Chapter 6

Phospho-Akt overexpression is prognostic and can be used to tailor the synergistic interaction of the novel Akt inhibitor perifosine with gemcitabine in pancreatic cancer

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Submitted
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Abstract

There is increasing evidence of a constitutive activation of Akt in pancreatic-ductal-adenocarcinoma (PDAC), associated with poor prognosis and chemoresistance. Therefore, we evaluated the expression of phospho-Akt in PDAC tissues and cells, and investigated the therapeutic potential of the novel Akt inhibitor perifosine in combination with gemcitabine in PDAC cells.

Phospho-Akt was overexpressed in 60% of PDACs as determined by immunohistochemistry, which was correlated with shorter survival in 50 resected PDAC patients. mRNA and protein expression levels varied considerably in 14 PDAC cells, as assessed by RT-PCR, immunocytochemistry and ELISA. Perifosine inhibited cell growth in monolayer cell cultures and spheroids, and synergistically enhanced the antiproliferative activity of gemcitabine, with combination index values of 0.4 and 0.8 in cells characterized by high phospho-Akt expression (LPC028 and CFPAC-1), while this combination was antagonistic in LPC006 cells, with low phospho-Akt expression. The synergistic effect was associated with reduction of the expression of the ribonucleotide reductase subunit-M2, potentially facilitating gemcitabine cytotoxicity. Moreover, perifosine decreased cell migration and invasion, which was additionally reduced by the perifosine/gemcitabine combination. The combination enhanced the percentages of cells in S-phase, and significantly increased apoptosis, associated with induction of caspase-3/6/8/9, PARP and BAD, and inhibition of Bcl-2 and NF-kB.

In summary, these data provide novel insights into the ability of perifosine to interfere with cell-proliferation, induce apoptosis, reduce migration/invasion and synergistically interact with gemcitabine in cells with phospho-Akt overexpression. Furthermore these results support the analysis of phospho-Akt expression as a biomarker for the rational development of this innovative therapeutic approach.

Key words: Pancreatic ductal adenocarcinoma, Akt, perifosine, gemcitabine
Introduction

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal solid tumors. Despite extensive preclinical and clinical research, the prognosis of this disease has not improved, with a 5-year survival rate below 5% [1]. This dismal outcome can partially be explained by the lack of biomarkers for screening and diagnosis at earlier stages, and by the resistance to currently available chemotherapy regimens [2].

Activating KRAS mutations occur early, followed by loss of p16, and then later, inactivation of TP53 and SMAD4 [3]; however, targeting these events has proven to be very difficult. Studies on new drugs targeting additional oncogenic pathways and/or downstream effectors are warranted. The phosphatidylinositol-3 kinase (PI3K)/Akt pathway emerged as one of the core signaling pathways in PDAC [4]. In particular, the serine/threonine kinase Akt, which is coded in three highly homologous isoforms (Akt1, Akt2 and Akt3), is overexpressed in more than 40% of PDAC patients [5,6]. Mechanisms underlying aberrant Akt activation in cancer include direct alterations such as mutations, amplification, or overexpression, but also activation of upstream signaling events, such as activation of HER-2/neu signaling or PTEN mutation/loss [7].

The PI3K/Akt pathway plays a key role in cell proliferation, survival and motility [8]. Deregulation of components involved in this pathway could confer resistance to chemotherapy [9,10], while blockage of Akt signaling result in programmed cell death and inhibition of tumor growth [11].

Activation of Akt is a frequent event in PDAC and has been correlated to its poor prognosis [12,13]. Fahy and collaborators showed that inhibition of the PI3K/Akt pathway sensitizes pancreatic cancer cells by increasing apoptosis both in vitro and in vivo [14].

Currently, several inhibitors of Akt are under investigation, but none is in clinical use. The synthetic oral alkylphospholipid perifosine is a pan-Akt inhibitor [15] that has been evaluated in clinical trials for several tumors, including colon [16], breast [17], head and neck [18], and prostate cancer [19]. Promising activity has been reported in sarcoma and Waldenström macroglobulinemia [16]. However, the disappointing response rates of most solid tumors to perifosine as a single agent should prompt further studies into its mechanism of action as well as on novel synergistic combinations.

Perifosine prevents translocation of Akt to the cell membrane by blocking the pleckstrin homology (PH) domain of Akt [15], leading to inactivation of downstream pathway and inhibition of cell proliferation. Previous studies demonstrated perifosine activity against different cancer types, in vitro and in vivo [20,21]. Recently, Pinton and collaborators showed that perifosine inhibited cell growth of malignant pleural mesothelioma cells by affecting EGFR and c-Met phosphorylation [22]. Another study showed that perifosine decreased the AEG-1 gene expression along with inhibition of Akt/GSK3/c-Myc signaling pathway in gastric cancer [23]. Perifosine and curcumin synergistically increased the intracellular level of reactive oxygen species and ceramide, and downregulated the expression of cyclin-D1 and Bel-2 in colorectal cancer cells [24]. However, studies on the activity and the molecular mechanisms triggered by perifosine in PDAC cells are still missing.

