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Targeting PAM signaling pathway in the treatment of pancreatic ductal adenocarcinoma

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Abstract

Oncogenic KRAS signaling is the main driving force behind pancreatic ductal adenocarcinoma (PDAC); however, targeting this pathway has proven to be difficult. Conversely, the PI3K/Akt pathway represents an exciting new target, because it has been associated with poor prognosis and chemoresistance, and several inhibitors are under development. In particular, perifosine prevents Akt traslocation to the cell membrane, while MK-2206 is an Akt allosteric inhibitor and BEZ-235 is a dual PI3K/mTOR inhibitor. Therefore, we investigated the prognostic role of phospho (p)-Akt1 in PDAC tissues, as well as the molecular mechanisms underlying the interaction of Akt inhibitors with gemcitabine, using PDAC cells, primary cultures and spheroids.

Immunohistochemistry of tissue microarrays with specimens from radically-resected patients (n=100) revealed a correlation between high p-Akt1 expression and worse outcome. Patients with low p-Akt1 expression (as detected by digital scoring) had a median overall survival (OS) of 16.2 months (95% CI, 14.8-20.1), while patients with high expression had a median OS of 12.0 months (95% CI, 9.0-14.9, P=0.03). Parallel immunocytochemistry studies revealed high expression levels in LPC028 primary cells, while LPC006 where characterized by low p-Akt1.

Akt inhibitors reduced cancer cell growth in monolayers and spheroids, and synergistically enhanced the antiproliferative activity of gemcitabine in LPC028 (e.g., combination index CI of 0.2, in the gemcitabine-perifosine combination for 72h, at fixed IC50 ratio)., while this combination was antagonistic in LPC006 cells. The synergistic effect was paralleled by a 5-fold reduction in the expression of the main gemcitabine target ribonucleotide reductase. Inhibition of Akt decreased cell migration and invasion, which was additionally reduced by the combination with

gemcitabine. However, the combination of Akt inhibitors with gemcitabine increased apoptosis, associated with induction of caspasi-3/6/8/9, PARP and BAD, and inhibition of Bcl-2 and NF-KB in LPC028, but not in LPC006 cells.

The Akt signaling is involved in the expression/localization of the key glucose transporter Glut1, and increased glucose metabolism was associated to resistance to axitinib (Hudson et al., Cell Death Dis, 2014). Remarkably, the resistant LPC006 cells were characterized by overexpression of Glut1, which was not reduced after exposure to Akt inhibitors. However, the novel Glut1 inhibitor PGL-13 enhanced perifosine and perifosine/gemcitabine-induced cell death.

In conclusion, our findings support the analysis of phosphor-Akt1 expression as both a prognostic and predictive biomarker, for the rationale development of new combination therapies targeting the Akt pathway in PDAC. Finally, inhibition of Glut1 might overcome resistance to these therapies and warrants further studies.

Introduction

PI3K/AKT/mTOR SIGNALING PATHWAY

PI3K/Akt/mTOR (PAM) pathway is often altered in patients with cancer. This pathway controls several biological activities within the cell, and its activation is one of the fundamental downstream molecular events following tyrosine kinase growth factor receptor activation [1,2,3].

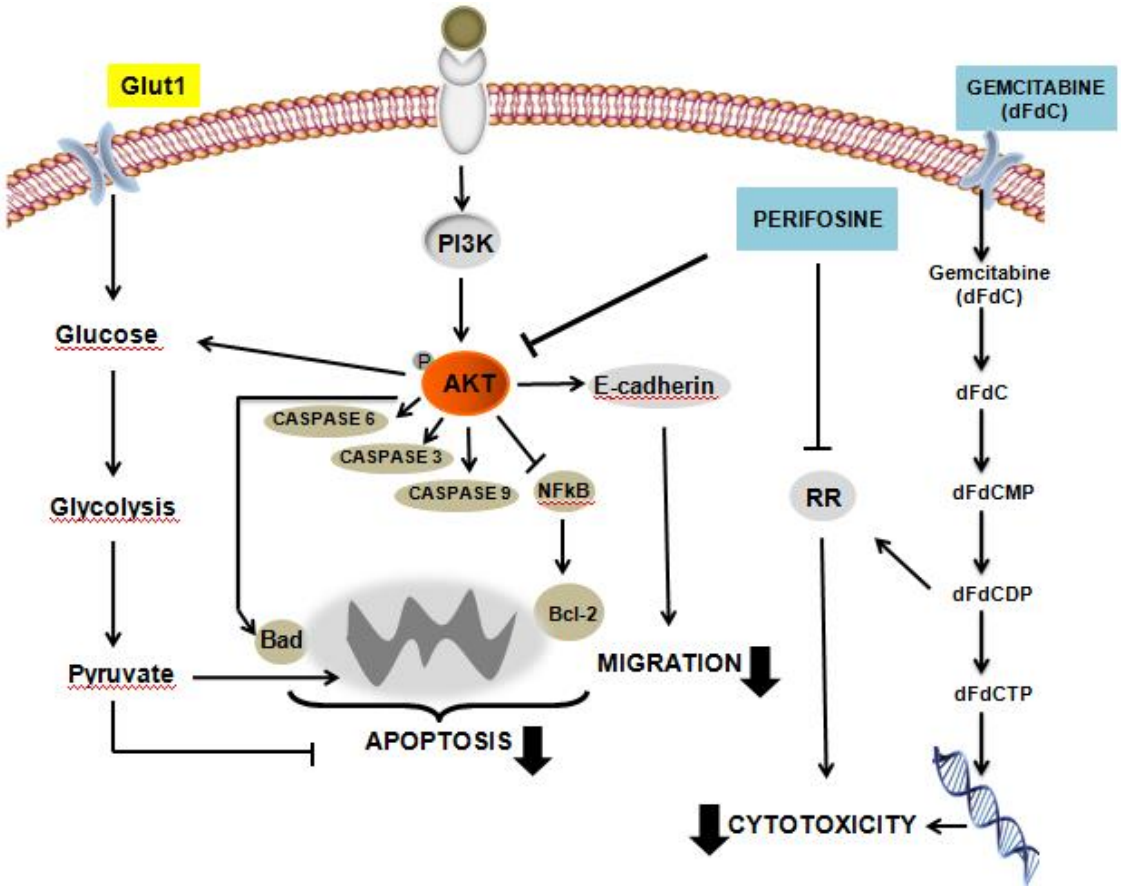

FIGURE 1: Activation mechanism of PI3K/AKT/mTOR signaling pathway

The first event is the activation of PI3K; The PI3Ks, a family of lipid kinases, can be divided into three classes according to the structure, mode of regulation and lipid substrate specificity, of which the class I PI3K is related to cancer [4].

Within class IA, the genes PIK3CA, PIK3CB, and PI3KCD, encode the homologous p110α, p110β, and p110δ isozymes respectively. Class IB consists of PIK3CG, which encodes p110γ. p110α and p110β are ubiquitously expressed while the expression of p110δ and p110γ is generally restricted to hematopoietic and immune cells. Class IA PI3Ks are heterodimeric proteins made up of a p110 catalytic subunit and a p85 regulatory subunit, and are involved in carcinogenesis.

PI3K is activated upstream by the binding of a growth factor or ligand to its cognate growth factor receptor tyrosine kinases (RTKs), which include members of the human epidermal growth factor receptor (HER) family, and the insulin and insulin-like growth factor 1 (IGF-1) receptor, among others [5]. PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3), which in turn leads to phosphorylation of Akt, a serine/threonine kinase. PIP3 acts as a docking site for AKT, which is the central mediator of the PI3K pathway and phosphoinositide-dependent kinase 1 (PDK1). Phosphorylation of AKT stimulates protein synthesis and cell growth by activating mTOR via effects on the intermediary tuberous sclerosis 1/2 complex (TSC1/2) [6,7].

Phosphatase and tensin homologue deleted on chromosome ten (PTEN) is a tumor suppressor, which has inhibitory effects on the pathway by dephosphorylating PIP3 to PIP2 [8]. PIP3 levels are hence closely regulated by the opposing activities of PTEN and PI3K [9]. The role of inositol polyphosphate 4-phosphatase type II (INPP4B), another tumor suppressor, is increasingly recognized. INPP4B is also involved in dephosphorylation of PIP3 to PIP2 [10].

mTOR, a serine/threonine protein kinase, is a downstream effector of PI3K and Akt. It comprises two different complexes, mTOR complex 1 (mTORC1) and mTOR

complex 2 (mTORC2), which are structurally similar but functionally different. mTORC1 is the target of rapamycin and rapamycin analogues, such as everolimus, and leads to cell anabolic growth by promoting mRNA translocation and protein synthesis [11] and also has roles in glucose metabolism and lipid synthesis. Its downstream substrate S6 kinase 1 can phosphorylate the activation function domain 1 of the ER, which is responsible for ligand-independent receptor activation [12]. mTORC2 on the other hand, organizes the cellular actin cytoskeleton and regulates AKT phosphorylation. Rapalogues exert their effect mainly on mTORC1 and the incomplete inhibition can lead to feedback loops causing paradoxical activation of Akt and proliferative effects via other downstream targets.

PAM PATHWAY IN PANCREATIC DUCTAL ADENOCARCINOMA

In pancreatic ductal adenocarcinoma (PDAC) PAM pathway plays a crucial role because deregulation of components involved in this pathway could confer resistance to chemotherapy, while blockage of Akt signaling results in programmed cell death and inhibition of tumor growth. Activation of Akt is a frequent event in PDAC and has been correlated to its poor prognosis [13,14].

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 [15].

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 [16].

PANCREATIC DUCTAL ADENOCARCINOMA

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal solid tumors. Despite extensive preclinical and clinical research, the prognosis of this disease has not significantly improved, with a 5-year survival rate around 7% [17].

This dismal outcome can partially be explained by the lack of biomarkers for screening and diagnosis at earlier stages, and by the resistance to most currently available chemotherapy regimens. This resistance has been attributed to both the desmoplastic tumor microenvironment and to the strong inter- and intra-tumor heterogeneity in terms of complexity of genetic aberrations and the resulting signaling pathway activities, as well as to resistance mechanisms that quickly adapt the tumor to drugs [18].

Therefore, there is an urgent need to better understand the molecular pathology of PC in order to improve patient selection for current treatment options, and to develop novel therapeutic strategies. Genomic analyses of pancreatic cancer reveal a complex mutational landscape with four common oncogenic events in well-known cancer genes (KRAS, TP53, SMAD4 and CDKN2A), among of genes mutated at low prevalence. Despite this heterogeneity, oncogenic point mutations of individual genes aggregate into core molecular pathways including DNA damage repair, cell cycle regulation, TGF-β signalling, chromatin regulation and axonal guidance [19,20]. Increasingly sophisticated analyses are revealing biologically important events with clinical significance, including whole-genome sequencing, which sub-classifies PC into 4 on the basis of the differential expression of transcription factors and downstream targets important in lineage specification and differentiation during pancreas development and regeneration:

- ✓ Squamous
- ✓ Pancreatic progenitor
- ✓ Immunogenic
- ✓ Aberrantly differentiated endocrine exocrine

AKT INHIBITORS

Several inhibitors of Akt are under investigation, but three are the farthest along and showed the most promise in early clinical research: the pan-Akt and PI3K inhibitor perifosine (KRX-0401, Aeterna Zentaris/Keryx), the allosteric pan-Akt inhibitor MK-2206 (Merck), and the dual PI3K/mTOR inhibitor dactolisib (NVP-BEZ235, Novartis).

In particular, the synthetic oral alkylphospholipid perifosine [21,22] has been evaluated in clinical trials for several tumors, including colon, breast, head and neck and prostate cancer [23,24,25,26]. Unfortunately, it failed the phase III clinical trials for treatment of colon cancer and relapsed refractory multiple myeloma (www.clinicaltrials.gov). These failures together with the disappointing response rates to perifosine as a single agent in most solid tumors, including PDAC, prompt further studies into its mechanism of action [27] as well as on synergistic combinations.

Perifosine prevents translocation of Akt to the cell membrane by blocking the pleckstrin homology (PH) domain of Akt [28] 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* [29].

Recently, Pinton and collaborators showed that perifosine inhibited cell growth of malignant pleural mesothelioma cells by affecting EGFR and c-Met phosphorylation [30]. Another study showed that perifosine decreased the *AEG-1* gene expression along with inhibition of Akt/GSK3/c-Myc signaling pathway in gastric cancer [31]. Perifosine and curcumin synergistically increased the intracellular level of reactive oxygen species and ceramide, and downregulated the expression of cyclin-D1 and Bcl-2 in colorectal cancer cells [32].

Finally, perifosine also inhibits the anti-apoptotic mitogen-activated protein kinase (MAPK) pathway and modulates the balance between the MAPK and pro-apoptotic stress-activated protein kinase (SAPK/JNK) pathways, thereby inducing apoptosis [33].

Aim of the Thesis

The aim of my project is to investigate the expression of phospho-Akt1 in PDAC tissues and cells, and to evaluate the effects of growth inhibition by Akt inhibitors, using PDAC cell lines and primary cultures growing as monolayer or as spheroids. Moreover, characterize several key factors, affecting cell cycle perturbation, apoptosis induction, as well as inhibition of cell migration and invasion and modulation of key factors in glucose metabolism in PDAC cells exposed to perifosine and perifosine/gemcitabine combination.

Materials and Methods

DRUGS AND CHEMICALS

Perifosine was provided by Æterna Zentaris Inc. (Frankfurt am Main, Germany), NVP-BEZ235 was purchased from Selleck Chemicals (Houston, TX) while gemcitabine and MK-2206 were generous gifts from Eli-Lilly (Indianapolis, IN), and Merck (Whitehouse Station, NJ), respectively. The drugs were dissolved in Dimethyl sulfoxide (DMSO) 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

Seven 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 [28]. 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.

GROWTH INHIBITION STUDIES

The cell growth inhibitory effects of perifosine, MK-2206 and NVP-BEZ235 were evaluated in the PANC-1 and LPC028 cells. Further studies evaluated perifosine and gemcitabine combination 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.

TISSUE MICROARRAYS (TMAS), IMMUNOHISTOCHEMISTRY (IHC) AND IMMUNOCYTOCHEMISTRY (ICC)

Phospho-Akt protein expression was evaluated in slides from four formalin-fixed, paraffin-embedded PDAC-specific TMAs build with neoplastic cores from a cohort of radically-resected patients (n=100), using the TMA Grand Master (3DHistec, Budapest, Hungary) instrument, and stained according to standard procedures with the 587F11 rabbit monoclonal antibody (1:50 dilution; Cell-Signaling, Beverly, MA). Visualization was obtained with BenchMark Special Stain Automation system (Ventana Medical Systems, Tucson, AZ). Two pathologists reviewed all the slides, assessing the amount of tumor and tissue loss, background staining and overall interpretability before the phospho-Akt reactivity evaluation. Staining results were evaluated using a computerized high-resolution acquisition system (D-Sight, Menarini, Florence, Italy), including the analysis of positive cells number and staining intensity which resulted in values expressed as arbitrary units" (a.u.). All patients have provided a written in- formed consent. This study was approved by the

Local Ethics Committee of the University of Pisa. Date of approval: July 3, 2013 (file number 3909).

For 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 using a digital system based on staining intensity and on the number of positively stained cells.

