acetic acid twice for 1 h and against 200 mL H_2O overnight). Proteins were quantified by Bradford protein assay (Bio-Rad, Munich, Germany) and stored at -80℃. 50 μg of cell or tissue lysates were separated on SDS-polyacrylamide gels and electroblotted onto polyvinylidene difluoride membranes (Amersham Pharmacia Biotech, Freiburg, Germany). Membranes were then incubated in blocking solution [50 g/L dry milk in 10 mmol/L Tris-HCl, 140 mmol/L NaCl, 1 g/L Tween-20 (TBS-T)], followed by incubation with the primary antibody at 4℃ overnight (50 g/L BSA in TBS-T). The membranes were then washed in TBS-T and incubated with horseradish peroxidase (HRPO)-conjugated secondary antibodies for 1 h at room temperature. Antibody detection was performed with an enhanced chemoluminescence reaction (SuperSignal West Dura, Pierce, Rockford, USA).

Cell cycle analysis

Cells were seeded in T-25 flasks (2×10^5), treated with various concentrations of NVP-LAQ824 or NVP-LBH589 or vehicle control (50 mL/L DMSO in D5W) for 72 h, washed with PBS, trypsinized, centrifuged, and fixed in 750 mL/L ice-cold ethanol-phosphate-buffered saline containing 10 g/L EDTA. DNA was labeled with 100 mL/L propidium iodide. Cells were sorted by FACScan analysis, and cell cycle profiles were determined using ModFitLT V2.0 software (Becton Dickinson, San Diego, USA). Each experiment was performed in triplicate.

Animal studies

Tumors were induced by injecting 5×10^6 HPAF-2 or L3.6pl cells in 200 μL PBS sc into the flank region of NMRI nude mice. Treatment was started when an average tumor volume of 150 mm^3 was reached (usually after 2 wk). The verum groups received either NVP-LBH589 (25 mg/kg, 5 × weekly) or gemcitabine (5 mg/kg, 1 × weekly) or a combination of both (NVP-LBH589 at 25 mg/kg, 5 × weekly plus gemcitabine at 5 mg/kg, 1 × weekly) ip, whereas the control group received placebo (carrier solution 50 mL/L DMSO in D5W) only. Treatment was continued for 28 consecutive days, tumors were measured daily with a Vernier caliper and tumor volumes were calculated using the formula tumor volume = $0.5 \times L \times W^2$, where L represents the length and W the width of the tumor. When treatment was finished, animals were sacrificed and tumors excised and weighed.

TUNEL POD test

Terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (*in situ* cell death detection kit, POD) was used to detect apoptosis in paraffin sections from mouse tumor tissue. TUNEL was carried out following the manufacturer's instructions (Roche, Penzberg, Germany) as previously described[28]. Apoptotic cells (red) were counted under a light microscope after fluorescence signal conversion using peroxidase-conjugated antibody and peroxidase substrate (DAB, Roche, Penzberg, Germany). The number of positive cells was counted by an experienced pathologist (M.N.) in a total of 8 high power fields (HPFs) and expressed as mean percentage of total cells in these fields of the tumor. Necrotic tumor cells were excluded from the cell count.

Immunohistochemical staining

For MIB-1 staining, we used paraffin sections following a protocol that has been described elsewhere[29]. The number of positive cells was counted by an experienced pathologist (M.N.) in a total of 4 HPFs and expressed as mean percentage of total cells in these fields of the tumor.

Statistical analysis

Statistical calculations were performed using SPSS, version 10.0 (SPSS Inc., Chicago, USA). Numeric data were presented as mean value with SD or SEM. Inter-group comparisons were performed with the Student *t*-test and ANOVA. $P < 0.05$ was considered significant.

RESULTS

Inhibition of cell growth

After 3 d of incubation, 7 of 8 tested cell lines were sensitive to NVP-LAQ824 (mean IC_{50} (3 d) = 0.18 ± 0.24 μmol/L) and even more to NVP-LBH589 (mean IC_{50} (3 d) = 0.09 ± 0.14 μmol/L). Only cell line Capan-2 demonstrated an IC_{50} (3 d) value > 1 μmol/L for both compounds. Inhibition of cell growth was more pronounced if incubation time was extended to 6 d with a mean IC_{50} value of 0.06 ± 0.07 μmol/L for NVP-LAQ824 and 0.03 ± 0.02 μmol/L for NVP-LBH589. After 6 d of incubation, cell line Capan-2 also became responsive (Figure 1 and Table 1). In addition, DMSO alone (the solvent for NVP-LAQ824 and NVP-LBH589) had no influence on cell growth (data not shown).

Immunoblotting

Treatment of cell lines HPAF-2 and L3.6pl with 0.1 μmol/L NVP-LAQ824 or 0.1 μmol/L NVP-LBH589 for 24 h resulted in acetylation of histone H4 (Figure 2A and B). The same treatment caused an induction of $p21^{WAF-1/CIP-1}$ expression (Figure 2C and D). A dose increase to 0.2 μmol/L NVP-LAQ824 or NVP-LBH589 corresponded with an increase in histone H4 acetylation and $p21^{WAF-1/CIP-1}$ levels. Histone H4 acetylation was higher in treated HPAF-2 than L3.6pl cells, whereas $p21^{WAF-1/CIP-1}$ expression was slightly higher in treated L3.6pl cells.