Therefore, the aims of current study were (1) to investigate the expression of phospho-Akt in PDAC tissues and cells, and (2) to evaluate the growth inhibition by perifosine,
gemcitabine and their combination, using monolayer cell cultures and cells growing as spheroids in serum-free-CSC-medium. Moreover, we characterized several key factors, affecting cell cycle perturbation, apoptosis induction, as well as inhibition of cell migration and invasion in PDAC cells exposed to perifosine and perifosine/gemcitabine combination.

**Materials and Methods**

**Drugs and chemicals**
Perfosine was provided by Åeterna Zentaris Inc. (Frankfurt am Main, Germany), while gemcitabine was a gift from Eli-Lilly (Indianapolis, IN). The drugs were dissolved in ethanol or sterile water, and diluted in culture medium before use. RPMI-1640 medium, foetal bovine serum (FBS), penicillin (50 IU/ml) and streptomycin (50 µg/ml) were from Gibco (Gaithersburg, MD). All other chemicals were purchased from Sigma-Aldrich (Zwijndrecht, The Netherlands).

**Cell culture**
Eight PDAC cell lines (PL45, MIA-PaCa2, HPAF-II, CFPAC-1, Bxpc3, HPAC and PANC-1) and the human immortalized pancreatic duct epithelial-like cell line hTERT-HPNE were obtained from the American Type Culture Collection, whereas seven primary PDAC cultures (LPC006, LPC028, LPC033, LPC067, LPC111, LPC167 and PP437) were isolated from patients at the University Hospital of Pisa (Pisa, Italy), as described previously [25]. The cell lines were tested for their authenticity by PCR profiling using short tandem repeats by BaseClear (Leiden, The Netherlands). The cells were cultured in RPMI-1640, supplemented with 10% heat-inactivated FBS and 1% streptomycin/penicillin at 37 °C, and harvested with trypsin-EDTA in their exponentially growing phase.

**Quantitative Reverse-Transcriptase Polymerase-Chain-Reaction (qRT-PCR)**
Total RNAs were extracted from cells using the TRI REAGENT-LS (Invitrogen, Carlsbad, CA), according to the manufacturers’ protocol. RNA was also extracted from seven primary tumors, after laser micro-dissection with a Leica-LMD7000 instrument (Leica, Wetzlar, Germany), using the QIAamp RNA Micro Kit (Qiagen, Hilden, Germany), as described [25]. RNA yield and purity were checked at 260 to 280 nm with NanoDrop-1000 Detector (NanoDrop Technologies, Wilmington, DE). One microgram of RNA was reverse-transcribed using the DyNAmo Synthesis Kit (Thermo Scientific, Vantaa, Finland). qRT-PCR was performed with specific TaqMan® primers and probes for Akt1, human equilibrative nucleoside transporter-1 (hENT1), deoxycytidine kinase (dCK), cytidine deaminase (CDA), ribonucleotide reductase subunit-M1 (RRM1), and subunit-M2 (RRM2), and E-cadherin which were obtained from Applied Biosystems TaqMan Gene expression products (Hs00920503_m1, Hs01085706_m1, Hs00984403_m1, Hs01040726_m1, Hs00156401_m1, Hs00168784_m1, Hs01072069_g1 and Hs01023894_m1, respectively). The cDNA was amplified using the ABI-PRISM 7500 instrument (Applied Biosystems, Foster City, CA). Gene expression values were normalized to β-actin, using a standard curve of cDNAs obtained from Quantitative PCR Human Reference RNA (Stratagene, La Jolla, CA), as described earlier [26].

**Tissue microarrays (TMAs), immunohistochemistry and immunocytochemistry**
Phospho-Akt protein expression was evaluated in slides from two formalin-fixed, paraffin-
embedded PDAC-specific TMAs build with neoplastic cores from a cohort of radically-rectected patients (n=50), using the TMA Grand Master (3DHistec, Budapest, Hungary) instrument, and stained according to standard procedures with the 587F11 rabbit polyclonal antibody (1:50 dilution; Cell-Signaling, Beverly, MA). Visualization was obtained with BenchMark Special Stain Automation system (Ventana Medical Systems, Tucson, AZ). Staining results were evaluated using a four-tier system including the analysis of positive cells number and staining intensity. All slides were reviewed by two pathologists, who evaluated also the amount of tissue loss, background staining and overall interpretability before the phospho-Akt reactivity evaluation. A parallel digital analysis of Akt reactivity was performed using a computerized high-resolution acquisition system (D-Sight, Menarini, Florence, Italy).

For immunocytochemistry (ICC), the cells were grown in a Chamber Slides System (Lab-Tek, Collinsville, IL). After 24 hours, the cells were fixed with 70% ethanol for 10 minutes, followed by incubation with the antibody described above (4°C overnight, 1:30 dilution in PBS). Cells were stained with the avidin-biotin-peroxidase complex (UltraMarque HRP Detection, Greenwood, AR). Negative controls were obtained by replacing the primary antibody with PBS. The sections were reviewed and scored blindly using a system based on staining intensity and on the number of positively stained cells, as described previously [26].

**Growth inhibition studies**

The cell growth inhibitory effects of perifosine, gemcitabine and their combination were studied in CFPAC-1, PANC-1, LPC028 and LPC006 cells. These cells were treated for 72 hours with perifosine (1-500 μM), gemcitabine (1-500 nM), and simultaneous combination at a fixed ratio based on IC50 (i.e. concentration of a drug required for 50% inhibition of cell growth) of each drug. The plates were then processed for the sulforhodamine-B assay, as described [26].