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). 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), E-cadherin*, and the *glucose transporter 1* (*SLC2A1*/*Glut1*) which were obtained from Applied Biosystems TaqMan Gene expression products (Hs00920503_m1, Hs01085706_m1,

Hs00984403_m1, Hs01040726_m1, Hs00156401_m1, Hs00168784_m1, Hs01072069_g1, Hs01023894_m1, and Hs00892681_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).

WESTERN BLOTTING (WB)

In order to evaluate the modulation of Akt, phospho-Akt, PARP, BAD, Bcl-2, NF-kB and Glut1 protein expression in PDAC cells treated for 24 hours with perifosine, gemcitabine and their combination, Western blot analyses were executed as described previously [30]. 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 the rabbit anti-Glut-1 (ab652, 1:500, diluted in the blocking solution, from Abcam, Cambridge, UK) 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.

EVALUATION OF SYNERGISTIC/ANTAGONISTIC INTERACTION WITH GEMCITABINE

The pharmacological interaction between perifosine and gemcitabine was evaluated by the median drug effect analysis method. 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).

EFFECTS ON MULTICELLULAR SPHEROIDS

LPC006 and LPC028 spheroids were established by seeding 10^4 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: $V=(4/3)\times\pi\ (D/2)3$.

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 2 fold greater than Western blotting. The levels of Akt and phospho-Akt were measured in cells seeded in a 96 well-plate at a density of 10^5 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. 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 10^5 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, LPC028, 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. 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 FACSCalibu. Additional studies were performed with the Annexin-V/PI assay, plating the cells in 6-well plates at a density of 1.5×10^5. 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 $CaCl_2$). 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, 10^6 LPC006, LPC028, CFPAC-1 and PANC-1 cells were exposed to the drugs for 24 hours at their IC50s. Fluorescence was measured at 350 nm excitation and 460 nm emission (Spectrafluor Tecan, Salzburg, Austria). Relative caspase activity was normalized with respect to the untreated cells.

ANALYSIS OF MODULATION OF GLUT1 BY FLOW CYTOMETRY

To quantitatively detect the expression of membrane-bound Glut1, cells were fixed with 80% ethanol, incubated with anti-Glut1 antibody (Abcam), and then stained with the appropriate FITC-conjugated anti-rabbit IgG antibody (BD Pharmingen™, BD Biosciences, San Jose, CA). Quantification of FITC fluorescence intensity was performed using a FACSCanto flow cytometer (BD Biosciences).

EVALUATION OF THE CYTOTOXIC AND PRO-APOPTOTIC EFFECTS INHIBITION OF GLUT1 INHIBITION COMBINED WITH AKT INHIBITORS

The Akt signaling is involved in the modulation of Glut1 expression/localization, and a recent study showed that increased glucose metabolism was associated to resistance to the tyrosine kinase inhibitor axitinib, and this resistance was overcame by Glut1 silencing. Therefore, we performed additional cytotoxicity studies using the novel Glut1 inhibitor PGL13. This compound was tested in the LPC006 cells, at a concentration of 30 μM, which effectively reduced glucose influx. The cells were exposed to PGL13 for 72 hours, alone or in combination with IC50 concentration values of perifosine, gemcitabine, and their combination. Cell growth inhibition was then assessed by counting the cells after staining with trypan blue, in comparison to untreated cells. Parallel evaluation of apoptosis induction was performed by fluorescence microscopy with bisbenzimide staining.

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) and progression-free-survival (PFS) were calculated from the date of pathological diagnosis (i.e. the date of surgery) to the date of death and tumor progression, respectively. OS and PFS 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 PHOSPHO-AKT AND AKT1 MRNA EXPRESSION IN PDAC TISSUES AND CELLS

The protein expression of phospho-Akt was successfully evaluated by IHC in 100 human PDACs collected in two TMAs. The main clinical characteristics of these patients are reported in the Table 1.

Table 1. Outcome according to clinical characteristics in the 100 PDAC patients enrolled in the present study

Characteristics		**N (=%)**	**OS months (95% CI)**	***P***
No. Patients	All	100	14.0 (12.1-15.8)	
Age, years	≤65	43	15.2 (13.3-16.8)	*0.361*
	>65	57	14.1 (11.1-17.0)	
Sex	Male	47	13.0 (11.1-14.9)	*0.814*
	Female	53	15.0 (11.9-18.1)	
Resection status	R0	56	15.2 (12.3-21.6)	*0.474*
	R1	44	13.5 (11.2-31.0)	
Lymph node	No	10	18.5 (7.6-32.4)	*0.521*
	Yes	90	14.2 (12.2-15.9)	
Grading	1-2	36	15.5 (11.5-18.1)	*0.097*
	3	64	12.1 (8.4-15.8)	

IHC has showed a variable protein expression with some specimens characterized by a strong and diffuse staining, while other tissues had only a few scattered positive cells with a weak staining, as respectively exemplified by the middle and left panels in figure2.

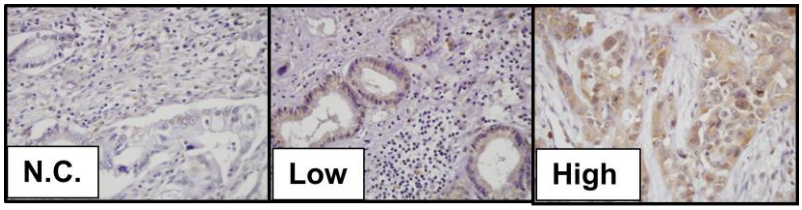

Figure 2: Representative examples (original magnification, 40X) showing the variable expression of phospho-Akt in paraffin-embedded PDAC samples collected in 4 TMAs (with 4 cores for each of the 100 patients). N.C., negative control.

Patients were categorized according to their high vs. low phospho-Akt expression compared to the median value (30 a.u.) calculated by digital scoring (Fig.3, black line).

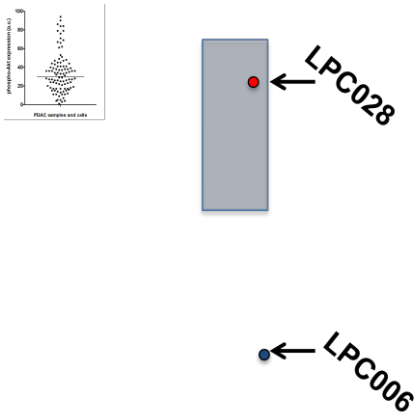

Figure 3: Expression values of phospho-Akt observed across the cohort of PDAC patients, obtained by digital quantification. Phospho-Akt showed positive cytoplasmatic and nuclear staining in most tissue sections, with intense staining in 14 out of 100 samples. The staining intensities of the LPC028 and LPC006 cells were included in the "very high" and "low" Akt expression groups, respectively

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 had a median OS of 16.2 months (95%CI, 14.8-20.1), while patients with a high expression had a median OS of 12.0 months (95%CI, 9.0-14.9, P=0.03, fig 4).

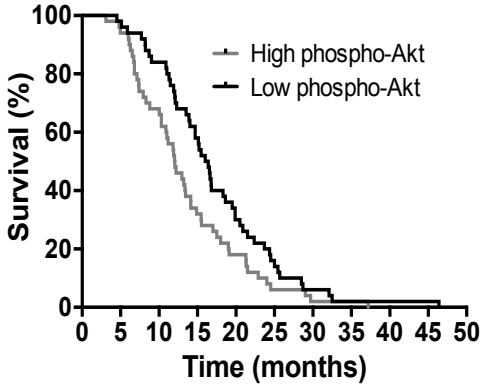

Figure 4: Kaplan–Meier survival curves according to the expression of phospho-Akt in 100 radically-resected PDACs showing that patients with high expression of phospho-Akt had a significantly shorter survival compared to patients with low phospho-Akt expression

However, only a trend toward a significant association was found between phospho-Akt expression and PFS (P=0.08, Fig.5).

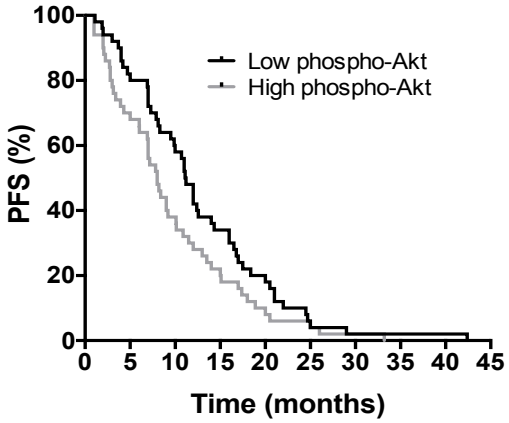

Figure 5: Kaplan-Meier PFS curves according to the expression of phospho-Akt in 100 radically-resected PDACs

An additional analysis was performed categorizing the patients with respect to a threshold expression of 57 a.u., which identified 14 cases with higher expression compared to all the others (defined as “very-high” phospho-Akt expression, Fig.3, blue square).

Using these categories, we observed a significant correlation between high phospho-Akt protein expression and both significantly shorter OS ($P<0.01$, fig. 6), and PFS (fig. 7).

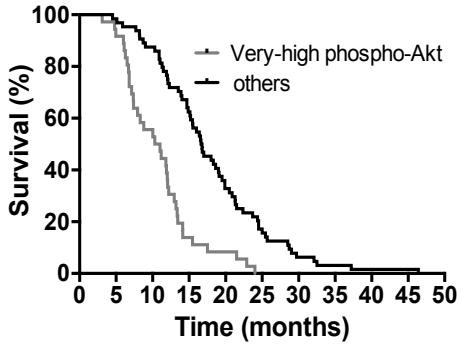

Figure 6: Kaplan–Meier survival curves according to the expression of phospho-Akt in 100 radically-resected PDACs, showing that patients with "very high" expression of phospho-Akt had a significantly shorter survival compared to patients with low phospho-Akt expression

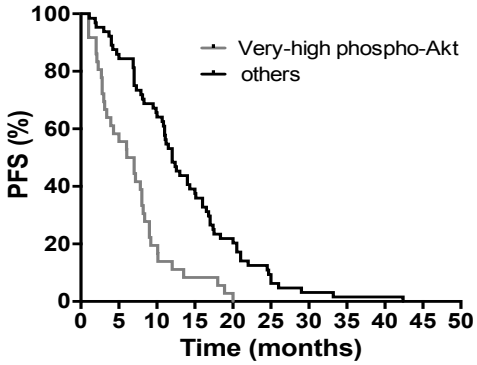

Figure 7: Kaplan-Meier PFS curves according to the expression of phospho-Akt in 100 radically-resected PDACs, showing that patients with "very high" expression had a significantly worste a PFS compared to patients with low phospho-Akt expression.

Parallel ICC studies revealed that the LPC006 cells had a significantly lower phospho-Akt expression compared to LPC028 cells, which were indeed included in the category of "low" and "very-high" phospho-Akt expression, respectively (Fig.3, blue and red circles). 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 0.9 arbitrary unit (a.u.) in LPC006 cells to 24.0 a.u. in LPC028 and PANC-1 cells (Fig. 8).

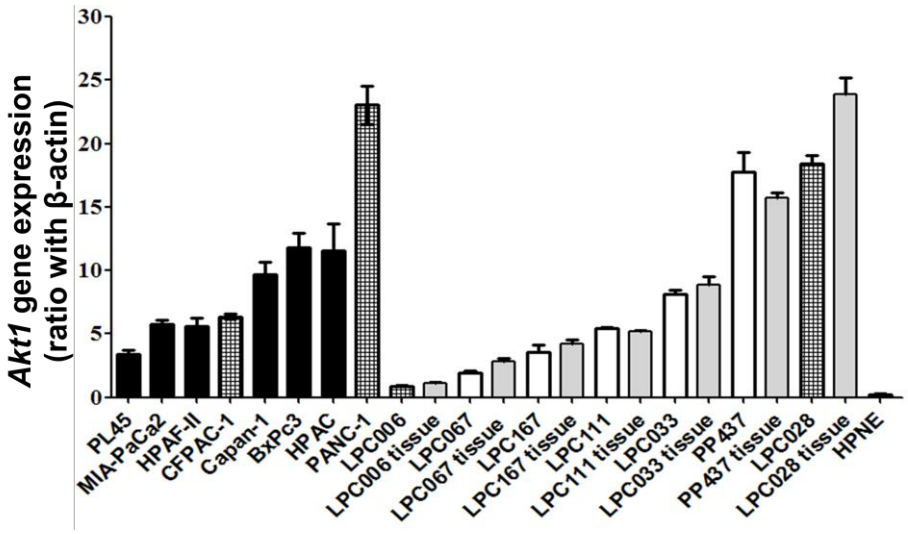

Figure 8: *Akt1* mRNA expression in ATCC cell lines (black-bars), primary tumor cultures (white-bars), and their originator tissues (gray-bars). Dashed bars identify the cells that were selected for further *in vitro* studies

The mean and median expression in the tumor cells (8.7±0.2 and 8.4 a.u., respectively) were significantly higher ($P<0.01$) than the expression detected in hTERT-HPNE cells (0.3 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 ($R^2>0.9$, $P<0.05$), suggesting that these cells represent optimal preclinical models for our pharmacological studies. Moreover, Western blot analysis revealed that the LPC006 and CFPAC-1 cells had a lower Akt and phospho-Akt expression compared to PANC-1 and LPC028 cells (fig.9).

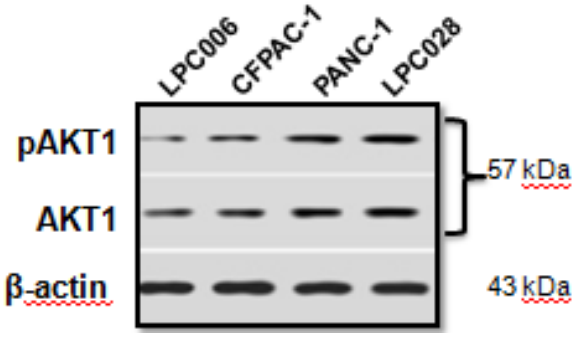

Figure 9: Representative Western blot pictures of phospho-Akt and Akt expression in LPC006, CFPAC-1, PANC-1 and LPC028 cells

Therefore, we selected for further studies two primary cell cultures (LPC006 and LPC028) which were representative of "low" and "very-high" expression values, as well as two cell lines, PANC-1 and CFPAC-1, with high and intermediate expression values of Akt1 mRNA, respectively.

PERIFOSINE INHIBITS CELL GROWTH AND INTERACTS SYNERGISTICALLY WITH GEMCITABINE IN PDAC CELLS WITH HIGH EXPRESSION OF PHOSPHO-AKT

The cytotoxic activity of three different Akt inhibitors (perifosine, MK-2206, and NVP-BEZ235) was evaluated in the PANC-1 cell line (fig.10).

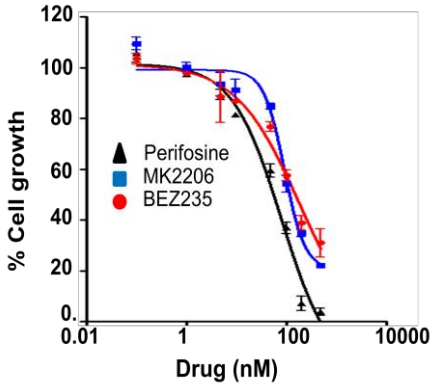

Figure 10: Growth inhibitory effects in PANC-1 cells after 72 h exposure to perifosine, MK-2206 and NVP-BEZ235

All these compounds caused a concentration-dependent inhibition of proliferation, with IC50 values ranging from 5.1 μM (perifosine) to 15.8 μM (NVP-BEZ235).