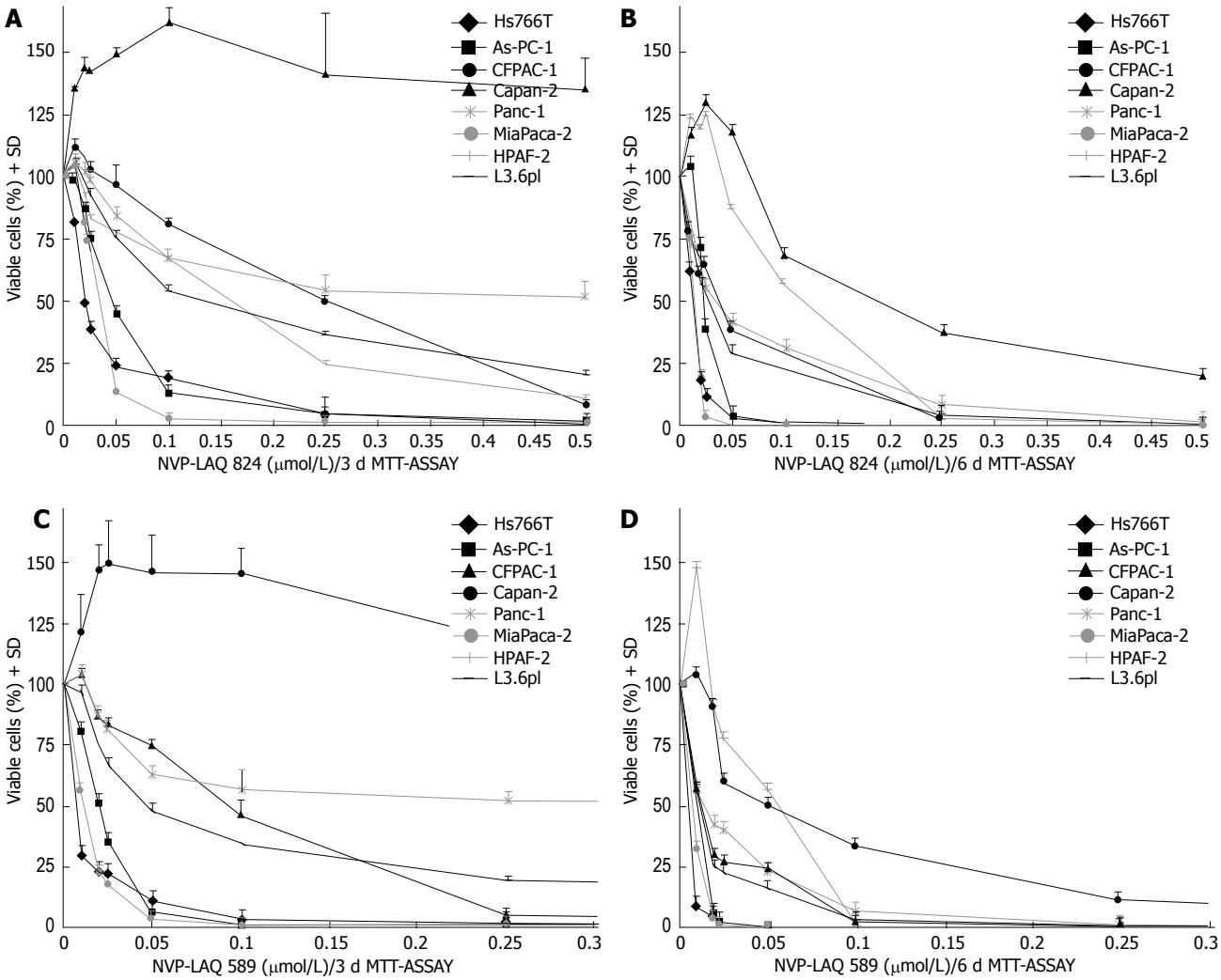

Figure 1 *In vitro* treatment of pancreatic cancer with NVP-LAQ824 and NVP-LBH589 (MTT assay). **A**: 3-d incubation with NVP-LAQ824 (n = 3); **B**: 6-d incubation with NVP-LAQ824 (n = 3); **C**: 3-d incubation with NVP-LBH589 (n = 3); **D**: 6-d incubation with NVP-LBH589 (n = 3).

Table 1 Inhibition of cell growth by NVP-LAQ824 and NVP-LBH589

Cell line	IC_{50} (μmol/L)			
	NVP-LAQ824		NVP-LAQ589	
	3 d	6 d	3 d	6 d
MiaPaca-2	0.03	0.01	0.01	0.01
As-PC-1	0.05	0.02	0.02	0.01
Panc-1	0.70	0.04	0.40	0.02
Hs766T	0.02	0.01	0.01	0.01
CFPAC-1	0.25	0.04	0.09	0.02
HPAF-2	0.16	0.12	0.07	0.06
L3.6pl	0.05	0.02	0.03	0.04
Capan-2	> 1	0.19	> 1	0.05

Cell cycle analysis

Treatment of cell lines HPAF-2 and L3.6pl with 0.1 μmol/L NVP-LAQ824 or NVP-LBH589 for 72 h resulted in G2/M arrest. This arrest was, in general, more pronounced if the dose of NVP-LAQ824 or NVP-LBH589 was increased to 0.2 μmol/L. Percentual G2/M arrest was lower for 0.2 μmol/L than 0.1 μmol/L only for the treatment of HPAF-2 cells with NVP-LBH589. This phenomenon may derive from the fact, that at the same time the sub-G1-peak was much higher for 0.2 μmol/L. For both concentrations, the effect of NVP-LBH589 was stronger than the effect of NVP-LAQ824 with the aforementioned exception of 0.2 μmol/L NVP-LBH589 in HPAF-2 cells (Figure 3). In addition, incubation with NVP-LAQ824 or NVP-LBH589 for 72 h resulted in a dose-dependent significant increase in the sub-G1-peak, which was higher for NVP-LBH589 than NVP-LAQ824 and higher in L3.6pl than in HPAF-2 cells. This result correlated well with the fact that IC_{50} values in the cell growth inhibition experiment (Figure 1) were lower for L3.6pl in comparison to HPAF-2 cells.