**Evaluation of synergistic/antagonistic interaction of perifosine with gemcitabine**

The pharmacological interaction between perifosine and gemcitabine was evaluated by the median drug effect analysis method as described previously [26]. In this regard, the combination index (CI) was calculated to compare cell growth inhibition of the combination and each drug alone. Data analysis was carried out using CalcuSyn software (Biosoft, Oxford, UK).

**Effect of perifosine and gemcitabine in multicellular spheroids**

LPC006 and LPC028 spheroids were established by seeding 104 cells per ml in DMEM/F12+GlutaMAX-I (1:1) with insulin-transferrin-selenium (1:1000, Invitrogen), in 24-well ultra low attachment plates (Corning Incorporated, NY). The cytotoxic effects were evaluated by measuring the size and number of spheroids with the inverted phase contrast microscope Leica-DMI300B (Leica, Wetzlar, Germany), taking 9 pictures for each well. Spheroid volume (V) was calculated from the geometric mean of the perpendicular diameters D=(Dmax+Dmin)/2, as follows: V=(4/3)×π (D/2)^3.

**Western blot analysis**

In order to evaluate the modulation of Akt, phospo-Akt, PARP, BAD, Bcl-2, NF-kB protein expression in PDAC cells treated for 24 hours with perifosine, gemcitabine and their combination, Western blot analyses were executed as described previously [27].
Briefly, 40 μg of proteins was separated on a 10% SDS-polyacrylamide gel and transferred onto PVDF membrane (Immobilion®-FL, Millipore, Billerica, MA). The membrane was incubated overnight with rabbit anti-Akt, anti-phospho-Akt, described above, as well as with rabbit anti-BAD, anti-Bcl-2, anti-PARP, and anti-NF-kB (1:1000, diluted in the blocking solution; all from Santa Cruz Biotechnology, Santa Cruz, CA) and mouse anti-β-actin (1:10000; Sigma–Aldrich). The secondary antibodies were goat anti-rabbit-InfraRedDye® 800 Green and goat anti-mouse-InfraRedDye® 680 Red (1:10000, Westburg, Leusden, The Netherlands). Fluorescent proteins were monitored by an Odyssey Infrared Imager (LI-COR Biosciences, Lincoln, NE), equipped with Odyssey 2.1 software to perform a semi-quantitative analysis of the bands.

**Akt and phospho-Akt analysis by enzyme linked immunosorbent (ELISA) assay**

To investigate the inhibitory effects of perifosine on Akt [pS473] phosphorylation, a specific ELISA assay was performed using the Pierce AKT Colorimetric In-cell ELISA Kit (Thermo Scientific, Rockford, IL), which has a sensitivity approximately 2x greater than that of Western blotting. The levels of Akt and phospho-Akt were measured in cells seeded in a 96 well-plate at a density of 105 cells per well, and treated for 2, 4 or 24 hours with perifosine, gemcitabine and their combination at IC50 values. The absorbance was measured in a Synergy HT Multi-Detection Microplate Reader (BioTek, Bad Friedrichshall, Germany) at a wavelength of 450 nm.

**In vitro migration and invasion assays**

The ability of perifosine and its combination with gemcitabine to inhibit the migratory behaviour of PDAC cells was investigated by in vitro migration assay, as described [25]. The cells were exposed to the drugs at their IC50s. Images were taken at the beginning of the exposure (time 0), with those taken after 4, 6, 8, 20, and 24 hours.

Transwell chambers with polycarbonate membranes and 8-μm pores were used for invasion assays. These assays were carried out through coated transwell filters, with 100 μl of 0.1 mg/mL collagen I solution. A total of 105 cells were plated on the upper side of the filter and incubated with the drugs at IC50 concentrations in RPMI-1640 medium. After 24 hours cells migrated into the lower side were fixed with paraformaldehyde and stained with Giemsa in 20% methanol. The filters were photographed and cells were counted.

**Analysis of cell-cycle and cell death**

To investigate the effect of drugs on modulation of cell cycle, LPC006, CFPAC-1 and PANC-1 cells were treated for 24 hours with gemcitabine, perifosine and their combination at IC50 concentrations. Cells were stained by propidium iodide (PI) and cell cycle modulation was evaluated using a FACSCalibur flow cytometer (Becton Dickinson, San José, CA), equipped with the CELLQuest software for data analysis.

The ability of gemcitabine, perifosine and its combination with gemcitabine to induce cell death was evaluated by measuring sub-G1 regions during cell cycle analysis, as described above. Moreover, the cells displaying apoptotic features were analysed by fluorescence microscopy with bisbenzimide staining, as described previously [27]. Apoptosis induction was also assessed by 3,3’-dihexyloxacarbocyanine iodide (DiOC) labelling. DiOC is a lipophilic and green fluorescent dye, which can pass the plasma membrane, without being metabolized by the cell, and accumulate at the membrane of mitochondria of living cells. Shortly, the cells were stained with DiOC for 30 min, and analysed by FACSCalibur, as
Additional studies were performed with the Annexin-V/PI assay, plating the cells in 6-well plates at a density of 1.5x10^5. After 24 hours, the cells were treated with the drugs at their IC50, followed by 24-hour incubation. Then, the cell pellets were re-suspended in 100 ml of ice-cold binding buffer (0.1 M Hepes/NaOH (pH=7.4), 1.4 M NaCl, 25 mm CaCl2). The staining was performed according to the manufacturer’s instructions (Annexin-V/PI detection Kit-I, Becton Dickinson). Cells were stained by 5 µL Annexin V-FITC and 5 µL PI. Samples were gently vortexed and incubated for 15 minutes at room temperature. Then, 400 µL of binding buffer was added to the cells. The samples were analyzed by FACSCalibur using excitation/emission wavelengths of 488/525 and 488/675 nm for Annexin-V and PI, respectively.

