Supplemental information

Supplemental methods

qRT-PCR

Total RNAs were extracted using the QIAamp RNA Micro Kit (Qiagen, Hilden, Germany), according to the manufacturers' protocol. To evaluate whether the expression of LDH-A was similar in the primary cells and their originator tumors, we also extracted RNA from these 7 tumors, after laser microdissection with a Leica-LMD6000 instrument (Leica, Wetzlar, Germany), as described (Giovannetti *et al*, 2010).

cDNAs were synthesized using the DyNAmo cDNA Synthesis Kit (Thermo Scientific, Vantaa, Finland), according to the manufacturers' instruction. The qRT-PCR reactions were executed with TaqMan® primers and probes for LDH-A, human equilibrative nucleoside transporter-1 (hENT1), deoxycytidine kinase (dCK), EZH2, CD133, metalloproteinase-2 (MMP2), and metalloproteinase-9 (MMP9), (Hs00191940_m1, Hs01085706_m1, Hs01040726_m1, Hs01016789_m1, Hs01009250_m1, Hs00234422_m1, and Hs00957562_m1, respectively). The cDNAs were amplified using the ABI-PRISM 7500 instrument (Applied Biosystems). Gene expression values were normalized to β-actin, using a standard curve of cDNAs obtained from Human-Reference Total-RNA (Stratagene, La Jolla, CA), as described previously (Avan *et al*, 2012). Since previous studies reported that β-actin expression varied under hypoxic conditions (Zhong *et al*, 1999; Foldager et al, BMC Mol Biol. 2009) we checked the stability of β-actin expression levels under hypoxic conditions in our PDAC cells, showing minimal variations in PCR results.

Inhibition of LDH-A expression by siRNA anti-LDH-A

PANC-1 and LPC006 were plated in duplicate at a density of 2×10^5 cells/well in 6 well plates. After 24 hours, the cells were treated with siRNA anti-LDH-A or negative control (Silencer® Select Negative Control #1 siRNA, Ambion) in a final RNA concentration of 25 nmol/L, according to the manufacturer's instructions. The modulation of LDH-A by siRNA anti-LDH-A, was investigated by qRT-PCR and Western blotting, after 48 and 72 hours.

Analysis of LDH-A enzymatic activity

For the analysis of LDH-A activity in PDAC cells, 2×10^6 cells/ml were resuspended in 200 mM Tris Buffer pH 8.5, sonicated on ice for 3 cycles of 5 seconds with intervals of 30 seconds, and diluted 1:200 in a solution containing 200 mM Tris pH 8.5, 10 mM pyruvate (dissolved in water), 1.7 mM NADH (dissolved in 1.5 M Tris) and H_2O_2. Pyruvate was used as a substrate and NADH as cofactor for the production of lactate and NAD^+ in a stoichiometric fashion. The absorbance of NADH was read at 360 nm using a Tecan plate reader, for 25 minutes. Enzymatic activity of LDH-A was evaluated using Lambert-Beer`s Law as a V_{max} of μmol lactate/min/mg protein and measured by non-linear regression analysis with Prism 5.0 software (GraphPad PRISM, Intuitive Software for Science, San Diego, CA).

Evaluation of the pharmacological interaction of NHI compounds with gemcitabine

The median-drug effect analysis method was employed for evaluating the drug interaction of NHI-1 and NHI-2 with gemcitabine. The cell growth inhibition of the

combination was compared with the cell growth inhibition of each drug alone using the combination index (CI). Combination studies were focused on simultaneous treatment, testing at least six different concentrations of gemcitabine, using a fixed concentration of the NHI compounds (1 μM in hypoxia, and 10 μM in normoxia). The plates were incubated for 72 hours under both normoxic and hypoxic conditions. Data analysis was carried out using CalcuSyn software (Biosoft, Oxford, UK). CI values at fraction affected (FA) of 0.5, 0.75 and 0.9 were averaged for each experiment, and these values were used to calculate the mean between experiments.