Higher IC50 values were obtained in the LPC006 cells, with IC50 of 22.5, 31.7 and 45.5 μM for perifosine, NVP-BEZ235 and MK-2206, respectively. According to the lowest IC50 values detected in these assays we selected perifosine for the following studies on the pharmacological interaction of Akt inhibitors with gemcitabine. The cell growth inhibitory effects of perifosine, gemcitabine and their combination in LPC028 and LPC006 cells are shown in figure 11, while the data for CFPAC-1 and PANC-1 are reported in the Supplemental figure 12.

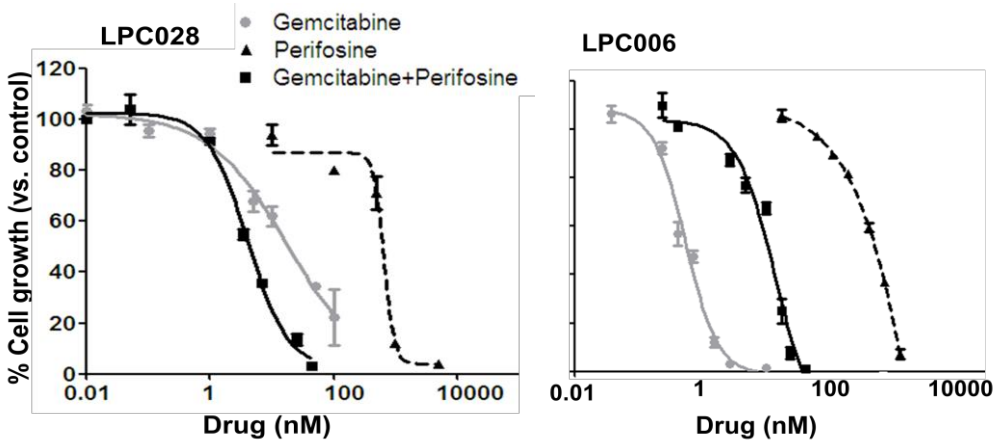

Figure 11: Growth inhibitory effects after 72 hours exposure to perifosine, gemcitabine or their combination at a fixed ratio based on IC50 values in LPC028 and LPC006 cells. On the X-axis the drug concentrations for the combination are referred to gemcitabine

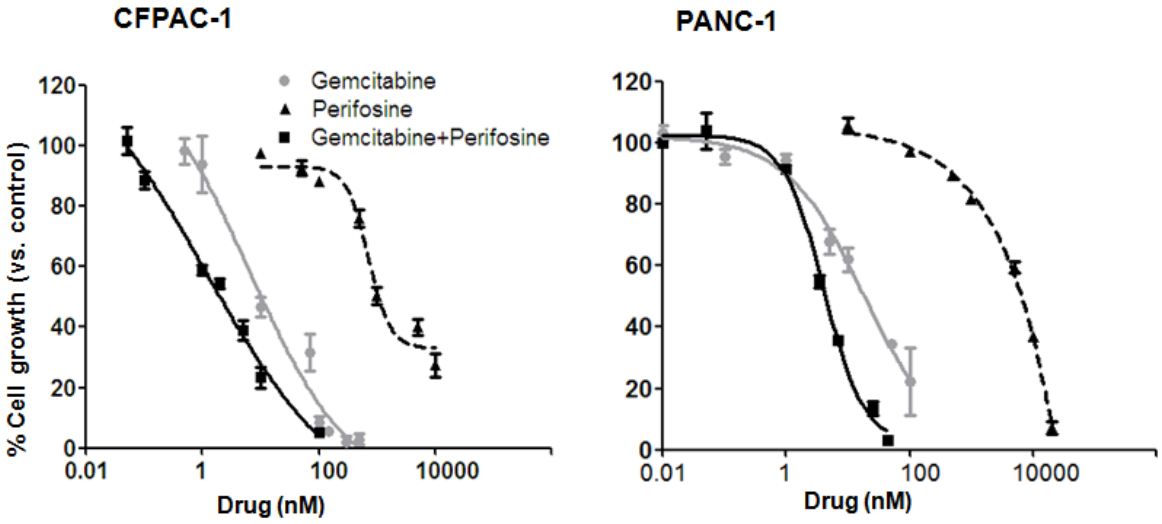

Figure 12: Growth inhibitory effects after 72 hours exposure to perifosine, gemcitabine or their combination at a fixed ratio based on IC50 values in CFPAC-1 and PANC-1 cells. On the X-axis the drug concentrations for the combination are referred to gemcitabine

Since the CI method recommends a ratio of concentrations at which drugs are equipotent, combination studies were performed using fixed ratios with IC values at IC50s. Perifosine enhanced the antiproliferative activity of gemcitabine, especially in the LPC028 and PANC-1 cells, by decreasing the IC50s of gemcitabine from 4.3±1.1 nM and 17.2±2.1 nM to 1.4±0.5 nM and 4.0±1.1 nM, respectively. The median drug-effect analysis revealed a slight-to-moderate synergism in CFPAC-1, and a strong synergism in the PANC-1 and LPC028 cells, with CI values of 0.8, 0.5 and 0.2, respectively (fig.13).

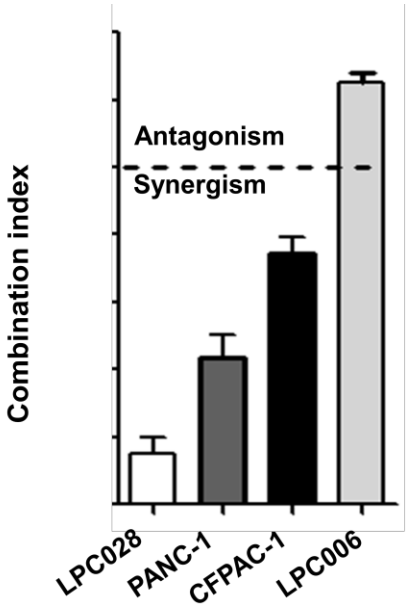

Figure 13: Mean CI of the perifosine/gemcitabine combination. CI values at FA of 0.5, 0.75 and 0.9 were averaged for each experiment, and this value was used to calculate the mean between experiments

Conversely, the combination of perifosine and gemcitabine was antagonistic in the LPC006 cells (CI>1.2). To evaluate whether these effects were observed also in three-dimensional (3-D) models and 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 3-D culture models are generally more chemo-/radio-resistant than two-dimensional monolayer cell cultures, supporting their use for drug testing [34]. 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, characterized by 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 14-15).

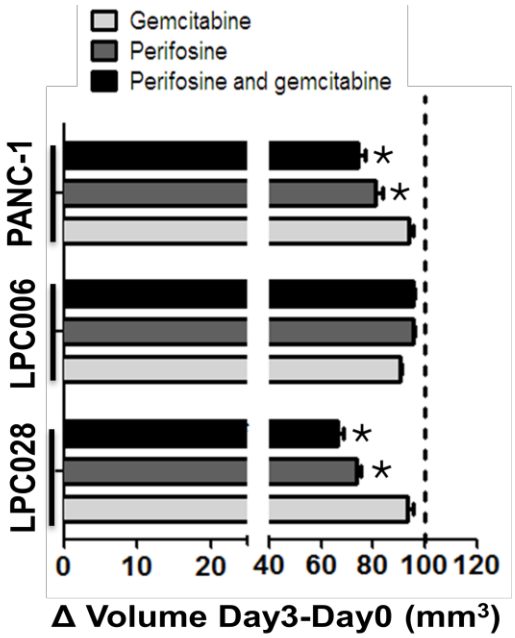

Figure 14: Effect of perifosine and gemcitabine and their combination, at IC50 values, on the volumes of PDAC spheroids after 72 hours exposure

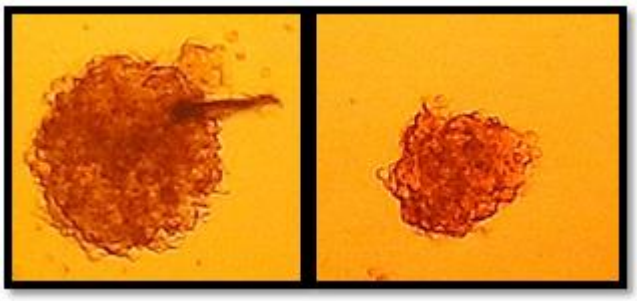

Figure 15: Representative images of untreated spheroid versus spheroid treated with perifosine and gemcitabine (original magnification, 40X)

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 [35], 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 figure 16, perifosine significantly reduced the expression of p-Akt in LPC028, CFPAC-1 and PANC-1 cells (e.g 40%, 25%, and 30% reduction, respectively). Similarly, the combinations of perifosine and gemcitabine markedly suppressed Akt phosphorylation, with a degree of inhibition ranging from -35% (CFPAC-1 cells) to -45% (LPC028 cells). Conversely, phospho-Akt levels were not affected by perifosine, gemcitabine or their combination in the LPC006 cells (fig.16).

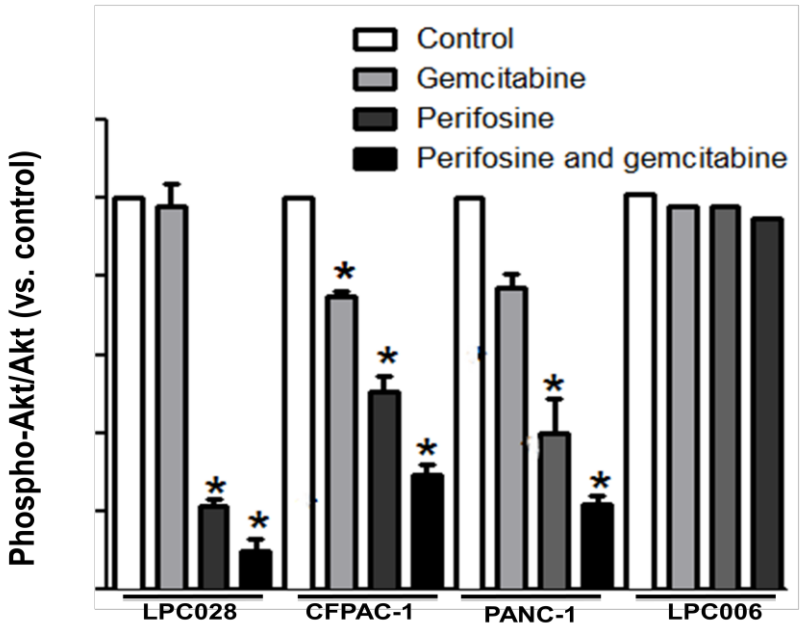

Figure 16: Standard curves for pAKT (pS473) and total AKT obtained using a specific ELISA assay

RRM1 and *RRM2* encode for the catalytic and the regulatory subunits of ribonucleotide reductase and is a key molecular target of gemcitabine [36].

Previous studies demonstrated that the expression of *RRM2* is modulated by the Akt/c- MYC pathway [37]. 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 or increased CDA expression). Therefore, we evaluated the mRNA expression of several gemcitabine determinants in the LPC006 and LPC028 cells. 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 (fig.17).

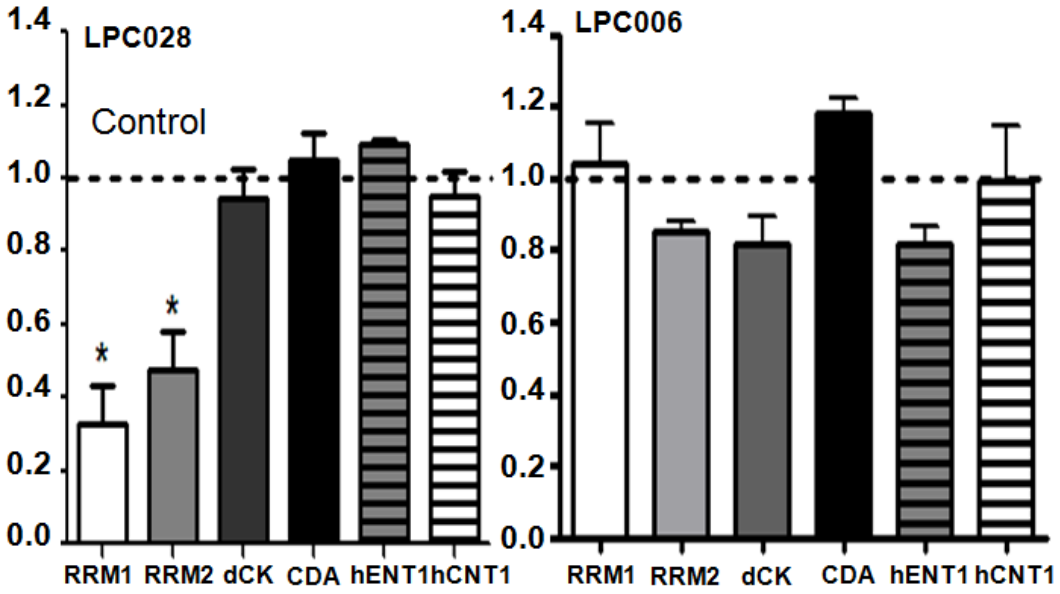

Figure 17: Expression of gemcitabine key determinants in LPC028 (left panel) and LPC006 (right panel) cells treated with perifosine at IC50 versus untreated cells, as determined by qRT-PCR

These results can at least in part explain the synergistic interaction 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, LPC006, CFPAC-1 (fig.18) and PANC-1 (fig.19).

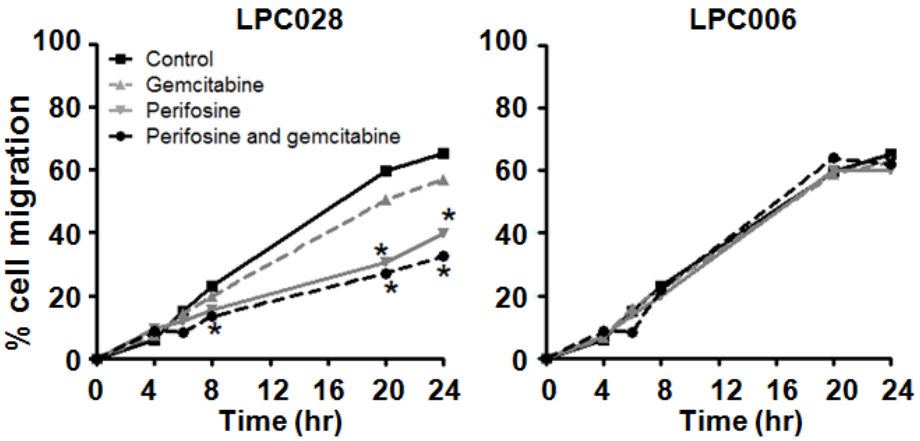

Figure 18: Results of wound-healing assay in LPC028 and LPC006 cells exposed to perifosine, gemcitabine or to their combination, at IC50 values for 24 hours

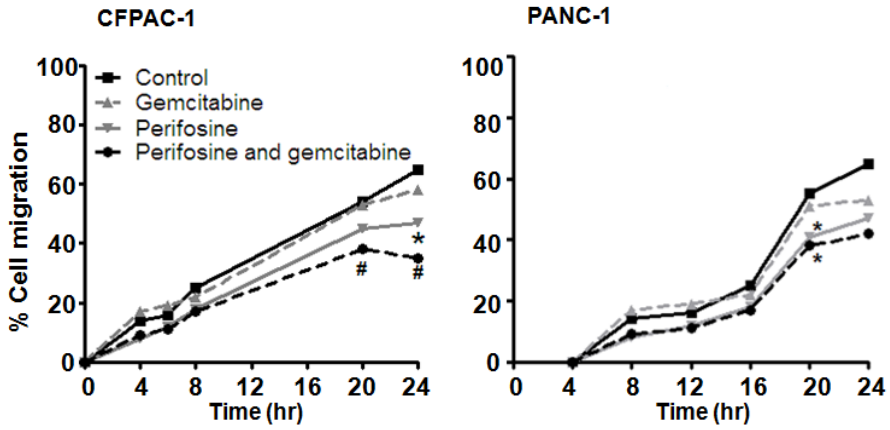

Figure 19: Results of wound-healing assay in CFPAC-1 and PANC-1 cells exposed to perifosine, gemcitabine or to their combination, at IC50 values for 24 hours

LPC028 showed a significant reduction of migration starting after 8 hours exposure to perifosine with a reduction of the scratch-area of about 50%, and the perifosine/gemcitabine combination additionally reduced cell migration ($P<0.05$, fig.19 left panel), while gemcitabine alone did not affect cell migration. No modulation of cell migration was observed in the LPC006 cells (fig.19 right panel).