**Caspase activity assay**

The effects of perifosine, gemcitabine and their combination on the activity of caspase-3, -6, -7, -8, -9 were determined by specific fluorometric assay kits (Zebra Bioscience, Enschede, The Netherlands), according to the manufacturer’s instructions. Briefly, 106 LPC006 and LPC028 cells were exposed to the drugs for 24 hours at their IC50s. Fluorescence was measured at 350 nm excitation and 460 nm emission (Spectra fluor Tecan, Salzburg, Austria). Relative caspase activity was normalized with respect to the untreated control cells.

**Statistical analysis**

All experiments were performed in triplicate and repeated at least twice. Data were expressed as mean values±SEM. and analyzed by Student’s t-test or ANOVA followed by Tukey's multiple comparison test. For the analysis of the correlation of phospho-Akt expression and clinical data, the overall survival (OS) was calculated from the date of pathological diagnosis (i.e. the date of surgery) to the date of death. OS curves were constructed using Kaplan-Meier method, and differences were analyzed using log-rank test. Data were analyzed using SPSS v.20 statistical software (IBM, Chicago). Statistical significance was set at P<0.05.

**Results**

**Correlation with outcome and Akt expression in PDAC tissues and cells**

The protein expression of phospho-Akt was evaluated by IHC in 50 human PDACs collected in TMAs. The main clinical characteristics of these patients are reported in Supplemental Table 1. Sixty% of these specimens showed high expression of phospho-Akt, compared to the tissues with low or negative expression (Fig. 1A). No association was observed between phospho-Akt and age, sex, grading, resection, and lymph node infiltration (data not shown). Patients with low phospho-Akt expression (N=20) had a median OS of 19.9 months (95%CI, 17.8-22.0), while patients with a high expression had a median OS of 12.0 months (95%CI, 9.0-14.9, P=0.033, Fig. 1B).

The mRNA expression of Akt1 was detectable in all PDAC cells by qRT-PCR, as well as in the originator tissues of the primary tumor cell cultures. This expression value differed among the cells, ranging from 0.93 arbitrary unit (a.u.) in LPC028 cells to 24.01 a.u. in PANC-1 cells (Fig. 1C). The mean and median expression in the tumor cells (8.74±0.17...
Figure 1. Akt/phospho-Akt expression in PDAC tissues and cells. (A) Representative IHC pictures in PDAC tissues, showing the variable expression of phospho-Akt; (B) Kaplan–Meier survival curves according to the expression of phospho-Akt in 50 radically-resected PDACs, showing that patients with high expression of phospho-Akt had a significantly shorter survival compared to patients with low phospho-Akt expression; (C) Akt1 mRNA expression in ATCC cell lines (black-bars), primary tumor cultures (white-bars), and their originator tissues (gray-bars), Columns, mean values obtained from three independent experiments, bars, SEM; (D) ICC pictures of phospho-Akt expression in LPC006 and LPC028 cells.

and 8.38 a.u., respectively) were significantly higher (P<0.01) than the expression detected in hTERT-HPNE cells (0.28 a.u.). Notably, Akt1 gene expression in the 7 primary tumor cells and their laser-microdissected originator tumors showed a similar pattern and were highly correlated with Spearman analysis (R2>0.9, P<0.05), suggesting that these cells represent optimal preclinical models for our pharmacological studies. Moreover, ICC revealed that the LPC006 cells had a significantly lower Akt and phospho-Akt expression compared to LPC028 and CFPAC-1 cells (Fig. 1D). Therefore, LPC028, CFPAC-1, and LPC006 cells were selected for further studies due to their levels of Akt mRNA and protein expression, which were representative of high, intermediate, and low values, respectively.

Perifosine inhibits cell growth and interacts synergistically with gemcitabine in PDAC cells with high expression of phospho-Akt

The cell growth inhibitory effects of perifosine, gemcitabine and their combination in
LPC028, CFPAC-1, and LPC006 cells are shown in Fig. 2A-C. Based on these results, combination studies were performed using a fixed ratio of perifosine and gemcitabine at IC50s. Perifosine enhanced the antiproliferative activity of gemcitabine, especially in the LPC028, and CFPAC-1 cells, by decreasing the IC50s of gemcitabine from 4.3±1.1 nM and 17.2±2.1 nM to 1.4±0.54 nM and 4.0±1.1 nM, respectively. The median drug-effect analysis revealed a slight-to-moderate synergism in CFPAC-1 cells, and a strong synergism in the LPC006 cells, with CI values of 0.8 and 0.4, respectively (Fig. 2D). Conversely, the combination of perifosine and gemcitabine was antagonistic in the LPC006 cells (CI>1.2). In order to investigate the mechanisms underlying these different interactions, several biochemical analyses were performed, as detailed below.