Chimeric mouse model

Tumors were induced in nude mice by subcutaneous injection of HPAF-2 and L3.6pl cells. These cell lines were selected because they had the best growth capability in our nude mice in a pilot study. Treatment of mice consisted of ip injections with NVP-LBH589, gemcitabine, NVP-LBH589 plus gemcitabine (COMBO) or placebo (50 mL/L DMSO in D5W). Three days after commencement of NVP-LBH589 or COMBO treatment, HPAF-2 cell tumors showed a signifi-

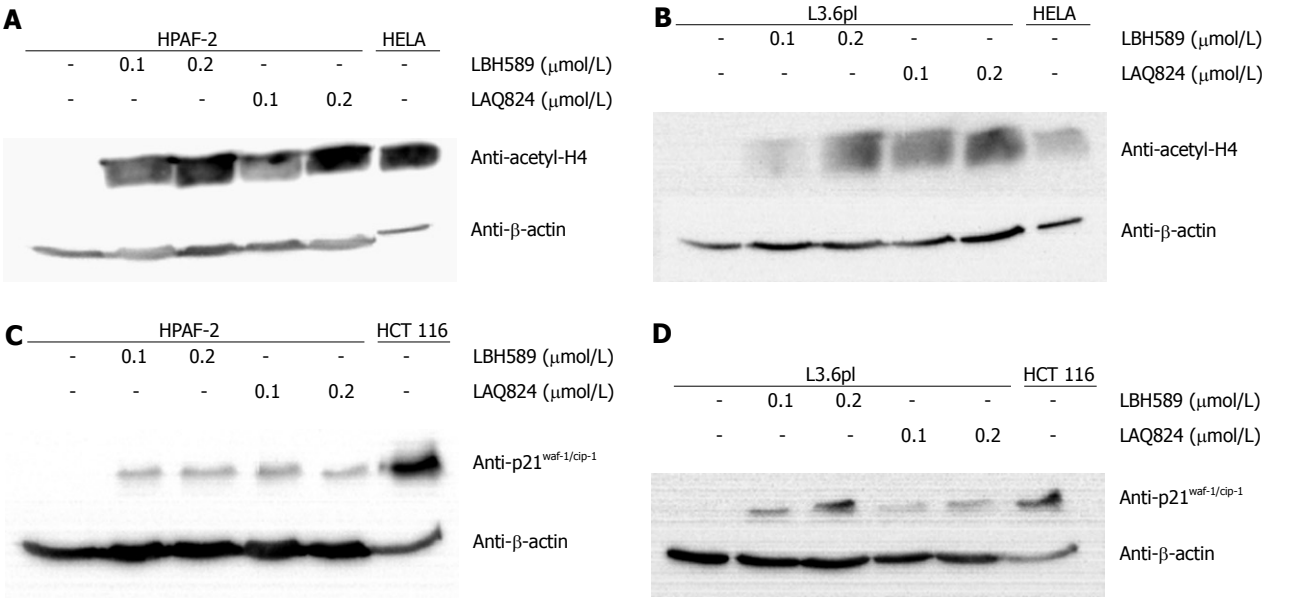

Figure 2 Mechanism of drug action after *in vitro* treatment with NVP-LAQ824 and NVP-LBH589 for 24 h. **A** and **B**: Acetylation of histone H4. Protein extracts from HELA cells that were treated with 5 mmol/L sodium butyrate served as positive controls; **C** and **D**: $p21^{WAF-1/CIP-1}$ expression. Cell lysate from HCT 116 colon cancer cells served as positive control; **A**-**D**: Staining with β-actin antibody confirmed equal protein loading.