Cell death and apoptosis analysis

Adherent and floating cells from the plates treated as described above were harvested and transferred to round-bottom test (12×75mm) tubes (Becton Dickinson, Mount View, CA). Cell cycle analyses were then performed using a FACSCalibur Flow Cytometer (Becton Dickinson, San José, CA, USA) and the data analysis was carried out with FACSdiva software 6.0 (Becton Dickinson). Apoptosis was determined with the FITC Annexin V/PI Apoptosis detection kit (Becton Dickinson). Cells were then trypsinized, harvested, transferred to test tubes and centrifuged at 1200 rpm for 10 min. The pellets were resuspended in 100 μl of ice-cold binding buffer (0.1 M Hepes/NaOH (pH 7.4) 1.4 M NaCl, 25 μM $CaCl_2$). Annexin staining protocol was performed according to the manufacturer`s protocol, with minor modifications. Cells were stained with both 5 μl Annexin V FITC and 5 μl PI and incubated for 15 min at RT in the dark before adding 400 μl of binding buffer to each tube. Samples were then analyzed by flow cytometry in FACSCalibur Flow Cytometer. A total of 10,000 events were collected using excitation/emission wavelengths of 488/525 and 488/675 nm for Annexin V and PI, respectively. This combination allowed the differentiation

among early apoptotic cells (Annexin V positive, PI negative), necrotic cells (Annexin V positive, PI positive), and viable cells (Annexin V negative, PI negative). The quantitative analysis of the data was carried out with FACSdiva software 6.0, as described above.

Invasion assay

The invasion assay for PANC-1 and LPC006 were done through coated transwell filters, with 300 µl of 0.1 mg/mL collagen I solution placed in the top compartment and incubated at 37 °C for 2 hours. Cells (5×10^4/well) were seeded on the upper side of the filter and incubated with 10 or 1 µM NHI compounds in RPMI medium and 0.5% FBS. The lower chamber was filled with medium containing 0.5% FBS. After 24 hours cells on the upper side of the filters were mechanically removed. Cells migrated to the lower side were fixed with paraformaldehyde and stained with Giemsa in 20% methanol. The filters were photographed and cells were counted.

To exclude the possibility that the antiproliferative effects of the drugs might affect the results of both the migration and the invasion assay we evaluated cell survival after 24 hours, counting the cells with a haemocytometer. In these experiments we detected only minimal antiproliferative effects (i.e., below 5%).

Liquid chromatography–mass spectrometry (LC-MS/MS) measurement of phosphorylated gemcitabine

Analysis by LC-MS/MS was used to determine the total phosphorylated gemcitabine ucleosides, which were calculated from the difference before and after alkaline phosphatase treatment. Approximately 2×10^6 cells were seeded into 6-well plates, and exposed to 1 µM gemcitabine, alone or together with 1 µM NHI compounds, for 24

hours, prior to being snap frozen as a pellet. Cell pellets were re-suspended in a known aliquot of phosphate buffer and precipitated with excess isopropyl alcohol. The supernatant was removed and evaporated to dryness via freeze-drying. The dry samples were reconstituted in 200 µL of water and 20 µL aliquots were used for LC/MS-MS analysis. The remaining samples were treated quantitatively with alkaline phosphatase (4 units) at 37 °C overnight. Chromatography was conducted using a Dionex Ultimate 3000 micro HPLC system coupled via a Turbo spray ionization source to an Applied Biosciences SCIEX API 3000 mass spectrometer (Applied). Data analysis was performed with version 1.52 Analyst software (AB Sciex, Nieuwerkerk aan den Ijssel, The Netherlands) controlled by Dionex Mass Spectrometry Link software version 2.8, combined with Chromeleon management software modules (Thermo Scientific).