**Perifosine and its combination with gemcitabine reduce the size of PDAC spheroids**

Previous studies illustrated that three-dimensional (3-D) culture models are generally more chemo-/radio-resistant than two-dimensional monolayer cell cultures, supporting the use of 3-D models for drug testing [29].

![Figure 2. Inhibition of cell proliferation in PDAC cells. Growth inhibitory effects after 72 hours exposure to perifosine, gemcitabine or their combination at a fixed ratio based on IC50 values in LPC028 (A), CFPAC-1 (B) and LPC006 cells (C). On the X-axis the drug concentrations for the combination are referred to gemcitabine; (D) Mean CI of the perifosine/gemcitabine combination. CI values at FA of 0.5, 0.75 and 0.9 were averaged for each experiment, and this value was used to calculate the mean between experiments, as explained in the Materials and Methods section; (E) Effect of perifosine and gemcitabine and their combination, at IC50 values, on the volumes of PDAC spheroids after 72 hours exposure. Modulation of phospho-Akt and gemcitabine determinants in PDAC cells. Columns or Points, mean values obtained from three independent experiments; bars, SEM. *Significantly different from controls.](image)
In order to explore whether perifosine would be active in 3-D PDAC models, we evaluated this drug in spheroids of LPC006 and LPC028 cells, with low and high phospho-Akt expression, respectively. Perifosine remarkably increased the disintegration of LPC028 spheroids, which were significantly (P<0.05) reduced in size compared to the untreated spheroids (Fig. 2E). The combination of perifosine with gemcitabine additionally reduced the size of the LPC028 spheroids with respect to the spheroids treated with the single drugs. In contrast, no changes were observed in the LPC006 spheroids, further supporting the antagonistic interaction of perifosine with gemcitabine in this PDAC model.

**Modulation of phospho-Akt and gemcitabine determinants in PDAC cells**

Perifosine inhibits the phosphorylation of Akt by blocking the PH-domain in different cancer cell lines [15], but no data have been reported yet on PDAC cells. Therefore, we evaluated the expression of phospho-Akt (at serine residue 473), normalized to the total Akt levels, both in untreated cells and in cells treated with perifosine, gemcitabine, and their combination, after 2, 4 or 24 hours exposure. Perifosine was more effective after 24 hours than after 2 or 4 hours (data now shown). As shown in Fig. 3A, perifosine significantly reduced the expression of p-Akt in LPC028 and CFPAC-1 cells (e.g. 25% and 41% reduction, respectively). Similarly, the combinations of perifosine and gemcitabine markedly suppressed Akt phosphorylation, with a degree of inhibition ranging from -36.6% (CFPAC-1 cells) to -43.8% (LPC028 cells). Conversely, phospho-Akt levels were not affected by perifosine, gemcitabine or their combination in the LPC006 cells (Fig. 3A).

Previous studies demonstrated that the expression of RRM2 is modulated by the Akt/c-MYC pathway [30] and this enzyme is a key target of gemcitabine [31]. However, the alterations in the expression or function of other enzymes, involved in the transport, metabolism and catabolism of gemcitabine can also lead to resistance (e.g., decreased dCK

![Figure 3. Modulation of phospho-Akt and gemcitabine determinants.](image)

(A) Effect of 24-hour exposure to gemcitabine, perifosine or their combination, at IC50 values, on the expression of phospho-Akt, normalized to the expression of total Akt, as determined by a specific ELISA assay; (B) Perifosine significantly reduced the expression of RRM1 and RRM2 in LPC028 cells treated with perifosine at IC50 versus untreated cells, as determined by qRT-PCR. Minimal changes were observed for the expressions of nucleoside transporters (hENT1 and hCNT1), and enzymes of gemcitabine metabolism (dCK) or catabolism (CDA). Columns, mean values obtained from three independent experiments; bars, SEM. *Significantly different from controls.
or increased CDA expression [31-33]). Therefore, we evaluated the mRNA expression of several gemcitabine determinants in the LPC006 and LPC028. As shown in Fig. 3B, the expression of RRM1 and RRM2 was significantly reduced (approximately 2-fold) in LPC028 cells treated with perifosine versus untreated cells, while only minimal variations were observed for hCNT1, hENT1, dCK and CDA expression. No significant changes were observed in the LPC006 cells (data not shown). These results can at least in part explain the synergistic effect of perifosine with gemcitabine in PDAC cells with high phospho-Akt expression.

Perifosine and its combination with gemcitabine inhibit cell migration/invasion and up-regulate the expression of E-cadherin

To determine the effects of perifosine, gemcitabine and their combination on migratory behavior, a scratch mobility assay was performed in LPC028, CFPAC-1, and LPC006 cells (Fig. 4A-C).

CFPAC-1 showed a significant reduction of migration starting after 20 hours exposure to perifosine (about 20% reduction compared to control; P<0.05; Fig. 4A), while gemcitabine alone did not affect cell migration. In particular, the percentages of cellular migration in CFPAC-1 were approximately 61%, 58%, 40% and 38%, in untreated, gemcitabine, perifosine and combination treated cells, respectively.