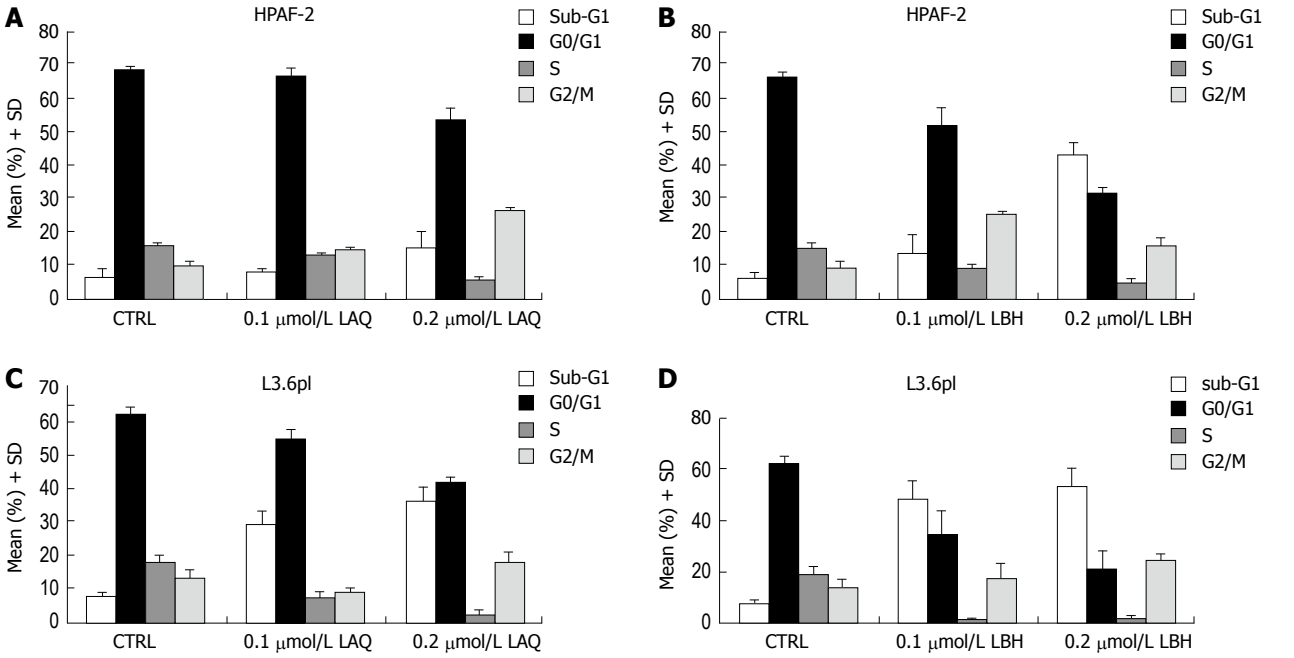

Figure 3 Cell cycle analysis. **A**: Treatment of cell line HPAF-2 with 0.1 or 0.2 μmol/L NVP-LAQ824 for 72 h (n = 3); **B**: Treatment of cell line HPAF-2 with 0.1 or 0.2 μmol/L NVP-LBH589 for 72 h (n = 3); **C**: Treatment of cell line L3.6pl with 0.1 or 0.2 μmol/L NVP-LAQ824 for 72 h (n = 3); **D**: Treatment of cell line L3.6pl with 0.1 or 0.2 μmol/L NVP-LBH589 for 72 h (n = 3).

cantly reduced volume in comparison to control (n = 7 for each group, $P < 0.05$). Treatment of mice with gemcitabine alone resulted in a significant reduction of tumor volume compared to control after 4 d from commencement of treatment. These differences were maintained until the end of the experiment. COMBO therapy was significantly more efficient than gemcitabine treatment alone on treatment day 7, 8, 13, 14, 15, and 16 and was significantly more efficient than NVP-LBH589 therapy alone on treatment day 7 and 14 ($P < 0.05$, Figure 4A). Treatment of L3.6pl tumors with NVP-LBH589 or COMBO resulted in a significantly reduced volume in comparison to control after 4 d ($P < 0.05$) and 3 d ($P < 0.05$) from commencement of therapy, respectively (n = 7 for each group). These differences were also maintained until the end of the experiment. Treatment of mice with gemcitabine alone resulted in a significant reduction of tumor volume compared to control at treatment day 12, 13, 16, 17, and 18 ($P < 0.05$). COMBO therapy was significantly more