LPC028, CFPAC-1 and PANC-1 cells treated with perifosine showed also a significantly reduced invasive potential, compared to untreated cells (fig. 20).

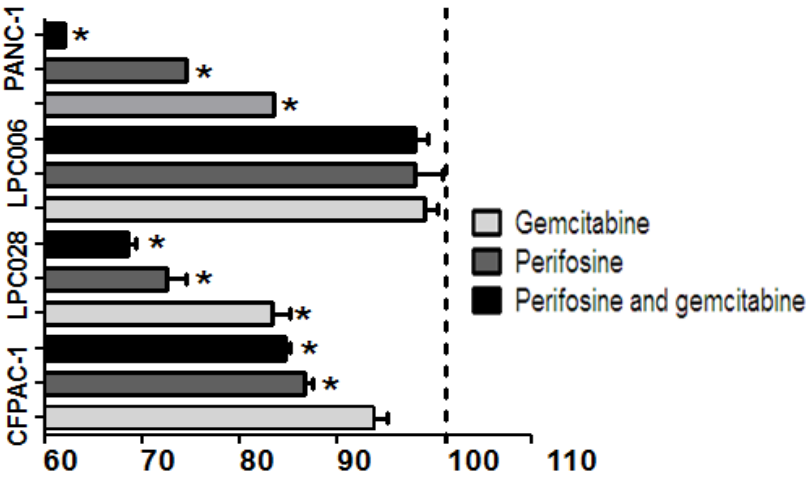

Figure 20: Results of invasion studies in the PDAC cells exposed for 24 hours to perifosine, gemcitabine or to their combination, at IC50 values (insert: representative pictures of LPC028 cells at 24 hours, original magnification 40X

In particular, the perifosine/gemcitabine combination was more effective in inhibiting invasion than perifosine-alone in LPC028 and PANC-1 cells, as shown by the significantly lower number of invading cells with Giemsa's stain. However, no modulation of cell invasion was observed in the LPC006 cells.

Since previous studies suggested that Akt signaling pathway suppressed E-cadherin expression [38], 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 in LPC028, CFPAC-1 and PANC-1 ($P<0.05$), while no changes were detected in LPC006 cells. Similarly, immunocytochemistry analysis in 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 the table 2.

Supplemental Table 1. Effects of gemcitabine and perifosine and their combination on cell cycle distribution and on cell death (Sub-G1)

	Treatment	G0/G1 Phase (%)	S Phase (%)	G2/M Phase (%)	Sub-G1
LPC0028	Control	54.1±1.5	18.7±1.7	27.1±2.0	1.9±0.1
	Gemcitabine	44.4±3.4	18.8±2.5	36.9±3.3	2.6±0.2
	Perifosine	51.2±1.8	26.1±1.4	29.7±1.7	9.3±1.1*
	Gemcitabine + Perifosine	36.9±0.9	48.9±0.6	30.2±2.1	17.6±1.2*
CFPAC-1	Control	57.5±1.2	9.2±1.3	33.3±1	1.9±0.1
	Gemcitabine	43.2±1.4	24.6±1.1	32.2±1.4	4.8±0.5
	Perifosine	16.3±0.8	59.1±1.4	24.6±0.3	9.2±1.1*
	Gemcitabine + Perifosine	11.9±2.2	41.2±2.7	28.8±1.2	15.8±1.2*
LPC006	Control	52.4±2.3	25.8±1.9	21.8±0.3	2.1±0.6
	Gemcitabine	38.3±1.5	30.1±2.1	31.6±1.9	11.9±0.8*
	Perifosine	55.0±2.1	22.3±1.9	22.6±1	4.3±1.1
	Gemcitabine + Perifosine	57.1±1.8	24.5±2.6	18.4±0.6	10.7±2.3*
PANC-1	Control	55.2±0.9	17.7±1.3	27.2±2.2	3.2±0.4
	Gemcitabine	46.4±2.8	17.8±2.8	35.9±5.6	8.6±1.2*
	Perifosine	51.0±2.4	23.1±1.9	25.9±1.1	10.3±1.2*
	Gemcitabine + Perifosine	32.2±0.8	47.0±2.1	30.8±2.1	18.9±3.2*

Note. Cells were exposed to IC50s values of gemcitabine, perifosine and their combination, for 72 hours. *Statistically significant compared to control (P<0.05)

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%. Comparable perturbations of cell cycle were observed in the CFPAC-1 and PANC-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 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 2). 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).

Furthermore, we evaluated the variation of mitochondrial membrane potential in LPC028, LPC006, PANC-1 and CFPAC-1. As shown in Figure 21, the combination perifosine gemcitabine causes an increase of mitochondrial membrane potential in LPC028, PANC-1 and CFPAC-1 cells.

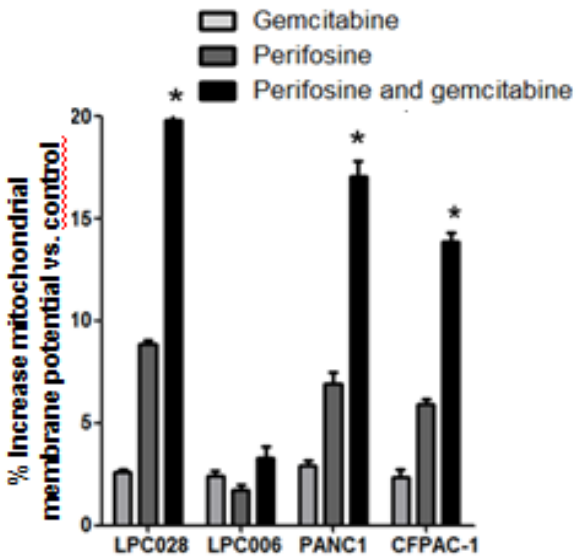

Figure 21: Mitochondrial membrane potential (as assessed by (DiOC) labelling) in LPC028, LPC006, PANC-1 and CFPAC-1 cells

Further analysis of cell death by the Annexin-V/PI assay confirmed the induction of apoptosis by perifosine. Perifosine increased both early and late apoptosis, as shown in Fig.22 for the LPC028 cells.

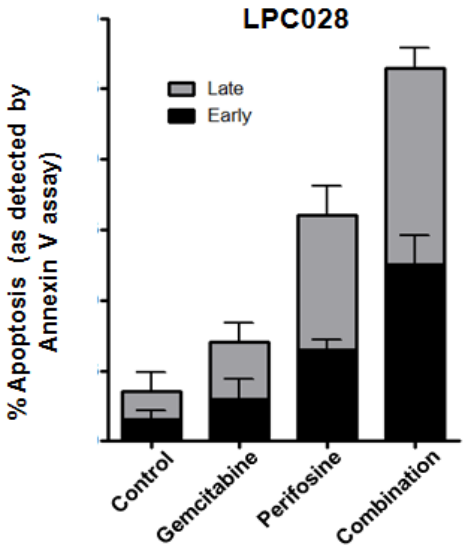

Figure 22: Annexin-V assay in LPC028

Moreover, the combination of perifosine and gemcitabine significantly increased the percentage of late apoptotic cells up to 26%. Similar results were observed in CFPAC-1 and PANC-1 cells (fig.23), whereas no apoptosis induction was detected in LPC006 cells (fig.24).

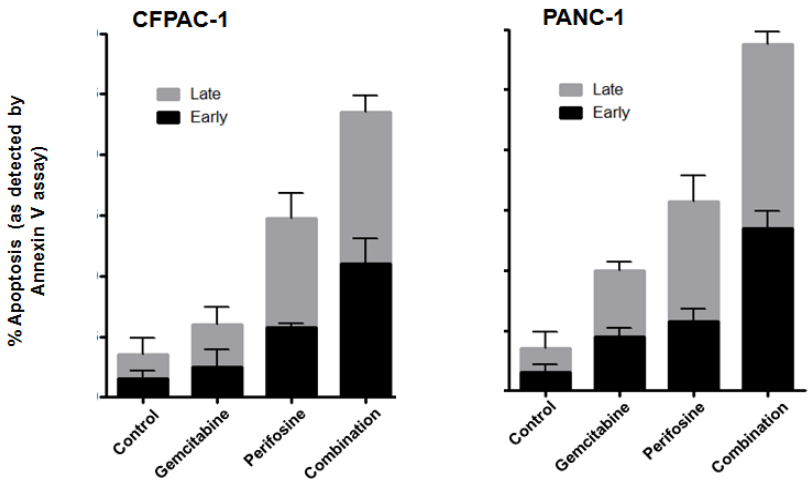

Figure 23: Annexin-V assay in LPC028 and LPC006 cells

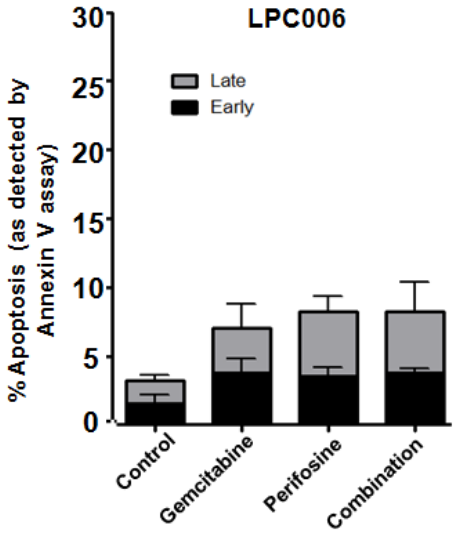

Figure 24: Annexin-V assay in LPC006

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 pro-apoptotic and anti-apoptotic proteins. As a shown figure 25, perifosine and its combination with gemcitabine were able to increase the activity of caspase-3/-6/-8/-9 in LPC028 but not in LP006.

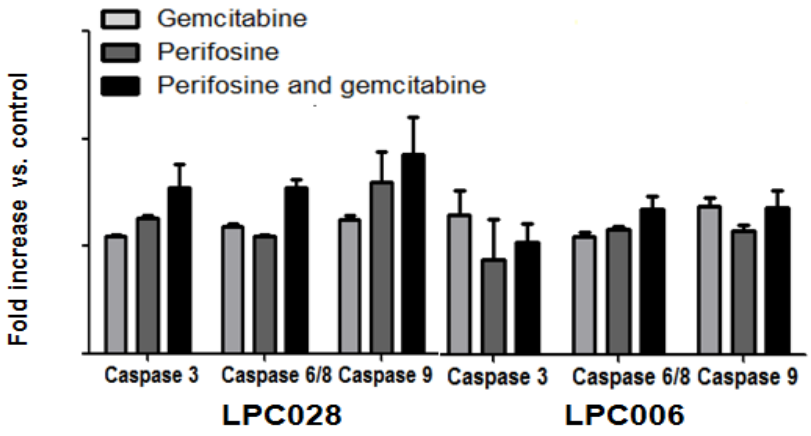

Figure 25: Modulation of caspase-3, caspase-6/-8/ and caspase-9 in LPC028 and LPC006 cells, as determined by a specific fluorometric assay described in the Materials and Methods section;

Also in CFPAC-1 and PANC-1 the levels of caspases were increased (fig.26).

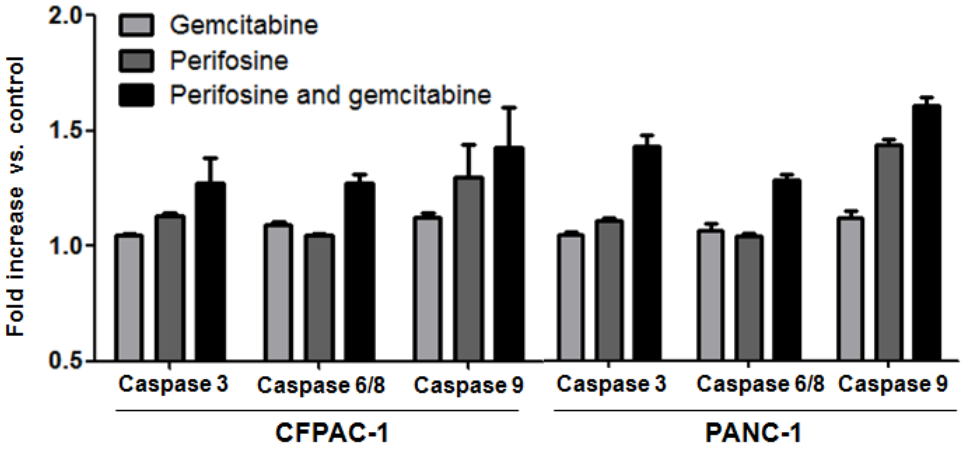

Figure 26: Modulation of caspase-3, caspase-6/-8/ and caspase-9 in CFPAC-1 and PANC-1 cells, as determined by a specific fluorometric assay

However, Western blot analyses demonstrated the modulation of other important apoptotic markers. In particular, perifosine and perifosine/gemcitabine combination increased the expression of PARP and BAD, while reducing Bcl-2 and NF-kB expression in LPC028 cells. Conversely, none of these proteins was affected by the exposure to perifosine and its combination with gemcitabine in the LPC006 cells (fig.28).