Inhibition of migration in LPC028 cells treated with perifosine was even more effective than in CFPAC-1 cells (Fig. 4B). In particular, perifosine significantly reduced cell migration with respect to LPC028 control cells already after 8 hours, with a reduction of the scratch-area of about 50%, and the perifosine/gemcitabine combination additionally reduced cell migration. LPC028 and CFPAC-1 cells treated with perifosine also showed a significantly reduced invasive potential, compared to the untreated cells (Fig. 4D). Moreover, the perifosine/gemcitabine combination was more effective than perifosine-alone in both LPC028 and CFPAC-1 cells. However, no modulation of cell migration neither of cell invasion was observed in the LPC006 cells (Fig. 4C).

Since previous studies suggested that Akt signaling pathway suppressed E-cadherin expression [34-36], we investigated whether perifosine could affect the level of this target at both mRNA and protein level. Perifosine and its combination with gemcitabine significantly enhanced E-cadherin mRNA expression (P<0.05; Fig. 4E). Similarly, immunocytochemistry analysis in CFPAC-1 and LPC028 cells illustrated a significant increase of E-cadherin protein staining after exposure to both perifosine and perifosine/gemcitabine combination (data not shown).

Perifosine and its combination with gemcitabine affect cell cycle

Perifosine, gemcitabine and their combination affected cycle distribution of PDAC cells, as summarized in Supplemental Table 2. Perifosine significantly (P<0.05) increased the percentages of LPC028 cells in S and G2/M phases (e.g., from 18.7 in the control to 26.1% in the S phase) after 72 hours, while reducing the percentage of the cells in G0/G1. Similarly, the perifosine/gemcitabine combination significantly decreased the cells in G1 phase, while increasing the cells in S phase, up to 48.9%. Similar perturbations of cell cycle were observed in the CFPAC-1 cells, suggesting that perifosine might favor gemcitabine activity through a significant increase of cells in the S phase. Opposite modulation of cell cycle was observed in LPC006 cells, with only a slight increase of the cells in the G0/G1
Figure 4. Effects of perifosine, gemcitabine and their combination on PDAC cells migration and invasion. Results of wound-healing assay in CFPAC-1 (A), LPC028 (B) and LPC006 (C) cells (insert: representative picture at 24 hours). Cells were exposed to perifosine, gemcitabine or to their combination, at IC50 values. (D) Results of invasion studies in the PDAC cells exposed for 24 hours to perifosine, gemcitabine or to their combination, at IC50 values (insert: representative picture at 24 hours); (E) Modulation of E-cadherin mRNA levels in LPC028 and CFPAC-1 after 24 hours exposure to perifosine, gemcitabine or to their combination, at IC50 values, as determined by q-RT-PCR. Columns or Points, mean values obtained from three independent experiments; bars, SEM. *Significantly different from controls; **Significantly different from gemcitabine.

phase and minimal modulations of the S and G2/M phase in cells exposed to perifosine/gemcitabine combination.

Perifosine and its combination with gemcitabine enhance cell death and apoptosis

Analysis of the sub-G1 region of cell cycle perturbation demonstrated that the treatment with perifosine enhanced cell death (Table 1). In particular, the LPC028 cells treated with the combination exhibited the largest sub-G1 signal (e.g., ≈20% in cells treated with perifosine/gemcitabine combination versus untreated cells). Further analysis with bisbenzimide staining using fluorescence microscopy in the cells exposed to perifosine, gemcitabine and their combination showed that these cells presented the typical apoptotic morphology, with cell shrinkage, nuclear condensation and fragmentation, and rupture of cells into debris. A total of 5-8% of apoptotic cells were observed after gemcitabine treatment, while perifosine-alone exposure was associated with a higher percentage (6-
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13%) of apoptotic cells. The perifosine/gemcitabine combination significantly increased the apoptotic index, compared to untreated cells and gemcitabine-treated cells (Fig. 5A). Similarly, analysis of mitochondria shrinkage showed that perifosine and its combination with gemcitabine significantly (P<0.05) increased cell death in CFPAC-1 and LPC028 (e.g., +18% increase of cell death in CFPAC-1 cells treated with perifosine/gemcitabine combination; P<0.05, Fig. 5B).

Further analysis of cell death by the Annexin-V/PI assay confirmed the induction of apoptosis in the LPC028 cells treated with perifosine. Perifosine increased both early and late apoptosis, as shown in Fig. 5C for the LPC028 cells. Moreover, the combination of perifosine and gemcitabine significantly increased the percentage of late apoptotic cells up to 36%. Similar results were observed in CFPAC-1 cells, whereas no apoptosis induction was detected in LPC006 cells (data not shown).