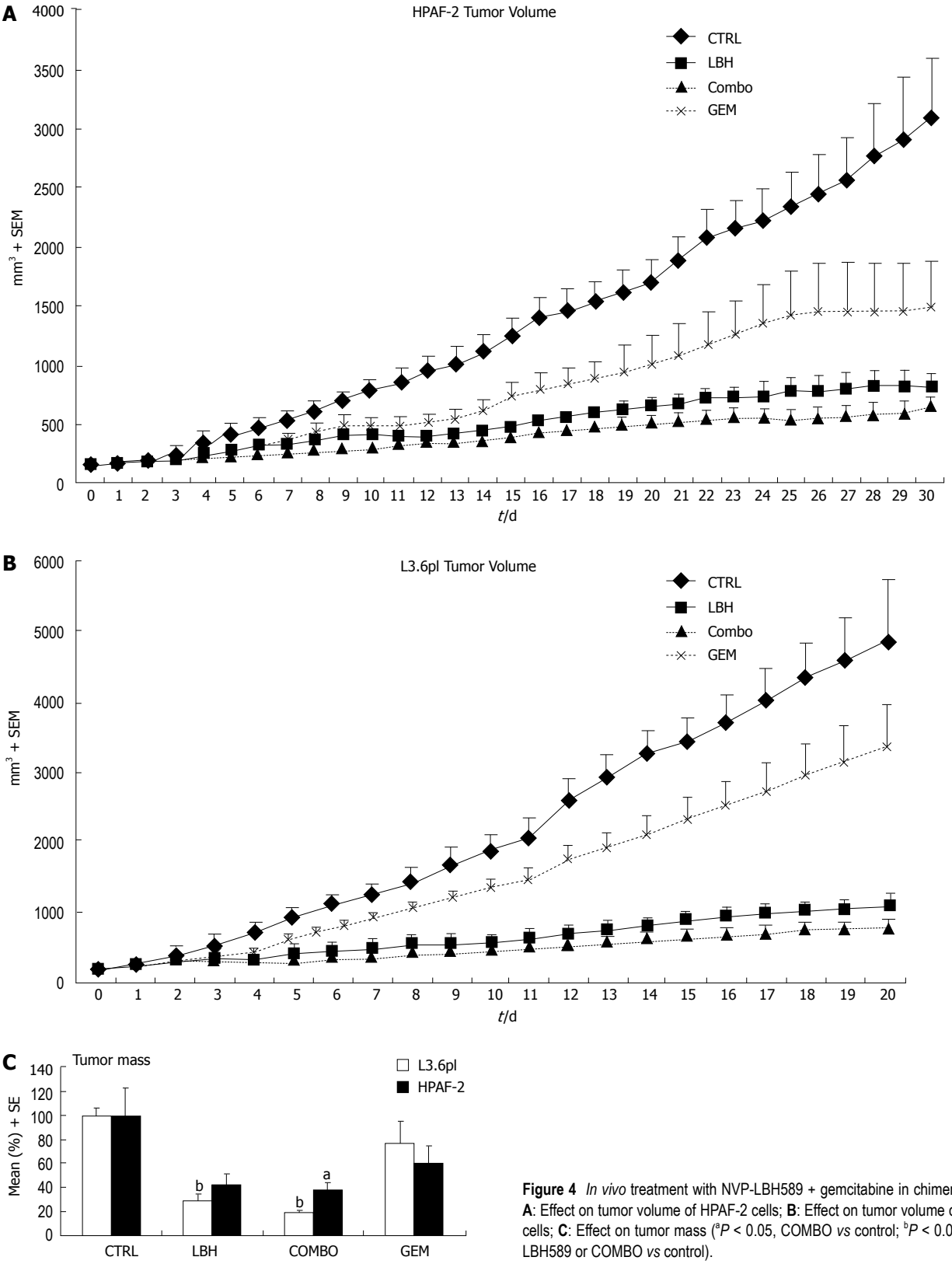

Figure 4 *In vivo* treatment with NVP-LBH589 + gemcitabine in chimeric mice. **A**: Effect on tumor volume of HPAF-2 cells; **B**: Effect on tumor volume of L3.6pl cells; **C**: Effect on tumor mass ($^{a}P < 0.05$, COMBO *vs* control; $^{b}P < 0.01$, NVP-LBH589 or COMBO *vs* control).

efficient than gemcitabine treatment alone on treatment day 3-20 and was significantly more efficient than NVP-LBH589 therapy alone on treatment day 3 ($P < 0.05$). NVP-LBH589 therapy was significantly more efficient than gemcitabine treatment alone on treatment day 5-20 ($P < 0.05$, Figure 4B). At the end of the experiment after 30 d, tumor mass in HPAF-2 cells bearing mice was significantly diminished as compared to placebo after treatment with COMBO (-63%, $P < 0.05$). In contrast, treatment of mice with gemcitabine (-24%, $P = 0.45$) or NVP-LBH589 alone (-58%, $P = 0.056$) did not result in any significant reduction of tumor mass as compared to control (Figure 4C). L3.6pl cell tumor mass in mice was significantly diminished after treatment