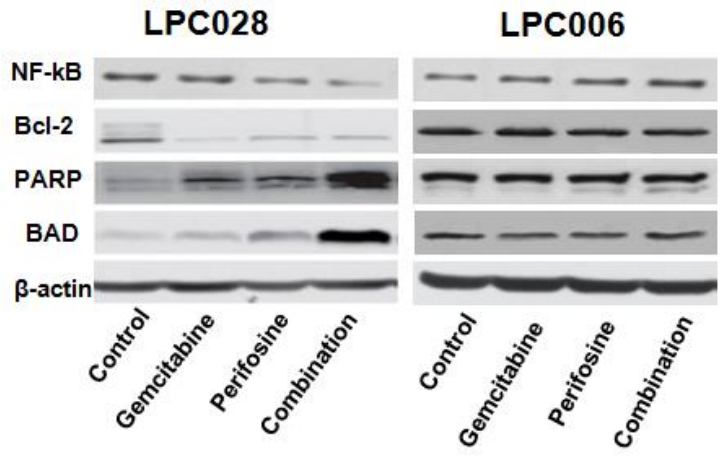

Figure 28: Representative Western blot pictures of apoptosis determinants in LPC006 and LPC028 cells

GLUT1 IS OVEREXPRESSED IN THE CELLS RESISTANT TO AKT INHIBITION, WHILE ITS INHIBITION SIGNIFICANTLY REDUCES CELL GROWTH AND INDUCES APOPTOSIS AFTER GEMCITABINE/PERIFOSINE TREATMENT

Since major oncogenic signaling pathways have been linked to increased glucose metabolism, and previous studies showed that stimulation of Akt1 induces Glut1 mRNA and protein accumulation [39] we evaluated the expression of this key glucose transporter in the LPC028 and LPC006 cells. As shown in the figure 29, *Glut1* mRNA levels were significantly reduced after treatment with perifosine alone

and in combination with gemcitabine in the LPC028 cells, whereas no modulation was detected in the LPC006 cells.

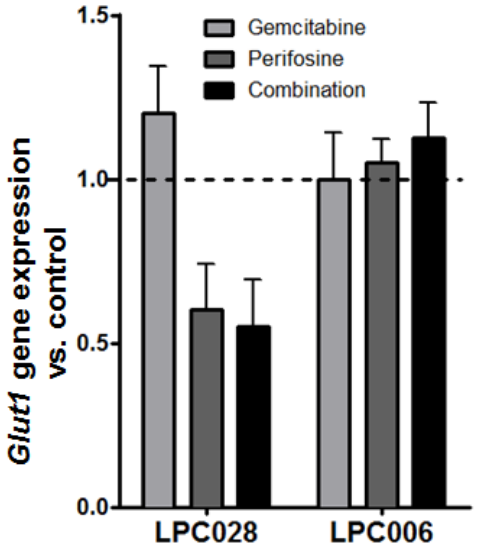

Figure 29: Modulation of *Glut1* mRNA levels in LPC028 and LPC006 cells after 24-hour exposure to perifosine, gemcitabine or to their combination, at IC50 values, as determined by qRT-PCR

However, since PI3K/AKT/mTOR signaling seems to play an essential role in trafficking of Glut1 from recycling endosomes and/or retention of Glut1 at the plasma membrane [40] , we performed further studies to evaluate the amount of membrane-bound Glut1 with FACS analysis (fig.30).

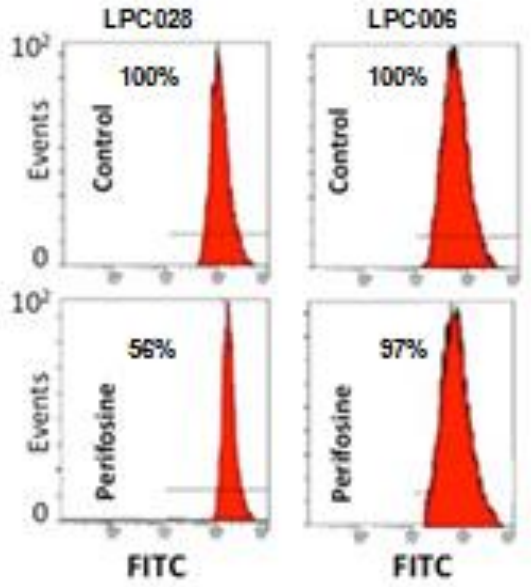

Figure 30: Representative of Glut1 membrane-bound expression in LPC006 and LPC028 cells exposed for 24 hours to perifosine at IC50s

Further studies with Western blot clearly demonstrated the overexpression of Glut1 in the LPC006 compared to the LPC028 and PANC-1 cells. Moreover Glut1 expression was not reduced by Akt inhibition (fig.31).

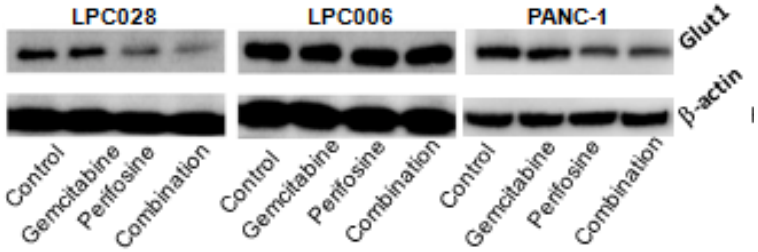

Figure 31: Representative Western blot pictures of Glut1 expression in LPC028, LPC006 and PANC-1 cells exposed for 24 hours to perifosine, gemcitabine or their combination at IC50s

We therefore investigated whether inhibition of Glut1 by the novel specific compound PGL13 can at least in part overcome the inherent resistance of the LPC006 cells to perifosine and other Akt inhibitors (fig.32).

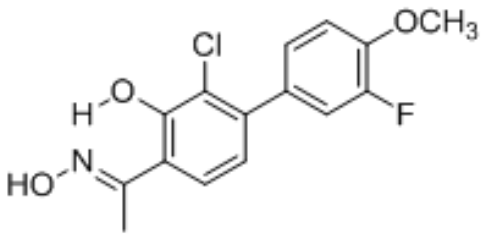

Figure 32: PGL13 structure

Remarkably, the Glut1 inhibitor alone caused only a slight reduction of cell growth (<10%), but its combination with perifosine reduced significantly the percentage of surviving cells compared to perifosine alone (fig.33).

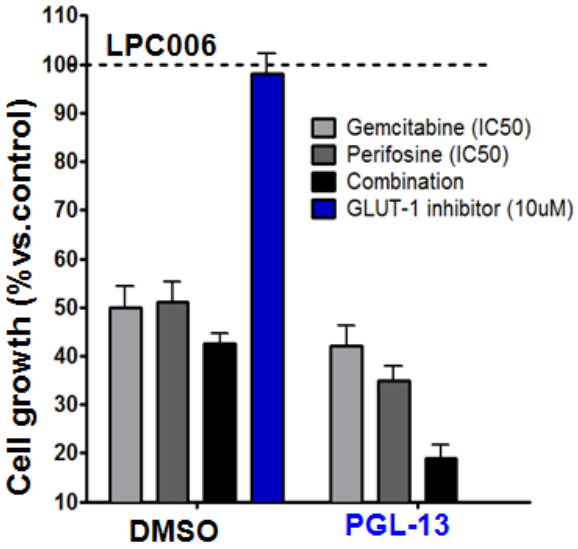

Figure 33: Cell growth inhibition in LPC006 cells after 72-hour exposure to perifosine, gemcitabine or to their combination, at IC50 values, together with DMSO or with the Glut1 inhibitor PGL13, at 30 □M.

Furthermore, the combination of PGL13 with both perifosine and gemcitabine led to a more dramatic drop in the number of surviving cells, up to –81%, compared to control which was associated with strong apoptosis induction, as detected by characteristic apoptotic nuclear morphological features with fluorescence microscopy. In particular the LPC006 cells exposed to PGL13 with both perifosine and gemcitabine had an apoptotic index of 27%, which was similar to the apoptotic index of the LPC028 cells treated with perifosine and gemcitabine (fig.34). The effect of a combined Akt inhibitor/anti-Glut1 treatment was further tested with MK-2206 and NVP-BEZ235, where it led to a -14% and -20% decrease in cell viability compared with these drugs alone. Thus, inhibition of Glut1 promoted anti-Akt-mediated cell death and this combined treatment shows promise for future investigation in the treatment of PDAC.

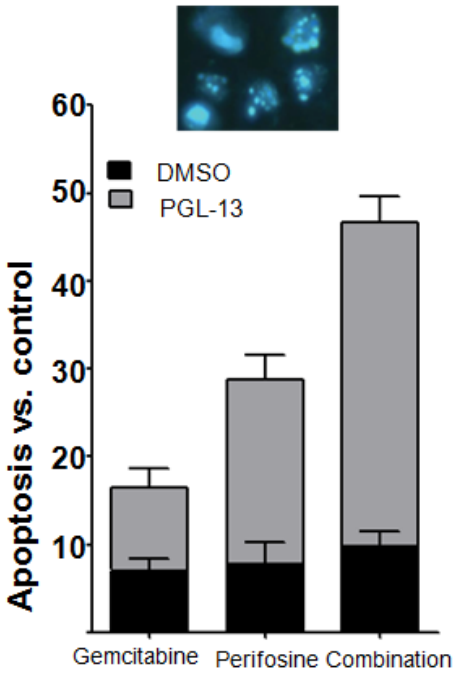

Figure 34: Apoptosis induction by perifosine, gemcitabine and their combination as assessed by bisbenzimide staining

Discussion

The present study supports a role for phospho-Akt as a prognostic factor in PDAC patients, and unravels its potential role as a target for the synergistic interaction of anti-Akt agents and gemcitabine through modulation of apoptotic and invasive processes.

Several studies demonstrated that PDAC tissues have increased activation of the PI3K/Akt, as assessed with the phosphorylation of Akt, and this has been associated with higher histological tumor grade [41] and worse prognosis [42,43].

In this project we further explored the clinical relevance of phospho-Akt by screening its expression in a homogeneous cohort of 100 surgically resected PDACs. In agreement with the previous studies, even considering several clinicopathological parameters, phospho-Akt expression was the only factor correlated to differential clinical outcome. However, a systematic review and meta-analysis of prognostic tissue biomarkers for PDAC, including phospho-Akt among the 22 markers associated with limitless replicative potential eligible for examination, showed that only Ki-67 maintained statistically significant associations with outcome [44]. These discrepancies might be attributed to the different experimental procedures used, including antigen retrieval technique, antibody characteristics and dilution, as well as observer variability in staining pattern description and cutoff point selection. Therefore, in the present study we have chosen an antibody that was previously validated in an immunohistochemical study on 102 colorectal cancer FFPE samples [45] and we have used image analysis software to calculate expression as a continuous parameter, in order to facilitate the identification of cutoff points. However, because of the typical dense stromal reaction and diffusely infiltrative growth pattern the data were always checked also by two experienced researchers.

This method allowed the assignment of the specimens to different categories, including a subset of tissues (about 14%) characterized by extremely high expression of phospho-akt, which could clearly influence the prognostic value. Indeed this cutoff point identified a group of patients with very poor outcome, who should be treated with more aggressive, novel therapeutic approaches. Of note, recent genomic studies showed that the PI3K/Akt signaling is among the core signaling pathways leading the intrinsic aggressiveness of PDAC, suggesting that in the "PDAC actionable genome" about 9 and 6% of the cases are Akt- and PI3K-dependent, respectively [46]. These data underline the potential importance of specific inhibitors of PI3K/Akt as novel effective therapeutics in a selected subpopulation of PDAC patients. Moreover, activation of this signaling pathway is associated with PDAC chemoresistance [47,48], supporting the hypothesis that Akt inhibitors might also be used to overcome resistance towards conventional cytotoxic agents. 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 [49].

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 [48]. More recently, several small molecules that inhibit the PI3K/Akt signaling entered clinical development, but more information on their activity in the preclinical setting is warranted. Our results demonstrate that perifosine is the targeted anti-PI3K/Akt antitumor agent demonstrating the most potent growth inhibitory effects in a panel of human PDAC cells characterized by distinct molecular properties. Limited published preclinical research focusing on this issue in

PDAC reported similar cytotoxic activity of perifosine in PANC-1, MIA PaCa-2, and AsPC-1 cells [28]. Sensitivity to perifosine in the PDAC cells also fell within the range of IC_{50} values previously reported in PDAC cell lines and spheroids for other Akt inhibitors, such as NVP-BEZ-235 [50,51]. Furthermore, perifosine interacted synergistically with gemcitabine 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. These results are in agreement with previous studies showing synergistic interaction of gemcitabine with perfifosine in PANC-1 cells and xenografts [28] as well as enhanced apoptotic cell death after combined treatment with paclitaxel in chemoresistant ovarian cancer cells [52]. However, most previous studies were performed in ATCC cell lines, which showed similar results [53], while, to more effectively develop targeted compounds, it will be helpful to understand why these agents fail when they do. Thus, 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 LPC0028 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 effects only in the cells with high phospho-Akt or intermediate/high values of *Akt1* mRNA, as determined by RT-PCR. Conversely this combination was antagonistic in the cells with low *Akt1*, and phospho-Akt expression. These data suggest that the expression and activation of Akt might therefore be used to tailor perifosine therapy.

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. The present study demonstrated also 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 RRM1 and RRM2 in the cells with a high expression of Akt, while this effect was not statistically significant in the cells with low Akt expression. RR is a key target of gemcitabine activity and previous studies correlated the expression of its subunits to gemcitabine sensitivity in PDAC cells [54,55].

Therefore, the synergistic interaction between perifosine and gemcitabine might be explained, at least in part, by the modulation of gemcitabine sensitivity through RRM1 and RRM2 suppression.

However, our results suggested that the synergistic interaction of perifosine with gemcitabine is associated with other important molecular mechanisms affecting PDAC aggressiveness. In agreement with previous observations showing the reduction of cell migration/invasion through Akt inhibition [56,57], 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 [38], we demonstrated that perifosine increased the expression of E-cadherin in LPC028, CFPAC-1 and PANC-1 cells. Since the Akt signaling pathway plays an important

role in cell survival process, its blockage can result in activation of programmed cell death [58]. 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 [59], 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 multiple methods, such as 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 critical factors involved in the apoptotic cascades. Previous studies showed that drug-induced Akt deactivation was associated with activation of pro-apoptotic factors, including caspase-9 and BAD, as well as with a parallel decrease in the expression of the anti-apoptotic factors Bcl-2 and NF-kB [60,24]. Our studies showed similar results after exposure of the PDAC cells to perifosine. Despite increasing evidence on the pivotal role of PI3K/Akt signaling in cancer, the strategies to hit PI3K/Akt/mTOR pathway have failed to demonstrate therapeutic activity in most ongoing clinical trials, and a previous phase II study testing perifosine in previously untreated patients with locally advanced, unresectable, or metastatic pancreatic adenocarcinoma, was terminated as a result of unacceptable adverse events [61].

Therefore, there is an urgent need to improve alternative strategies that might tailor these therapies to the individual characteristics of the patients and favor synergistic combination, reducing toxic doses, as well as prevent feedback loops involved in PDAC progression upon pan-PI3K treatment. For example, it is already known that

in PDAC cells, dual PI3K-mTOR inhibition induces rapid over-activation of MAPK pathway through a PI3K-independent pathway [62] and that drug resistance may be overcome by inhibition of parallel oncogenic-dependent pathways, such as with the dual MEK and PI3K/mTOR blockade [51].

One strategy to overcome resistance consists into identifying key molecular differences in the tumors that are less likely to respond. Oncogenic *KRAS* drives metabolic reprogramming in tumor cells by increasing aerobic glycolysis, and recent studies showed that subtypes of PDAC cells with distinct metabolite levels associated with glycolysis, lipogenesis, and redox pathways, confirmed at the transcriptional level. The glycolytic and lipogenic subtypes showed striking differences in glucose and glutamine utilization, as well as mitochondrial function, and corresponded to differences in cell sensitivity to inhibitors of glycolysis, glutamine metabolism, lipid synthesis, and redox balance [63].