Supplemental references

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Supplemental results

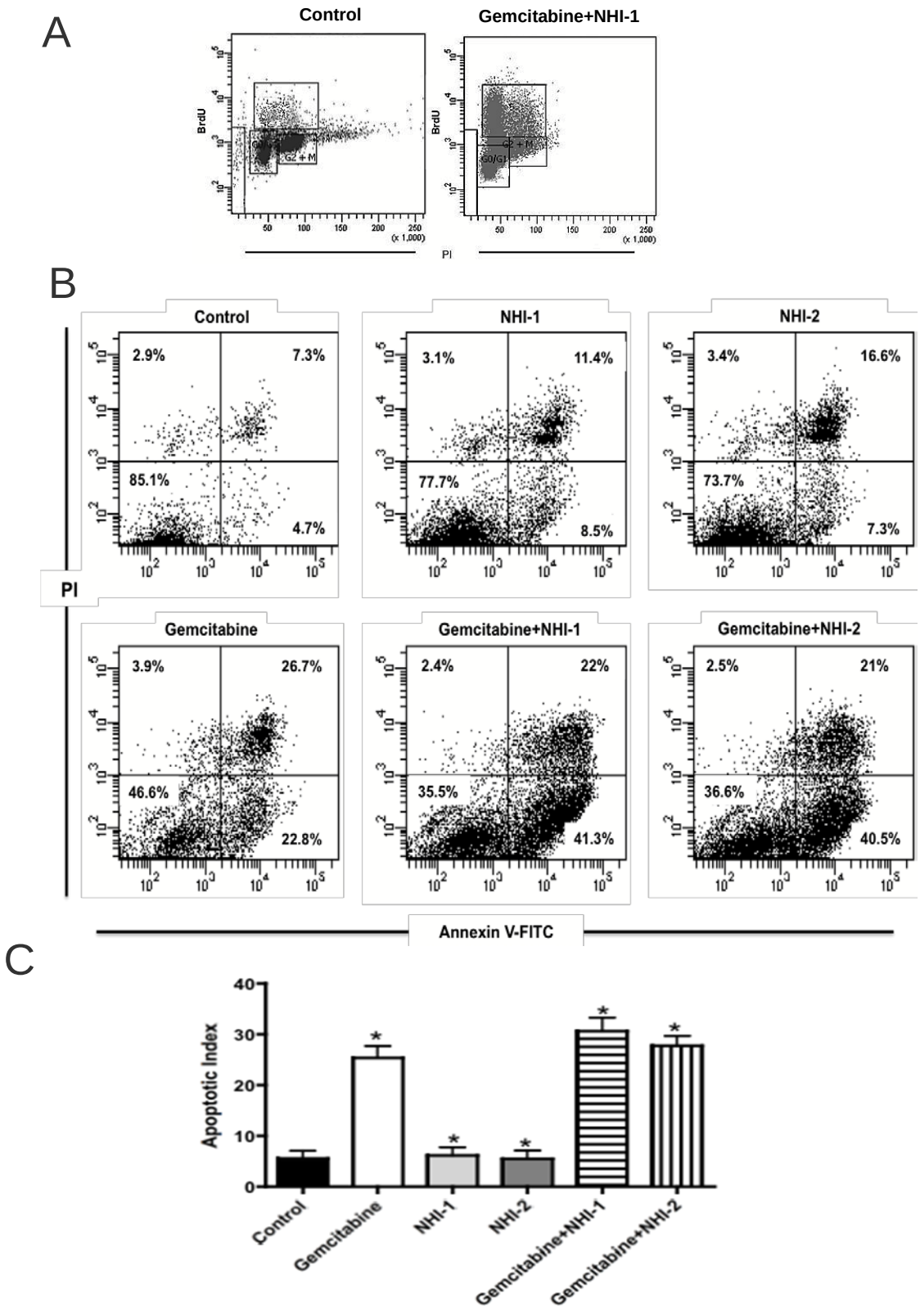
Supplemental Table 1. Enzyme inhibition potencies (IC_{50})

Compound	LDH-A (IC_{50}, μM)		LDH-B (IC_{50}, μM)	
	[NADH][a]	[Pyruvate][b]	[NADH][a]	[Pyruvate][b]
NHI-1	29.0 ± 3.0	73.4 ± 11.0	123.8 ± 0.7	> 200
NHI-2	14.7 ± 2.1	10.5 ± 2.5	55.8 ± 7.1	53.8 ± 4.4