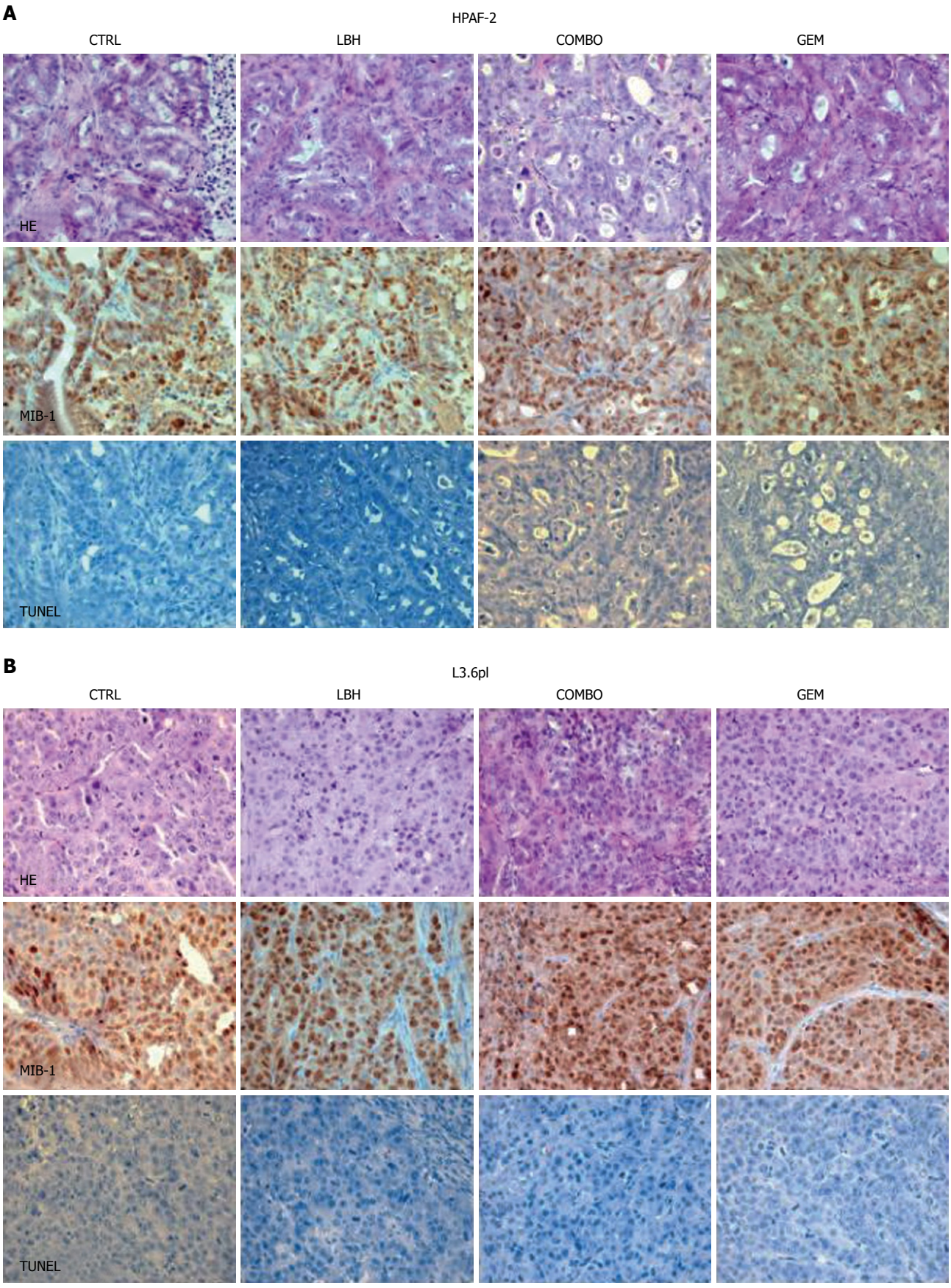

Figure 5 Hematoxylin-eosin (HE), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) staining of mouse tumors (SABC, x 40). **A**: Cell line HPAF-2; **B**: Cell line L3.6pl.

with either NVP-LBH589 (-70%, $P < 0.01$) or COMBO (-81%, $P < 0.01$), but not with gemcitabine (-24%, $P = 0.28$),

Table 2 MIB-1- and TUNEL-staining of mouse tumor specimens

Mean in %	HPAF-2		L3.6pl	
	MIB-1	Apoptosis	MIB-1	Apoptosis
CTRL	67.5	1.3	61.3	0
GEM	66.3	2.5	70	0
LBH	51.3	3.8	76.3	6.3
COMBO	55.0	3.8	78.8	3.8

respectively. In addition, the combination of NVP-LBH589 with gemcitabine was more effective at tumor mass reduction in comparison to gemcitabine alone ($P < 0.05$). The L3.6pl animal experiment was stopped at day 21 for ethical reasons, since animals suffered from tumor burden. Regarding side effects of the different drugs used in HPAF-2 cell tumor bearing mice, weight loss was 2%, 0%, 13%, and 6%, in the control, gemcitabine, NVP-LBH589, and COMBO groups. There was a statistically significant difference between the control and NVP-LBH589 group ($P < 0.05$) and between the gemcitabine and NVP-LBH589 group ($P < 0.01$). Concerning side effects of the different drugs used in L3.6pl cell tumor bearing mice, weight loss was 23%, 17%, 12%, and 25%, in the control, gemcitabine, NVP-LBH589, and COMBO groups. There was a statistically significant difference between the control and NVP-LBH589 group ($P < 0.05$).

In order to assess the anti-tumoral drug mechanism, paraffin sections of mouse tumors were stained with hematoxylin-eosin (H&E), MIB-1 (proliferation marker) and TUNEL (apoptosis marker) (Figure 5). Treatment with NVP-LBH589 and COMBO slightly reduced proliferation (reduced MIB-1 staining) and slightly induced apoptosis (increased TUNEL-staining) in HPAF-2 cell bearing mice, whereas proliferation was not decreased and apoptosis only slightly increased in L3.6pl cell bearing mice (Table 2).

DISCUSSION

Analyzing palliative treatment data, a novel approach for patients with metastatic pancreatic cancer is urgently required. Targeting HDACs may be a new option for this tumor entity. Preliminary studies have demonstrated *in vitro* activity of HDACIs in pancreatic cancer cell lines. Natoni *et al*[30] showed that treatment with sodium butyrate, a carboxyl acid class inhibitor of HDACs, resulted in marked down-regulation of anti-apotoctic Bcl-xL protein expression, mitochondrial membrane depolarization, cytochrome c release from mitochondria, activation of caspase-9 and -3, and apoptosis induction. Garcia-Morales *et al*[31] reported HDACIs induced apoptosis in the pancreatic cancer cell lines IMIM-PC-1, IMIM-PC-2, and RWP-1 that are normally resistant to other antineoplastic drugs. This finding was previously observed by Sato *et al*[32] for five normally chemotherapy-resistant cell lines when treated with FR901228, a cyclic peptide HDACI belonging to the depsipeptides class. Recently, another class of HDACIs, the hydroxamic acids, with representatives such as trichostatin A (TSA), suberoylanilide hydroxamic acid (vorinostat, SAHA), azelaic bis-hydroxamic acid (ABHA), scriptaid, oxamflatin, pyroxamide, m-carboxycinnamic acid bis-hydroxamide (CBHA), and the recently developed NVP-LAQ824, NVP-LBH589, and PXD101 have become the focus for further research, including pancreatic cancer. Gahr *et al*[33] used HDACI trichostatin A for *in vitro* treatment of pancreatic carcinoma cell lines YAP C and DAN G. They described an apoptosis rate of 71% and 66% after 72 h using a drug concentration of 1 μmol/L. Moore *et al*[34] tested trichostatin A in PaCa44 cells using microarrays containing 22283 probe sets. One prominent feature was the increased ratio between the levels of expression of pro-apoptotic (BIM) and anti-apoptotic (Bcl-xL and Bcl-W) genes. In addition, Cecconi *et al*[35] reported for the same cell line PaCa44 that trichostatin A caused cell cycle arrest at the G2 phase and induced apoptotic cell death. Another hydroxamic acid, SAHA, induced growth inhibition in three pancreatic cell lines BxPC3, COLO-357, and PANC-1 by upregulating p21 and sequestering it in the cytoplasm[36]. In our current study, we investigated the two novel cinnamic hydroxamic acid compounds NVP-LAQ824 and NVP-LBH589 for *in vitro* treatment of 8 different human pancreatic cancer cell lines. Cell-growth inhibition by NVP-LAQ824 and NVP-LBH589 was studied by MTT assay. Treatment with both compounds significantly suppressed the growth of 7 cancer cell lines after 3 d of incubation and all cancer cell lines after 6 d of incubation. We hypothezise that the lack of response of Capan-2 cells after 3 d of treatment may be based on the status of the tumor suppressor *p53*. A genetic profile of 10 different human pancreatic cancer cell lines (6 of the 8 cell lines used in our experiment being amongst them) created by a group from John Hopkins University (http://pathology2.jhu.edu/pancreas/geneticsweb/ profiles.htm) discovered *p53* mutations in almost all cell lines, but not in Capan-2 cells. On the other hand, it has been shown that acetylation and deacetylation of *p53* is likely to be part of the mechanism that controls its physiological activity. Whereas HDACs are capable of downregulating p53 function, HDAC inhibition can cause the opposite effect[37]. Interestingly, it has also been shown that HDAC inhibitors, such as FR901228 and trichostatin A, completely deplete mutant p53 in cancer cell lines and restore p53-like functions, which is highly toxic to cell lines with mutant p53[38]. Donadelli *et al* confirmed this finding in *p53* gene mutated pancreatic cancer cell lines which were treated with trichostatin A. The compound induced G2 phase arrest and apoptotic cell death by activation of $p21^{waf1}$, which is normally induced by *p53*[39].