**Perifosine and its combination with gemcitabine activate caspases and proapoptotic factors, and down-regulate Bcl-2 and NF-kB**

In order to investigate the molecular mechanisms underlying apoptosis induction, we explored several potential cellular targets of perifosine, focusing on activation of the “initiator” caspases, caspase-8 and -9, and the “effector” caspases, caspase-3, and -6. Moreover, we studied the expression of various proapoptotic and antiapoptotic proteins. As shown in Fig. 5D, perifosine and its combination with gemcitabine were able to increase the activity of caspase-3/-6/-8/-9 in Lpc028, as determined by specific fluorometric caspase activity assays. However, perifosine modulated also other apoptotic markers. In particular, this drug increased the expression of PARP and BAD, while reducing Bcl-2 and NF-kB expression, as measured by Western blot analysis in LPC028 cells (Fig. 5E).

| Table 1. Effects of gemcitabine and perifosine and their combination on cell cycle distribution |
|-------------|-----------|-----------|-----------|
| Treatment   | G0/G1 Phase (%) | S Phase (%) | G2/M Phase (%) |
| LPC0028     | 54.1±1.5 | 18.7±1.7 | 27.1±2.0 |
| Control     | 54.1±1.5 | 18.7±1.7 | 27.1±2.0 |
| Gemcitabine | 44.4±3.4 | 18.8±2.5 | 36.9±3.3 |
| Perifosine  | 51.2±1.8 | 26.1±1.4 | 29.7±1.7 |
| Gemcitabine + Perifosine | 36.9±0.9 | 48.9±0.6 | 30.2±2.1 |
| CFPAC-1     | 57.5±1.2 | 9.2±1.3 | 33.3±1 |
| Control     | 57.5±1.2 | 9.2±1.3 | 33.3±1 |
| Gemcitabine | 43.2±1.4 | 24.6±1.1 | 32.2±1.4 |
| Perifosine  | 16.3±0.8 | 59.1±1.4 | 24.6±0.3 |
| Gemcitabine + Perifosine | 11.9±2.2 | 41.2±2.7 | 28.8±1.2 |
| LPC006      | 52.4±2.3 | 25.8±1.9 | 21.8±0.3 |
| Control     | 52.4±2.3 | 25.8±1.9 | 21.8±0.3 |
| Gemcitabine | 38.3±1.5 | 30.1±2.1 | 31.6±1.9 |
| Perifosine  | 55.0±2.1 | 22.3±1.9 | 22.6±1 |
| Gemcitabine + Perifosine | 57.1±1.8 | 24.5±2.6 | 18.4±0.6 |

Cells were exposed to IC50s values of gemcitabine, perifosine and their combination, for 72 hours.
Figure 5. Apoptosis induction by perifosine, gemcitabine and their combination. (A) Effects of perifosine, gemcitabine and their combination on cell death, as determined by the analysis of Sub-G1 region in LPC028 and CFPAC-1 cells (A), mitochondrial membrane potential (DiOC staining) in LPC028 cells (B), and Annexin-V/PI (C) in LPC028 cells. Annexin V-FITC is a fluorescent marker for the detection of phosphatidyl choline residues in cell membranes. In combination with propidium iodide (PI), the Annexin-V/PI method allows discrimination between viable cells (Annexin V-/PI-, bottom left quadrant), cells in early apoptosis (Annexin V+/PI-, bottom right quadrant), cells in late apoptosis (Annexin V+/PI+, top right quadrant) and necrotic cells (Annexin V-/PI+, top left quadrant); (D) Modulation of caspase-3, caspase-6/-8/ and caspase-9 in LPC028 cells after 24-hour treatment with perifosine, gemcitabine or their combination at IC50s, as determined by a specific fluorometric assay; (E) Representative blots of the modulation of protein expression of PARP, BAD, Bcl-2 and NF-kB in LPC028 after 24 hours exposure to perifosine at IC50. Columns, mean values obtained from three independent experiments; bars, SEM. *Significantly different from controls; **Significantly different from gemcitabine.

Discussion
This is the first study evaluating the pharmacological interaction of the novel Akt inhibitor perifosine with gemcitabine in PDAC. Perifosine combination with gemcitabine was synergistic in PDAC cells with high phospho-Akt expression, but antagonistic in cells with low phospho-Akt expression. Synergism was associated with inhibition of migration/invasion and induction of apoptosis.

The PI3K/Akt is an important signaling pathway that mediates several cellular processes, including cell proliferation, growth, survival, and motility. PI3K is activated in the response
to a variety of growth stimuli through receptor tyrosine kinases and G-protein coupled receptors [8,10]. PI3K phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2) generating the second messenger phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 binds to Akt via its PH domain and mediates its translocation to the cell membrane (Fig.6).

The PI3K/Akt signaling pathway is constitutively activated in several PDACs, and immunohistochemical presence of phospho-Akt has been associated with worse prognosis and outcome [5,13,36]. In agreement with these studies we observed a significant correlation of phospho-Akt expression with shorter OS in our homogeneous cohort of radically-resected PDAC. However, the relative degree of Akt activation is quite variable across PDAC tissues and cells, and it is currently unknown whether evaluation of Akt activity may be useful in identifying PDAC that will be more sensitive to novel Akt inhibitors. Moreover, activation of this signaling pathway is associated with chemoresistance of pancreatic cancer [9,37], supporting the hypothesis that Akt inhibitors might also be used to overcome resistance towards conventional cytotoxic agents.