In the present study we demonstrated that the resistant LPC006 cells were characterized by overexpression of Glut1, which was not reduced after exposure to perifosine. Remarkably, the inhibition of Glut1 dramatically enhanced perifosine and perifosine/gemcitabine-induced cell death. Agents directly inhibiting Glut1 are in early-phase evaluations, and a few preclinical studies have demonstrated that Glut inhibitors led to diminish tumor growth *in vitro* and *in vivo* [64]. However, the altered expression of Glut1 might also influence the sensitivity of tumor cells to chemotherapy, since a recent study showed that the knockdown of *Glut1* sensitizes head and neck cancer cells to the chemotherapy drug cisplatin [65].

To our knowledge this is the first study showing that Glut1 inhibitors can restore the repression of aerobic glycolysis induced by PI3K/mTOR inhibitors in resistant cells,

and favor their synergistic interaction with cytotoxic compounds. These results should prompt further studies to understand how PDAC cell metabolism might affect sensitivity to new anti-signaling therapies and to identify promising therapeutic targets that might be exploited by combination therapies.

Conclusions

Our data support the analysis of phospho-Akt expression as both a prognostic and a predictive biomarker, for the rational development of novel therapies targeting the Akt pathway in PDAC. In particular we observed that phospho-Akt expression levels influence the antitumor activity of perifosine, as well as the synergistic interaction with gemcitabine, through its ability to attack key mechanisms involved in the proliferation, cell cycle control, apoptosis and migration/invasion properties. Finally, we demonstrated that inhibition of Glut1 overcame resistance to this combination treatment and might provide the basis for the development of new therapeutic approaches with Akt inhibitors in patients with PDAC.

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

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Abstract

Background: 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 molecular mechanisms influencing the therapeutic potential of Akt inhibition in combination with gemcitabine.

Methods: Phospho-Akt expression was evaluated by immunohistochemistry in tissue microarrays (TMAs) with specimens tissue from radically-resected patients ($n = 100$). Data were analyzed by Fisher and log-rank test. In vitro studies were performed in 14 PDAC cells, including seven primary cultures, characterized for their *Akt1* mRNA and phospho-Akt/Akt levels by quantitative-RT-PCR and immunocytochemistry. Growth inhibitory effects of Akt inhibitors and gemcitabine were evaluated by SRB assay, whereas modulation of Akt and phospho-Akt was investigated by Western blotting and ELISA. Cell cycle perturbation, apoptosis-induction, and anti-migratory behaviors were studied by flow cytometry, AnnexinV, membrane potential, and migration assay, while pharmacological interaction with gemcitabine was determined with combination index (CI) method.

Results: Immunohistochemistry of TMAs revealed a correlation between phospho-Akt expression and worse outcome, particularly in patients with the highest phospho-Akt levels, who had significantly shorter overall and progression-free-survival. Similar expression levels were detected in LPC028 primary cells, while LPC006 were characterized by low phospho-Akt. Remarkably, Akt inhibitors reduced cancer cell growth in monolayers and spheroids and synergistically enhanced the antiproliferative activity of gemcitabine in LPC028, while this combination was antagonistic in LPC006 cells. The synergistic effect was paralleled by a reduced expression of ribonucleotide reductase, potentially facilitating gemcitabine cytotoxicity. Inhibition of Akt decreased cell migration and invasion, which was additionally reduced by the combination with gemcitabine. This combination significantly increased apoptosis, associated with induction of caspase-3/6/8/9, PARP and BAD, and inhibition of Bcl-2 and NF-kB in LPC028, but not in LPC006 cells. However, targeting the key glucose transporter Glut1 resulted in similar apoptosis induction in LPC006 cells.

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Conclusions: These data support the analysis of phospho-Akt expression as both a prognostic and a predictive biomarker, for the rational development of new combination therapies targeting the Akt pathway in PDAC. Finally, inhibition of Glut1 might overcome resistance to these therapies and warrants further studies.

Keywords: Pancreatic ductal adenocarcinoma, Akt, Synergism, Gemcitabine

Background

Pancreatic ductal adenocarcinoma (PDAC) is among the most lethal solid tumors. Despite extensive preclinical and clinical research, the prognosis of this disease has not significantly improved, with a 5-year survival rate around 7% [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 most currently available chemotherapy regimens. This resistance has been attributed to both the desmoplastic tumor microenvironment and to the strong inter- and intra-tumor heterogeneity in terms of complexity of genetic aberrations and the resulting signaling pathway activities, as well as to resistance mechanisms that quickly adapt the tumor to drugs [2].

Oncogenic *KRAS* signaling is the main driving force behind PDAC. Activating KRAS mutations occur early, followed by loss of *p16*, and then later, inactivation of *TP53* and *SMAD4* [3, 4]; however, targeting these events has proven to be very difficult. Conversely, the phosphatidylinositol-3 kinase (PI3K)/Akt downstream pathway represents an exciting new target for therapeutic intervention, especially because it emerged among the core signaling pathways in PDAC [5, 6], and several known inhibitors are currently in clinical trials (www.clinicaltrials.gov).

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 [7]. 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 [8–11].

The PI3K/Akt pathway plays a key role in cell proliferation, survival, and motility [12]. Deregulation of components involved in this pathway could confer resistance to chemotherapy [13, 14], while blockage of Akt signaling results in programmed cell death and inhibition of tumor growth [15, 16]. Activation of Akt is a frequent event in PDAC and has been correlated to its poor prognosis [17, 18].

Several inhibitors of Akt are under investigation, but three are the farthest along and showed the most promise in early clinical research: the pan-Akt and PI3K inhibitor perifosine (KRX-0401, Aeterna Zentaris/Keryx), the allosteric pan-Akt inhibitor MK-2206 (Merck), and the dual PI3K/mTOR inhibitor dactolisib (NVP-BEZ235, Novartis).

In particular, the synthetic oral alkylphospholipid perifosine [19, 20] has been evaluated in clinical trials for several tumors, including colon [21], breast [22], head and neck, and prostate cancer [23, 24]. Unfortunately, it failed the phase III clinical trials for treatment of colon cancer and relapsed refractory multiple myeloma (www.clinicaltrials.gov). These failures, together with the disappointing response rates to perifosine as a single agent in most solid tumors, including PDAC, prompt further studies into its mechanism of action [6] as well as on synergistic combinations.

Perifosine prevents translocation of Akt to the cell membrane by blocking the pleckstrin homology (PH) domain of Akt [25] 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 [26]. Recently, Pinton and collaborators showed that perifosine inhibited cell growth of malignant pleural mesothelioma cells by affecting EGFR and c-Met phosphorylation [27]. Another study showed that perifosine decreased the *AEG-1* gene expression along with inhibition of Akt/GSK3/c-Myc signaling pathway in gastric cancer [28]. Perifosine and curcumin synergistically increased the intracellular level of reactive oxygen species and ceramide, and downregulated the expression of cyclin-D1 and Bcl-2 in colorectal cancer cells [29]. Finally, perifosine also inhibits the anti-apoptotic mitogen-activated protein kinase (MAPK) pathway and modulates the balance between the MAPK and pro-apoptotic stress-activated protein kinase (SAPK/JNK) pathways, thereby inducing apoptosis [30].

The aims of current study were to investigate the expression of phospho-Akt in PDAC tissues and cells, and to evaluate the effects of growth inhibition by Akt inhibitors, using PDAC cell lines and primary cultures growing as monolayer or as spheroids. Moreover, we characterized several key factors, affecting cell cycle perturbation, apoptosis induction, as well as inhibition of cell migration and invasion and modulation of key factors in glucose metabolism in PDAC cells exposed to perifosine and perifosine/gemcitabine combination.

Methods

Tissue microarrays (TMAs), immunohistochemistry (IHC), and immunocytochemistry (ICC)

Phospho-Akt protein expression was evaluated in slides from four formalin-fixed, paraffin-embedded PDAC-specific TMAs build with neoplastic cores from a cohort of radically resected patients ($n = 100$), using the TMA Grand Master (3DHistec, Budapest, Hungary) instrument, and stained according to standard procedures with the EP2109Y rabbit monoclonal antibody (1:50 dilution; Abcam, Cambridge, UK). Visualization was obtained with BenchMark Special Stain Automation system (Ventana Medical Systems, Tucson, AZ). Two pathologists reviewed all the slides, assessing the amount of tumor and tissue loss, background staining, and overall interpretability before the phospho-Akt reactivity evaluation. Staining results were evaluated using a computerized high-resolution acquisition system (D-Sight, Menarini, Florence, Italy), including the analysis of positive cells number and staining intensity which resulted in values expressed as arbitrary units (a.u.). All patients have provided a written informed consent. This study was approved by the Local Ethics Committee of the University of Pisa. Date of approval: July 3, 2013 (file number 3909).

For ICC, the cells were grown in a Chamber Slides System (Lab-Tek, Collinsville, IL). After 24 h, the cells were fixed with 70% ethanol for 10 min, 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 using a digital system based on staining intensity and on the number of positively stained cells, as described above.

Drugs and chemicals

Perifosine was provided by Æterna Zentaris Inc. (Frankfurt am Main, Germany), NVP-BEZ235 was purchased from Selleck Chemicals (Houston, TX), while gemcitabine and MK-2206 were generous gifts from Eli-Lilly (Indianapolis, IN) and Merck (Whitehouse Station, NJ), respectively. The drugs were dissolved in Dimethyl sulfoxide (DMSO) 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 cultures

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 [31]. 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 manufacturer's 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 [31].

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*), *E-cadherin*, and the *glucose transporter 1* (*SLC2A1*/*Glut1*) which were obtained from Applied Biosystems TaqMan Gene expression products (Hs00920503_m1, Hs01085706_m1, Hs00984403_m1, Hs01040726_m1, Hs00156401_m1, Hs00168784_m1, Hs01072069_g1, Hs01023894_m1, and Hs00892681_m1). 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 [32].

Growth inhibition studies

The cell growth inhibitory effects of perifosine, MK-2206 and NVP-BEZ235 were evaluated in the PANC-1, LPC028, and LPC006 cells. Further studies evaluated perifosine and gemcitabine combination in CFPAC-1, PANC-1, LPC028, and LPC006 cells. These cells were treated for 72 h 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 [32].

Evaluation of synergistic/antagonistic interaction with gemcitabine

The pharmacological interaction between perifosine and gemcitabine was evaluated by the median drug effect analysis method as described previously [32]. 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).

Effects on multicellular spheroids

LPC006 and LPC028 spheroids were established by seeding 10^4 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 $D = (D_{max} + D_{min})/2$, as follows: $V = (4/3) \times \pi (D/2)3$.

Western blot

In order to evaluate the modulation of Akt1, phospho-Akt1, PARP, BAD, Bcl-2, NF-kB, and Glut1 protein expression in PDAC cells treated for 24 h with perifosine, gemcitabine, and their combination, Western blot analyses were executed as described previously using the Akt1 sc-5298 mouse monoclonal (Santa Cruz, Biotechnology, Santa Cruz, CA) and the EP2109Y rabbit monoclonal antibody (1:500 dilution; Abcam), PARP sc-8007 mouse monoclonal (1:500 dilution; Santa Cruz), BAD sc-8044 mouse monoclonal (1:500 dilution;Santa Cruz), Bcl-2 sc-7382 mouse monoclonal (1:500 dilution; Santa Cruz), NF-kB sc-114 rabbit polyclonal (1:500 dilution; Santa Cruz), and Glut1 sc-1605 goat polyclonal (1:500 dilution; Santa Cruz) [33]. Briefly, 40 μg of proteins was separated on a 10% SDS-polyacrylamide gel and transferred onto polyvinylidene difluoride (PVDF) membrane (Immobilion®-FL, Millipore, Billerica, MA). The membrane was incubated overnight with mouse and rabbit anti-Akt1, anti-phospho-Akt1, as described above, as well as with mouse anti-BAD, anti-Bcl-2, anti-PARP, with rabbit anti-NF-kB (1:1000, diluted in the blocking solution; all from Santa Cruz Biotechnology, Santa Cruz, CA), goat anti-Glut-1 (ab652, 1:500, diluted in the blocking solution, from Abcam, Cambridge, UK), 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] and [Thr308] phosphorylation, specific ELISA assays were performed using the Pierce AKT Colorimetric In-cell ELISA Kit (Thermo Scientific, Rockford, IL), which has a sensitivity approximately twofolds greater than Western blotting. The levels of Akt and phospho-Akt were measured in cells seeded in a 96-well-plate at a density of 10^5 cells per well, and treated for 4 or 24 h with perifosine, gemcitabine, and their combination at IC_{50} 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 and MK-2206 and its combination with gemcitabine to inhibit the migratory behaviour of PDAC cells was investigated by in vitro migration assay, as described [31]. The cells were exposed to the drugs at their IC_{50s}. Images were taken at the beginning of the exposure (time 0), with those taken after 4, 6, 8, 20, and 24 h. 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 10^5 cells were plated on the upper side of the filter and incubated with the drugs at IC_{50} concentrations in RPMI-1640 medium. After 24 h, 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, LPC028, LPC006, CFPAC-1, and PANC-1 cells were treated for 24 h with gemcitabine, perifosine, and their combination at IC_{50} 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. 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 described [34]. Additional studies were performed with the Annexin-V/PI assay, plating the cells in 6-well-plates at a density of 1.5×10^5. After 24 h, the cells were treated with the drugs at their IC_{50}, followed by 24-h 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 $CaCl_2$). 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 min 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, 10^6 LPC006, LPC028, CFPAC-1, and PANC-1 cells were exposed to the drugs for 24 h at their IC_{50s}. Fluorescence was measured at 350 nm excitation and 460 nm emission (Spectrafluor Tecan, Salzburg, Austria). Relative caspase activity was normalized with respect to the untreated cells.

Analysis of modulation of Glut1 by flow cytometry

To quantitatively detect the expression of membrane-bound Glut1, cells were fixed with 80% ethanol, incubated with anti-Glut1 antibody (Abcam), and then stained with the appropriate FITC-conjugated anti-rabbit IgG antibody (BD Pharmingen™, BD Biosciences, San Jose, CA). Quantification of FITC fluorescence intensity was performed using a FACSCanto flow cytometer (BD Biosciences).

Evaluation of the cytotoxic and pro-apoptotic effects inhibition of Glut1 inhibition combined with Akt inhibitors

The Akt signaling is involved in the modulation of Glut1 expression/localization, and a recent study showed that increased glucose metabolism was associated to resistance to the tyrosine kinase inhibitor axitinib, and this resistance was overcame by Glut1 silencing [35]. Therefore, we performed additional cytotoxicity studies using the novel Glut1 inhibitor PGL13. This compound was tested in the LPC006 cells, at a concentration of 30 μM, which effectively reduced glucose influx in previous studies [36, 37]. The cells were exposed to PGL13 for 72 h, alone or in combination with IC_{50} concentration values of perifosine, gemcitabine, and their combination. Cell growth inhibition was then assessed by counting the cells after staining with trypan blue, in comparison to untreated cells. Parallel evaluation of apoptosis induction was performed by fluorescence microscopy with bisbenzimide staining, as described previously [33].