In previous *in vitro* studies, NVP-LAQ824 exhibited potent anti-proliferative activity against colon carcinoma (IC_{50} = 0.01 μmol/L), and biliary tract cancer (IC_{50} = 0.11 μmol/L) as well as against non-small cell lung carcinoma (IC_{50} = 0.15 μmol/L), prostate cancer (IC_{50} = 0.018-0.023 μmol/L), head and neck squamous

carcinoma (IC_{50} = 0.04-0.34 μmol/L), and human breast adenocarcinoma cells (IC_{50} = 0.03-0.039 μmol/L) after 72 h of exposure[16,40-42]. The *in vitro* effects of NVP-LAQ824 on hematologic malignancies have been examined in several human cell lines with a death rate of more than 90% following 48 h of drug incubation, with exposures as low as 0.1 μmol/L[43-45]. Our second compound NVP-LBH589, was even more effective *in vitro* for the treatment of human chronic myeloid leukemia blast crisis K562 and LAMA-84, multiple myeloma, and acute leukemia MV4-11 cells[15,46-48].

The *in vitro* anti-tumoral drug mechanism in our study was assessed by immunoblotting for acH4 (surrogate marker for histone acetylation) $p21^{WAF-1/CIP-1}$, and cell cycle analysis. Treatment with both compounds was associated with hyperacetylation of nucleosomal histone H4, increased expression of $p21^{WAF-1/CIP-1}$, cell cycle arrest at G2/M-checkpoint, and significant induction of apoptosis (increased sub-G1-peak). Therefore, our results are very consistent with the *in vitro* results of the aforementioned studies by Natoni *et al*[30], Garcia-Morales *et al*[31], Sato *et al*[32], Gahr *et al*[33], Donadelli *et al*[39], Cecconi *et al*[35], and Arnold *et al*[36].

Encouraged by our *in vitro* results, we decided to test the most effective drug NVP-LBH589 *in vivo* in comparison to placebo using the chimeric mouse model. The NVP-LBH589 dose of 25 mg/kg (5 d/wk) was selected according to a study testing different iv doses of NVP-LAQ824 between 5 and 100 mg/kg (5 d/wk) in a similar chimeric mouse model using the human colon cancer cell line HCT 116[16]. *In vivo* data for NVP-LBH589 using human prostate carcinoma cell PC-3 xenografts became available only after completion of our study, and showed tumor reduction at a dose of 10 mg/kg per day[49]. In our experiments, NVP-LBH589 significantly reduced tumor mass in comparison to placebo and potentiated the efficacy of gemcitabine. In accordance with our observations, Gahr *et al*[33] and Piacentini *et al*[50] showed that a combination with gemcitabine potentiated the *in vitro* effects of trichostatin A in pancreatic cancer cells, demonstrating a synergistic effect between both agents. This phenomenon has been shown for *in vitro* cotreatment with SAHA, too, where the compound rendered pancreatic cancer cells sensitive to the inhibitory and proapoptotic effects of gemcitabine[36]. In human breast cancer cell lines SKBR-3 and BT-474, NVP-LAQ824 also enhanced gemcitabine-induced apoptosis *in vitro*[41]. For head and neck squamous carcinoma cells, the combination of NVP-LAQ824 with gemcitabine was more effective *in vitro* than a combination with docetaxel, paclitaxel, or cisplatin, especially when the cytotoxic agent was used first for 24 h followed by 48 h of NVP-LAQ824[40]. Unfortunately, in the first recently published randomized, double-blind, placebo-controlled multicenter-phase Ⅱ trial, gemcitabine plus benzamide HDACI CI-994 (N-acetyldinaline) showed no advantage over gemcitabine alone in patients with advanced pancreatic cancer[51]. In this study, a total of 174 patients received combination therapy (CI-994, 6 mg/m^2 per day, day 1-21 plus gemcitabine, 1000 mg/m^2, day 1, 8 and 15 each 28-d cycle) or placebo plus gemcitabine (1000 mg/m^2, day 1, 8 and 15 each 28-d cycle). Median survival was 194 d (combination therapy) *vs* 214 d (gemcitabine) (P = 0.908). The objective response rate was 12% *vs* 14% when investigator-assessed and 1% *vs* 6%, respectively, when assessed centrally. Time to treatment failure did not differ between the two arms (P = 0.304). Quality of life scores at 2 mo were worse with the combination than with gemcitabine alone. Pain response rates were similar between the two groups. There was an increased incidence of neutropenia and thrombocytopenia with combination therapy. However, it is currently unknown whether these clinical observations are also true for the hydroxamic acids class of HDACIs. In addition, recent *in vitro* and *in vivo* data have shown synergistic effects of trichostatin A in combination with DNA methyltransferase inhibitors azacytidine[52,53] and zebularine[54] and proteasome inhibitor PS-341[55], suggesting alternative combination partners for HDACIs. Whereas upregulation of tumor suppressors DUSP6[52] and MUC 2[53] is the proposed mechanism for the additional effect of DNA methyltransferase inhibitors, it is inactivation of NFkappaB signalling, downregulation of anti-apoptotic Bcl-xL and disruption of MAP kinase pathway for combination with the proteasome inhibitor PS-341[55].