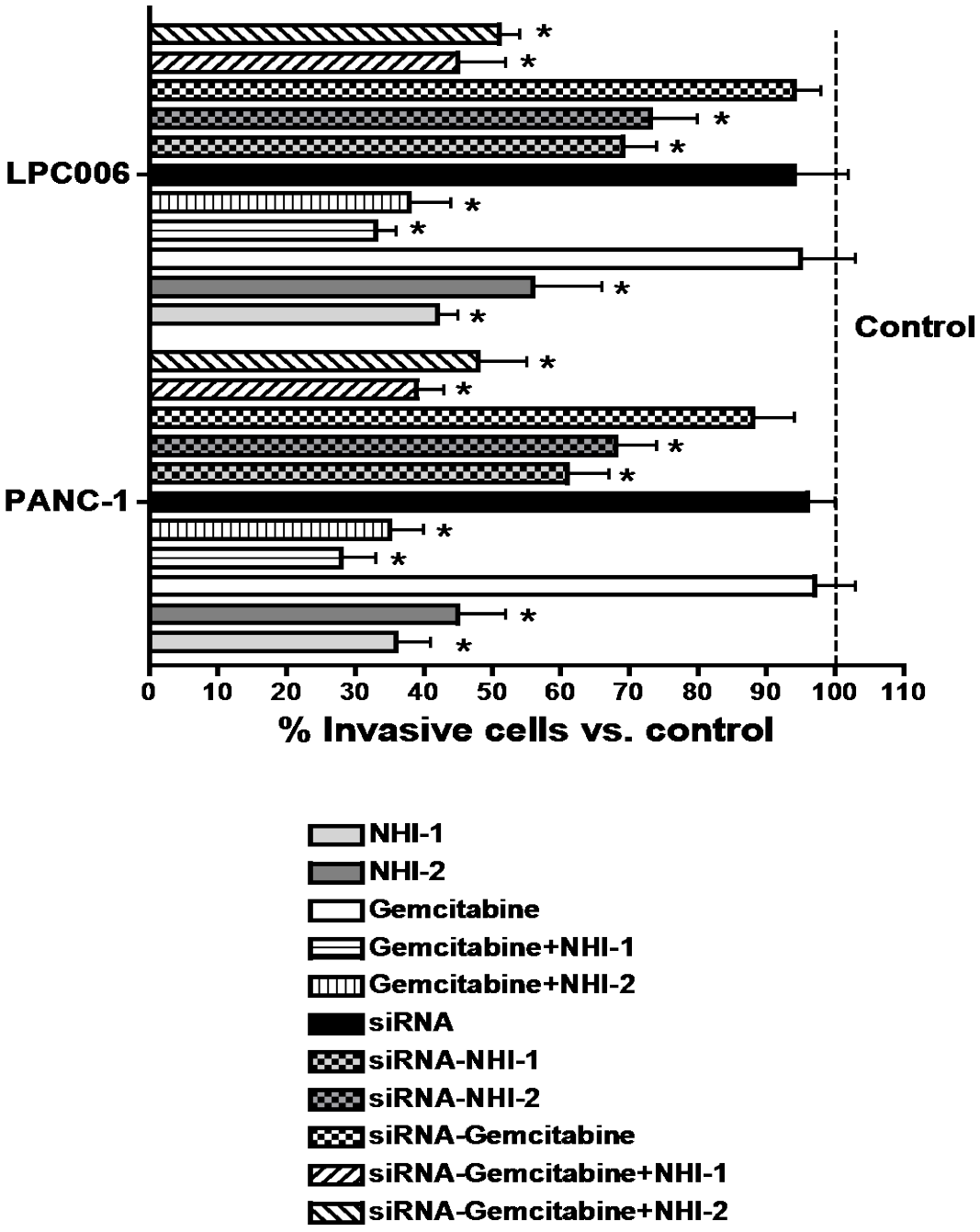
Note: Values are reported as the mean ± SD of 3 or more independent experiments [further details on these experiments are reported in the article by Granchi *et al*, *Org Biomol Chem* 2013]

[a]NADH-competition assay: saturating concentration (1440 μM) of sodium pyruvate and competitive concentration (40 μM) of NADH. [b]Pyruvate-competition assay: saturating concentration (150 μM) of NADH and competitive concentration (200 μM) of sodium pyruvate.

Supplemental figures



Supplemental Fig.1. Modulation of cycle and apoptosis induction by NHI compounds, gemcitabine, and anti-LDH-A specific siRNA. (A) Representative histograms of DNA content and BrdU incorporation in LPC006 cells (under hypoxic conditions) analyzed by flow cytometry, as described in the methods; (B) Representative histograms of cytofluorometric analysis of cell death and apoptosis by Annexin V/PI assay, illustrating the significant increase in apoptosis induction by the combination of NHI compounds with gemcitabine in LPC006 cells under hypoxic conditions. Samples were analyzed using excitation/emission wavelengths of 488/525 and 488/675 nm for Annexin V and PI, respectively; (C) Induction of apoptosis, as detected by bisbenzimide staining in LPC006 cells exposed for 72 hours to gemcitabine at IC50 concentration, 1 μM NHI-1 or NHI-2, and their combinations, in hypoxic conditions, after transfection with 4 μM siRNA anti-LDH-A. *Columns*, mean values obtained from three independent experiments. *Bars*, SEM. *P<0.05 vs. control (untreated) cells.



Supplemental Fig.2. Results of invasion studies in PANC-1 and LPC006 cells exposed for 24 hours to gemcitabine at IC50 concentration, 1 μM NHI-1 or NHI-2, and their combination in hypoxia, with and without transfection with anti-LDH-A siRNA. *Columns*, mean values obtained from three independent experiments. *Bars*, SEM. **P*<0.05 vs. control cells (i.e., 100%).