**Figure 6. Molecular mechanisms involved in the synergistic interaction of perifosine with gemcitabine.** The main upstream activator of Akt is phosphatidylinositol-3 kinase (PI3K). This kinase phosphorylates phosphatidylinositol-4,5-diphosphate (PIP2), which results in generation of phosphatidylinositol-3,4,5-triphosphate (PIP3). PIP3 interacts with the pleckstrin homology (PH) domain of Akt, leading to translocation of Akt to the cell membrane, and phosphorylation at Thr308 and Ser473. Perifosine inhibits Akt activation and enhances the growth inhibitory effects of gemcitabine through its pronounced pro-apoptotic, anti-invasive effects, as well as by inhibiting the cell proliferation, followed by modulation of ribonucleotide reductase (RR), potentially facilitating gemcitabine cytotoxicity.
Several Akt/PI3K inhibitors are being developed. The first-generation of these inhibitors includes LY294002 and wortmannin, which were tested to elucidate the value of Akt/PI3K as therapeutic target [38]. The inhibition of Akt using either wortmannin or LY294002 sensitized PDAC to the apoptotic effect of gemcitabine in vitro [14,39] and in vivo [39,40]. However, due to the unfavorable pharmaceutical properties, toxicity, and crossover inhibition of other lipid and protein kinases, these compounds were not used in clinical studies [41].

One of the most clinically advanced Akt inhibitors is perifosine that is structurally similar to miltefosine [42]. Perifosine has shown anti-tumor activity in a variety of preclinical models [16,17]. The clinical efficacy of perifosine is currently evaluated in phase I/II clinical trials in patients with advanced solid tumors. In one phase II trial for metastatic colon cancer the perifosine/capecitabine combination doubled time to progression, but the following phase III trial showed no benefit in OS when adding perifosine to capecitabine in the refractory colorectal cancer setting [43]. Similarly, a small phase II study in unselected patients with locally advanced, unresectable, or metastatic PDAC failed [44].

In the development of novel therapeutics, surrogate biomarkers are essential to demonstrate efficiency of kinase silencing and predict the efficacy of ongoing treatment. Therefore, in the present study, cell growth inhibitory effects of perifosine, gemcitabine and their combination were evaluated in several representative PDAC cells, including two primary PDAC cell cultures, LPC028 and LPC006, characterized by high and low Akt expression, respectively. For the LPC028 model we demonstrated that perifosine inhibited cell growth, both in monolayer cell cultures and in cells growing as spheroids, whereas LPC006 cells and spheroids were not affected. Similarly the perifosine/gemcitabine combination had synergistic effect only in the cells with high or intermediate values of Akt mRNA and phospho-Akt expression, as determined by RT-PCR and ICC. Conversely this combination was antagonistic in the cells with low phospho-Akt expression. Importantly, a specific ELISA showed that perifosine effectively reached and inhibited its targets only in the LPC028 cells, and the combination with gemcitabine additionally inhibited Akt activation in these cells.

Moreover, the present study demonstrated for the first time that perifosine interfered with pivotal determinants for the activity of gemcitabine. In particular, we observed that perifosine and its combination with gemcitabine significantly reduced the expression of RRM2 and RRM1 in the cells with a high expression of Akt, while this effect was not statistically significant in the cells with low Akt expression. RRM2 is a target of gemcitabine activity and a previous study correlated the expression of its subunits to gemcitabine sensitivity in PDAC cells [45], supporting the hypothesis that the synergistic effect between perifosine and gemcitabine might be explained at least in part by the modulation of gemcitabine sensitivity through RRM2 suppression.

Our studies suggested that the synergistic interaction of perifosine with gemcitabine is associated also with other important molecular mechanisms affecting PDAC aggressiveness. In agreement with previous observations showing the reduction of cell migration/invasion through Akt inhibition [46-47], we observed that perifosine and its combination with gemcitabine markedly reduced cell migration and invasion in PDAC cells. Several classes of proteins are involved in this invasive behavior, including cell-cell adhesion molecules like members of immunoglobulin and calcium-dependent cadherin families and integrins. In line with previous evidence on inverse relationship between Akt
and E-cadherin expression [34], our results demonstrated that perifosine increased the expression of E-cadherin in LPC028 and CFPAC-1.

Since the Akt signaling pathway plays an important role in cell survival process, its blockage can result in activation of programmed cell death [11]. Thus, we further evaluated the effect of perifosine on cell cycle perturbation and apoptosis induction. In agreement with previous findings on the effect of perifosine on cell cycle perturbation at the G1-S boundary [47-48], our results showed that perifosine increased the percentage of cells in the S phase, potentially favoring the cytotoxic activity of gemcitabine. This modulation of the cell cycle was associated with significant induction of apoptosis, as determined by analysis of sub-G1, mitochondria membrane potential and Annexin-V/PI. In order to investigate the mechanisms underlying the activation of programmed cell death, we checked the modulation of several factors involved in the apoptotic cascades. Previous studies showed that drug-induced Akt deactivation was associated with activation of proapoptotic factors, including caspase-9 and BAD, as well as with a parallel decrease in the expression of the antiapoptotic factors Bcl-2 and NF-kB [36,49-50]. Our studies showed similar results after exposure of the PDAC cells to perifosine.

In conclusion, our data provide novel insights into the antitumor activity of the novel Akt inhibitor perifosine in PDAC cells, showing its ability to attack key mechanisms involved in the proliferation, cell cycle control, apoptosis and migration/invasion properties of PDAC cells. The synergistic results were observed only in cells with Akt activation and may have critical implications for the rational development of the perifosine/gemcitabine combination, supporting further studies on biomarkers to guide this novel therapeutic approach. Identification and selection of patients with the highest likelihood of responding will indeed be crucial for the application of this regimen for the treatment of PDAC.
References


Biophys Acta 1998;1414:238-48