Regarding side effects of the different drugs used in our studies, there was no significant additional weight loss in the COMBO group as compared to placebo. Moreover, NVP-LBH589 alone only induced additional weight loss in the HPAF-2 cell experiment. Weight loss in general was apparently more pronounced in the L3.6pl than in the HPAF-2 cell experiment. This may be due to the fact that L3.6pl cells are a selected variant of COLO-357 cells with increased metastatic potential[24,56,57]. Regarding other studies, weight loss of animals was not previously reported for NVP-LAQ824[16], but for NVP-LBH589[42].

In order to assess *in vivo* anti-tumoral drug mechanisms, paraffin sections of mouse tumors were stained with hematoxylin-eosin (H&E), MIB-1 (proliferation marker) and TUNEL (apoptosis marker). Treatment with NVP-LBH589 and COMBO slightly reduced proliferation (reduced MIB-1 staining) and slightly induced apoptosis (increased TUNEL-staining) in HPAF-2 cell bearing mice, whereas proliferation was not decreased and apoptosis only slightly increased in L3.6pl cell bearing mice. Surprisingly, the calculated numbers were much smaller than expected from the *in vitro* experiments. This might be derived from the fact that other pathways, like inhibition of angiogenesis, which we were unable to study in our model due to insufficient tissue quality, may be more important for NVP-LBH589 action in the *in vivo* setting.

Our findings suggest that NVP-LBH589 and NVP-LAQ824 are active against human pancreatic cancer cells *in vitro*, mainly by inhibition of proliferation and induction of apoptosis. NVP-LBH589 is also active in the *in vivo* setting, although the precise mechanism of

drug action is not yet completely understood. Therefore, a clinical study testing NVP-LBH589 for the treatment of pancreaticobiliary cancer has just been initiated at our department.

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COMMENTS

Background

Pancreatic adenocarcinoma is essentially an incurable disease, with mortality closely approaching incidence. Single agent gemcitabine is currently considered the standard of care for the treatment of inoperable pancreatic cancer, providing a small but sizable benefit in survival and palliation of symptoms.

Research frontiers

In the past ten years, several molecular-targeting agents have been introduced in the clinical setting. Despite promising results in phase Ⅱ studies, randomized clinical trials exploring the new compounds, such as matrix-metalloprotease-inhibitors (MMPI), farnesyl transferase inhibitors (FTI), signal transduction inhibitors, and angiogenesis inhibitors, either alone or in combination with gemcitabine have been largely disappointing. Polo-like kinase 1 (PLK-1), death receptor 5 (DR5), and histondeacetylase (HDAC) inhibitors are currently under clinical evaluation as new treatment options.

Innovations and breakthroughs

In 2003, fixed-dose-rate (FDR) gemcitabine (1500 mg/m^2 at 10 mg/m^2 per minute) improved median survival time from 5.0 mo in the standard arm to 8.0 mo in a randomized study; However, grade 3 and 4 toxicity increased significantly. In 2005, investigators of a phase Ⅲ study found that the gemcitabine-capecitabine combination significantly improved overall survival over gemcitabine alone (hazard ratio 0.80; 95% CI 0.65-0.98; P = 0.026). Recently, a randomized phase Ⅲ placebo-controlled trial demonstrated that combining gemcitabine with EGFR inhibitor erlotinib was associated with a modest, but statistically significant survival benefit of 15 d.

Applications

The aim of our study was to investigate *in vitro* and *in vivo* treatment with the histone deacetylase inhibitors NVP-LAQ824 and NVP-LBH589 in pancreatic cancer. Our findings suggested that NVP-LBH589 and NVP-LAQ824 are active against human pancreatic cancer *in vitro*. In addition, NVP-LBH589 demonstrated significant *in vivo* activity and potentiated the efficacy of gemcitabine.

Terminology

Histones (positively charged proteins) are the major components of chromatin. Histone acetylation and deacetylation modulate chromosome structure and regulate gene transcription. Two families of enzymes, histone acetyltransferases (HATs) and histone deacetylases (HDACs), activate and repress gene expression, respectively. Aberrant HAT or HDAC activity is associated with various epithelial and hematologic cancers. HDACs may play an important role in human oncogenesis through HDAC-mediated gene silencing and interaction of HDACs with proteins involved in tumorigenesis. HDAC inhibition could potentially restore normal processes in transformed cells without affecting normal cells.

Peer review

This paper addresses the use of histone deacetylase inhibitors in the treatment of pancreatic cancer *in vitro* and *in vivo*. It represents an important experimental assessment of novel agents in the treatment of a cancer for which effective therapy is currently lacking. It's a very interesting paper.

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