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), and progression-free-survival (PFS) were calculated from the date of pathological diagnosis (i.e., the date of surgery) to the date of death and tumor progression, respectively. OS and PFS 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 phospho-Akt and Akt1 mRNA expression in PDAC tissues and cells

The protein expression of phospho-Akt was successfully evaluated by IHC in 100 human PDACs collected in two TMAs. The main clinical characteristics of these patients are reported in the Table 1. IHC showed a variable protein expression with some specimens characterized by a strong and diffuse staining, while other tissues had only a few scattered positive cells with a weak staining (as exemplified by the middle and lower panels in the Fig. 1a, respectively). Patients were categorized according to their high versus low phospho-Akt expression compared to the median value (30 a.u.) calculated by digital scoring (Fig. 1b, black line). 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 had a median OS of 16.2 months (95% CI, 14.8–20.1), while patients with a high expression had a median OS of 12.0 months (95% CI, 9.0–14.9, $P = 0.03$, Fig. 1c, upper panel). However, only a trend toward a significant association was found between phospho-Akt expression and PFS ($P = 0.08$, Additional file 1: Figure S1a).

An additional analysis was performed categorizing the patients with respect to a threshold expression of 57 a.u., which identified 14 cases with higher expression

Table 1 Outcome according to clinical characteristics in the 100 PDAC patients enrolled in the present study

Characteristics		N (=%)	OS months (95% CI)	P
No. patients	All	100	14.0 (12.1–15.8)	
Age, years	≤65	43	15.2 (13.3–16.8)	0.361
	>65	57	14.1 (11.1–17.0)	
Sex	Male	47	13.0 (11.1–14.9)	0.814
	Female	53	15.0 (11.9–18.1)	
Resection status	R0	56	15.2 (12.3–21.6)	0.474
	R1	44	13.5 (11.2–31.0)	
Lymph node	No	10	18.5 (7.6–32.4)	0.521
	Yes	90	14.2 (12.2–15.9)	
Grading	1–2	36	15.5 (11.5–18.1)	0.097
	3	64	12.1 (8.4–15.8)	

compared to all the others (defined as *very high* phospho-Akt expression, Fig. 1b, blue square). Using these categories, we observed a significant correlation between high phospho-Akt protein expression and both significantly shorter OS ($P < 0.01$, Fig. 1c, lower panel), and PFS (Additional file 1: Figure S1b).

Parallel ICC studies revealed that the LPC006 cells had a significantly lower phospho-Akt expression compared to LPC028 cells, which were indeed included in the category of *low* and very high phospho-Akt expression, respectively (Fig. 1b, blue and red circles). 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 0.9 arbitrary unit (a.u.) in LPC006 cells to 24.0 a.u. in LPC028 and PANC-1 cells (Fig. 1d). The mean and median expression in the tumor cells (8.7 ± 0.2 and 8.4 a.u., respectively) were significantly higher ($P < 0.01$) than the expression detected in hTERT-HPNE cells (0.3 a.u.). Notably, *Akt1* gene expression in the seven primary tumor cells and their

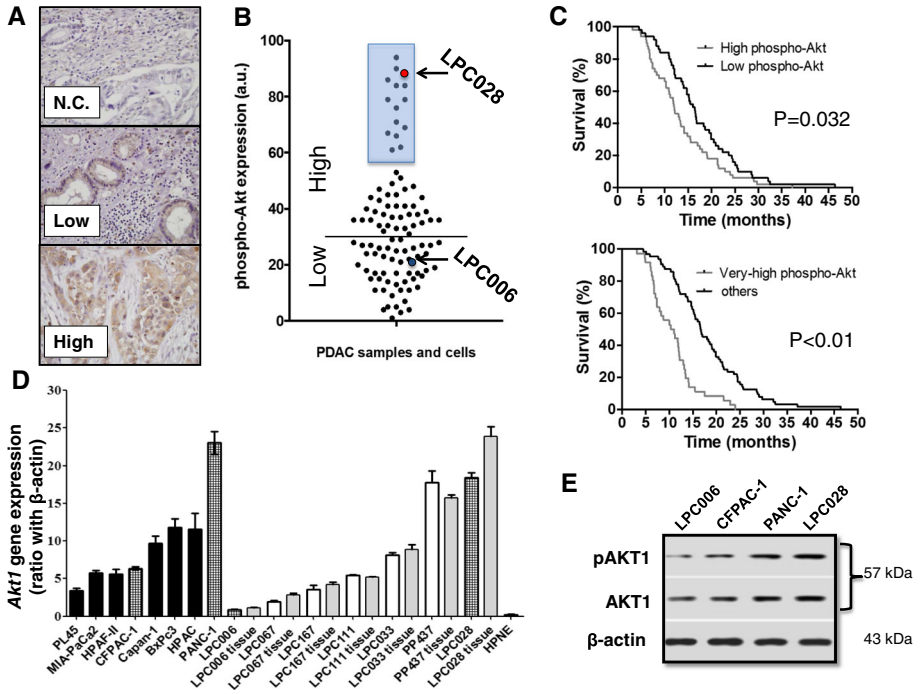

Fig. 1 Akt/phospho-Akt expression in PDAC tissues and cells. **a** Representative examples (original magnification, ×40) showing the variable expression of phospho-Akt in paraffin-embedded PDAC samples collected in four TMAs (with 4 cores for each of the 100 patients). *N.C.*, negative control. **b** Expression values of phospho-Akt observed across the cohort of PDAC patients, obtained by digital quantification. Phospho-Akt showed positive cytoplasmic and nuclear staining in most tissue sections, with intense staining in 14 out of 100 samples. The staining intensities of the LPC028 and LPC006 cells were included in the very high and low Akt expression groups, respectively. **c** Kaplan–Meier survival curves according to the expression of phospho-Akt in 100 radically resected PDACs, showing that patients with high expression (*upper panel*) and very high expression (*lower panel*) of phospho-Akt had a significantly shorter survival compared to patients with low phospho-Akt expression. **d** *Akt1* mRNA expression in ATCC cell lines (*black bars*), primary tumor cultures (*white bars*), and their originator tissues (*gray bars*). *Dashed bars* identify the cells that were selected for further in vitro studies; **e** Representative Western blot pictures of phospho-Akt1 and Akt1 expression in LPC006, CFPAC-1, PANC-1, and LPC028 cells. *Columns*, mean values obtained from three independent experiments, *bars*, SEM

laser-microdissected originator tumors showed a similar pattern and were highly correlated with Spearman analysis ($R^2 > 0.9$, $P < 0.05$), suggesting that these cells represent optimal preclinical models for our pharmacological studies. Moreover, Western blot analysis revealed that the LPC006 and CFPAC-1 cells had a lower phospho-Akt1/Akt1 ratio (0.3 and 0.6 a.u., respectively) expression compared to PANC-1 (0.8) and LPC028 (1.1) cells (Fig. 1e).

Therefore, we selected for further studies two primary cell cultures (LPC006 and LPC028) which were representative of low and very high expression values, as well as two cell lines, PANC-1 and CFPAC-1, with high and intermediate expression values of Akt1 mRNA, respectively.

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

The cytotoxic activity of three different Akt inhibitors (perifosine, MK-2206, and NVP-BEZ235) was evaluated in the PANC-1 cell line (Fig. 2a). All these compounds caused a concentration-dependent inhibition of proliferation, with IC_{50} values ranging from 5.1 (perifosine) to 15.8 μM (NVP-BEZ235). Higher IC_{50} values were obtained in the LPC006 cells, i.e., 22.5, 31.7 and 45.5 μM for perifosine, NVP-BEZ235, and MK-2206 (Additional file 1: Figure S2), respectively. According to the lowest IC_{50} values detected in these assays, we selected perifosine for the following studies on the pharmacological interaction of Akt inhibitors with gemcitabine.

The cell growth inhibitory effects of perifosine, gemcitabine, and their combination in LPC028 and LPC006 cells are shown in Fig. 2b, while the data for CFPAC-1 and PANC-1 are reported in the Additional file 1: Figure S3. Since the CI method recommends a ratio of concentrations at which drugs are equipotent, combination studies were performed using fixed ratios with IC values at IC_{50s}. Perifosine enhanced the antiproliferative activity of gemcitabine, especially in the LPC028 and PANC-1 cells, by decreasing the IC_{50s} of gemcitabine from 4.3 ± 1.1 and 17.2 ± 2.1 nM to 1.4 ± 0.5 and 4.0 ± 1.1 nM, respectively. The median drug-effect analysis revealed a slight-to-moderate synergism in CFPAC-1, and a strong synergism in the PANC-1 and LPC028 cells, with CI values of 0.8, 0.5, and 0.2, respectively (Fig. 2c). Conversely, the combination of perifosine and gemcitabine was antagonistic in the LPC006 cells (CI > 1.2). To evaluate whether these effects were observed also in three-dimensional (3D) models and

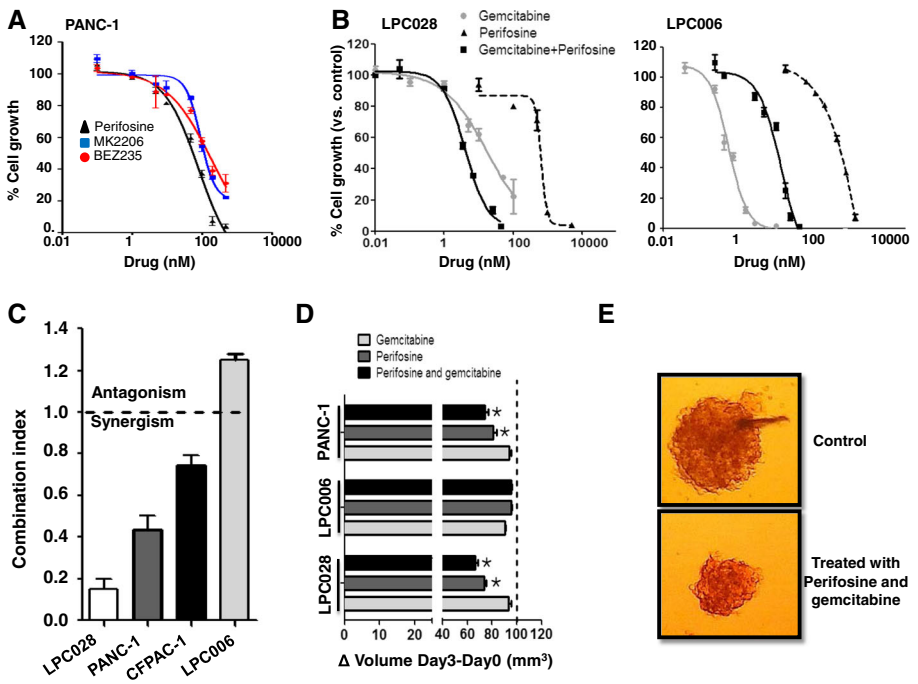

Fig. 2 Inhibition of cell proliferation in PDAC cells. **a** Growth inhibitory effects in PANC-1 cells after 72 h exposure to perifosine, MK-2206 and NVP-BEZ235. **b** Growth inhibitory effects after 72 h exposure to perifosine, gemcitabine, or their combination at a fixed ratio based on IC_{50} values in LPC028 and LPC006 cells. On the *X* axis, the drug concentrations for the combination are referred to gemcitabine. **c** 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 Methods section. **d** Effect of perifosine and gemcitabine. and their combination, at IC_{50} values, on the volumes of PDAC spheroids after 72 h exposure. **e** Representative images of untreated spheroid versus spheroid treated with perifosine and gemcitabine (original magnification, ×40). *Columns* and *points* mean values obtained from three independent experiments, *bars*, SEM; *Significantly different from controls

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 3D culture models are generally more chemo-/radio-resistant than two-dimensional monolayer cell cultures, supporting their use for drug testing [38]. In order to explore whether perifosine would be active in 3D PDAC models, we evaluated this drug in spheroids of LPC006, LPC028, and PANC-1 cells.

Perifosine remarkably increased the disintegration of LPC028 and PANC-1 spheroids, which were significantly ($P < 0.05$) reduced in size compared to the untreated spheroids (Fig. 2d–e). The combination of perifosine with gemcitabine additionally reduced the size of the LPC028 and PANC-1 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 [39], but no data have been reported yet on PDAC cells. Therefore, we evaluated the expression of phospho-Akt (at serine residue 473 (Ser473) and at threonine residues 308 (Thr308)), normalized to the total Akt levels, both in untreated cells and in cells treated with Akt inhibitors (perifosine and MK-2206), gemcitabine, and their combination. We observed a similar inhibition of the phosphorylation status after 4 or 24 h (Fig. 3a and Additional file 1: Figure S4) as well as in both residues (Additional file 1: Figure S5a, b). Perifosine significantly reduced the expression of p-Akt in LPC028, CFPAC-1, and PANC-1 cells (e.g., 40, 25, and 30% reduction, respectively). Regarding Ser473 phosphorylation, the combination of perifosine and gemcitabine was also able to significantly suppress Akt phosphorylation, with a degree of inhibition ranging from −35 (CFPAC-1 cells) to −45% (LPC028 cells). Conversely, both Ser473 and Thr308 phospho-Akt levels were not affected by perifosine, MK-2206, and their combination with gemcitabine in the LPC006 cells.

RRM1 and *RRM2* encode for the catalytic and the regulatory subunits of ribonucleotide reductase and is a key molecular target of gemcitabine [40]. Previous studies demonstrated that the expression of *RRM2* is modulated by the Akt/c- MYC pathway [41]. 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 or increased CDA expression [40]). Therefore, we evaluated the mRNA expression of several gemcitabine determinants in the LPC006, LPC028 and PANC-1 cells. As shown in Fig. 3b, the expression of *RRM1* and *RRM2* was significantly reduced (approximately 2-fold) in LPC028 and also in PANC-1 cells (Additional file 1: Figure S6) 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 (Fig. 3b). These results can at least in part explain the synergistic interaction of perifosine with gemcitabine in PDAC cells with high phospho-Akt expression.

Perifosine and its combination with gemcitabine inhibit cell migration/invasion and upregulate 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, LPC006 (Fig. 4a), CFPAC-1, and PANC-1 (Additional file 1: Figure S7). LPC028 showed a significant reduction of migration starting after 8 h exposure to perifosine with a reduction of the scratch-area of about 50%, and the perifosine/gemcitabine combination additionally reduced cell migration ($P < 0.05$; Fig. 4a left panel), while gemcitabine alone did not affect cell migration. No modulation of cell migration was observed in the LPC006 cells (Fig. 4a right panel). Similarly, the migration of these cells was not affected by MK-2206 alone and in combination with gemcitabine (Additional file 1: Figure S8).

LPC028, CFPAC-1, and PANC-1 cells treated with perifosine showed also a significantly reduced invasive potential, compared to untreated cells (Fig. 4b). In particular, the perifosine/gemcitabine combination was more effective in inhibiting invasion than perifosine-alone in LPC028 and PANC-1 cells, as shown by the significantly lower number of invading cells with Giemsa's stain. However, no modulation of cell invasion was observed in the LPC006 cells.

Since previous studies suggested that the Akt signaling pathway suppressed E-cadherin expression [42], 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 in LPC028, CFPAC-1, and PANC-1 ($P < 0.05$; Fig. 4c), while no changes were detected in LPC006 cells. Similarly, immunocytochemistry analysis in LPC028 cells illustrated a significant increase of E-cadherin protein staining after exposure to both perifosine and perifosine/gemcitabine combination (data not shown).

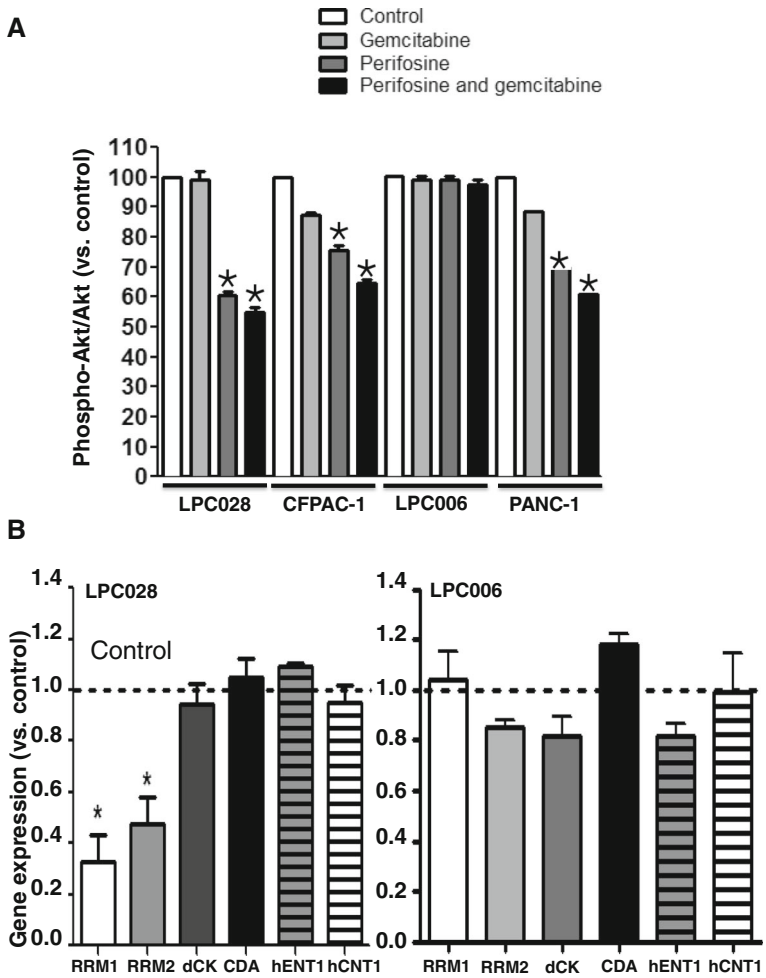

Fig. 3 Modulation of phospho-Akt and gemcitabine determinants. **a** Effect of 24-h exposure to gemcitabine, perifosine or their combination, at IC_{50} values, on the expression of phospho-Akt, normalized to the expression of total Akt, as determined by ELISA. **b** Expression of gemcitabine key determinants in LPC028 (*left panel*) and LPC006 (*right panel*) cells treated with perifosine at IC_{50} versus untreated cells, as determined by qRT-PCR. *Columns* mean values obtained from three independent experiments, *bars*, SEM. *Dashed line*, values in untreated samples (Control). *Significantly different from controls

Perifosine and its combination with gemcitabine affect cell cycle

Perifosine, gemcitabine and their combination affected cycle distribution of PDAC cells, as summarized in Additional file 2: Table S1. 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 h, 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%. Comparable perturbations of cell cycle were observed in the CFPAC-1 and PANC-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 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 (Additional file 2: Table S1). 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).

Moreover, we evaluated the variation of mitochondrial membrane potential in LPC028, LPC006, PANC-1, and

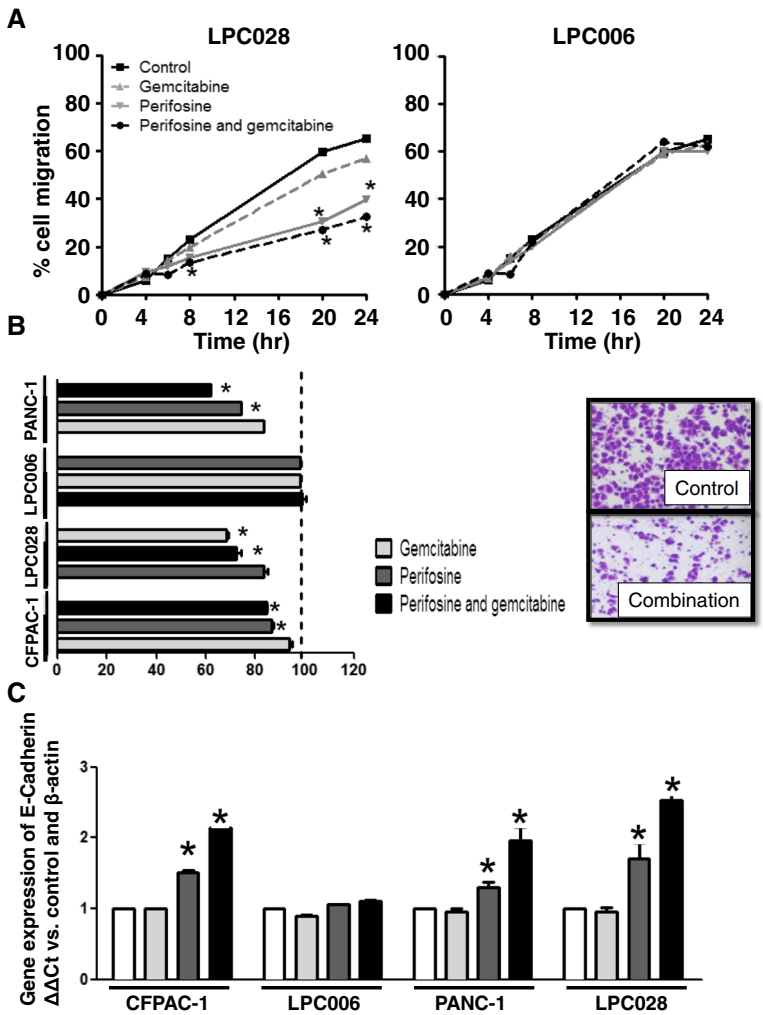

Fig. 4 Effects of perifosine, gemcitabine and their combination on PDAC cells migration and invasion. **a** Results of wound-healing assay in LPC028 and LPC006 cells exposed to perifosine, gemcitabine or to their combination, at IC50 values for 24 h. **b** Results of invasion studies in the PDAC cells exposed for 24 h to perifosine, gemcitabine, or to their combination, at IC_{50} values (insert: representative pictures of LPC028 cells at 24 h, original magnification ×40). **c** Modulation of *E-cadherin* mRNA levels in LPC028, LPC006, PANC-1, and CFPAC-1 cells after 24-h exposure to perifosine, gemcitabine, or to their combination, at IC_{50} values, as determined by qRT-PCR. *Columns* or *points* mean values obtained from three independent experiments; *bars*, SEM. *Significantly different from controls

CFPAC-1. As shown in Fig. 5a, the combination perifosine gemcitabine causes an increase of mitochondrial membrane potential in LPC028, PANC-1, and CFPAC-1 cells.

Further analysis of cell death by the Annexin-V/PI assay confirmed the induction of apoptosis by perifosine. Perifosine increased both early and late apoptosis, as shown in Fig. 5b (left panel) for the LPC028 cells. Moreover, the combination of perifosine and gemcitabine significantly increased the percentage of late apoptotic cells up to 26%. Similar results were observed in CFPAC-1 and PANC-1 cells (Additional file 1: Figure S9), whereas no apoptosis induction was detected in LPC006 cells (Fig. 5b right panel).

Perifosine and its combination with gemcitabine activate caspases and pro-apoptotic factors, and downregulate 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 pro-apoptotic and anti-apoptotic proteins. As shown in Fig. 5c, perifosine and its combination with gemcitabine were able to increase the activity of caspase-3/-6/-8/-9 in LPC028 as well as CFPAC-1 and PANC-1 (Additional file 1: Figure S10) but not in the LPC006 cells, as determined by specific fluorometric

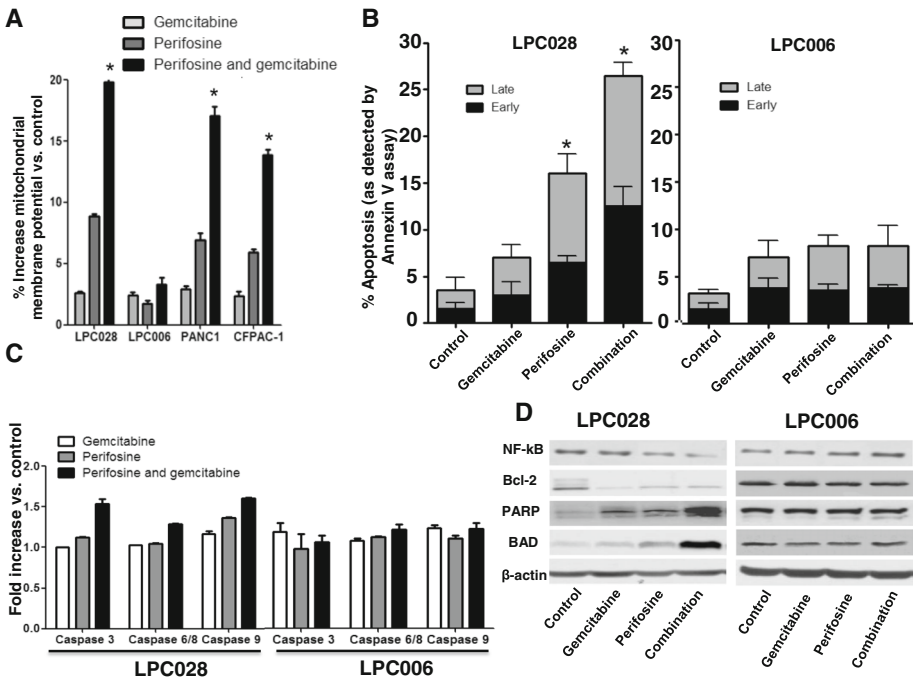

Fig. 5 Apoptosis induction by perifosine, gemcitabine and their combination. **a** Mitochondrial membrane potential (as assessed by (DiOC) labelling) in LPC028, LPC006, PANC-1, and CFPAC-1 cells. **b** Annexin-V assay in LPC028 and LPC006 cells. **c** Modulation of caspase-3, caspase-6/-8/ and caspase-9 in LPC028 and LPC006 cells, as determined by a specific fluorometric assay described in the Methods section. **d** Representative Western blot pictures of apoptosis determinants in LPC006 and LPC028 cells. All these results refer to cells exposed for 24 h to perifosine, gemcitabine. or their combination at IC_{50s}. *Columns*, mean values obtained from three independent experiments; *bars*, SEM. *Significantly different from controls

caspase activity assays. However, Western blot analyses demonstrated the modulation of other important apoptotic markers. In particular, perifosine and perifosine/gemcitabine combination increased the expression of PARP and BAD, while reducing Bcl-2 and NF-kB expression in LPC028 cells. Conversely, none of these proteins was affected by the exposure to perifosine and its combination with gemcitabine in the LPC006 cells (Fig. 5d).

Glut1 is overexpressed in the cells resistant to Akt inhibition, while its inhibition significantly reduces cell growth and induces apoptosis after gemcitabine/perifosine treatment

Since major oncogenic signaling pathways have been linked to increased glucose metabolism, and previous studies showed that stimulation of Akt1 induces Glut1 mRNA and protein accumulation, [43] we evaluated the expression of this key glucose transporter in the LPC028 and LPC006 cells. As shown in the Fig. 6a, *Glut1* mRNA levels were significantly reduced after treatment with perifosine alone and in combination with gemcitabine in the LPC028 and PANC-1 cells, whereas no modulation was detected in the LPC006 cells. However, since PI3K/AKT/mTOR signaling seems to play an essential role in trafficking of Glut1 from recycling endosomes and/or retention of Glut1 at the plasma membrane [44], we performed further studies to evaluate the amount of membrane-bound Glut1 with FACS analysis (Fig. 6b). In the LPC028 cells, we observed a significant reduction ($P < 0.05$) of the membrane-bound expression of Glut1 after treatment with perifosine (56% compared to untreated cells). Further studies with Western blot clearly demonstrated the overexpression of Glut1 in the LPC006 compared to the LPC028 and PANC-1 cells. A high expression of Glut-1 was also observed in PANC-1 cells (Fig. 6c). Moreover, Glut1 expression was not reduced by Akt inhibition (Fig. 6c). We therefore investigated whether inhibition of Glut1 by the novel specific compound PGL13 (Fig. 6d) can at least in part overcome the inherent resistance of the LPC006 cells to perifosine and other Akt inhibitors. Remarkably, the Glut1 inhibitor alone caused only a slight reduction of cell growth (<10%), but its combination with perifosine reduced significantly the percentage of surviving cells compared to perifosine alone (Fig. 6e). Furthermore, the combination of PGL13 with both perifosine and gemcitabine led to a more dramatic drop in the number of surviving cells, up to −81%, compared to control which was associated with strong apoptosis induction, as detected by characteristic apoptotic nuclear morphological

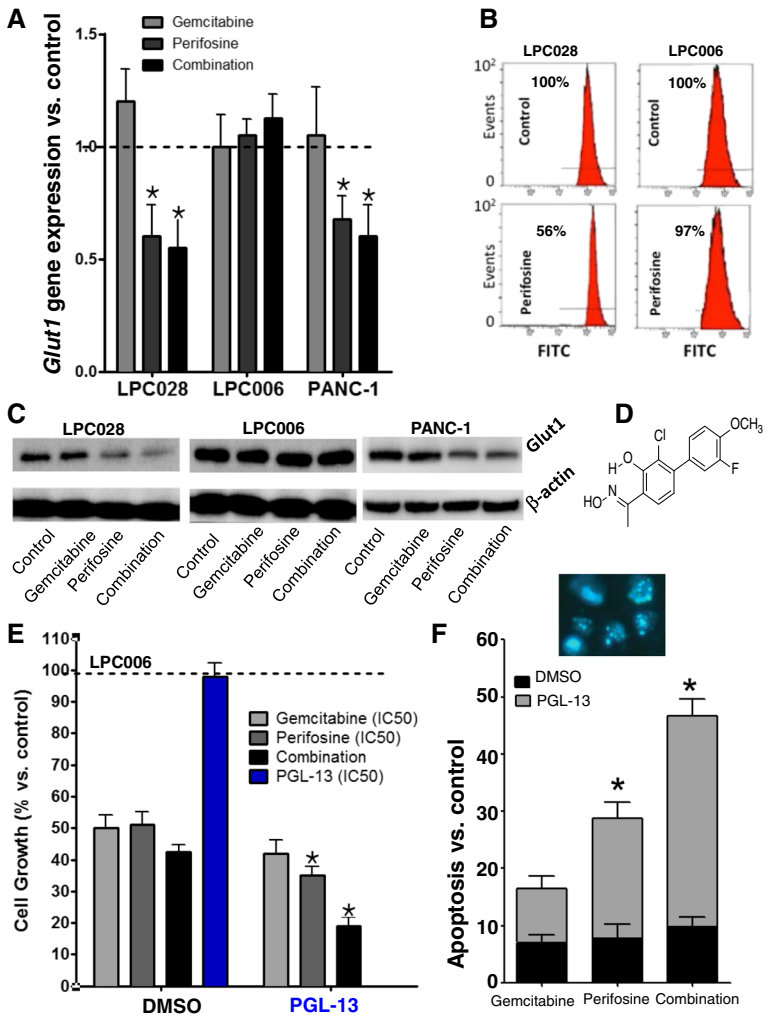

Fig. 6 Role of Glut1 expression and inhibition in cell growth and apoptosis induction by perifosine, gemcitabine, and their combination. **a** Modulation of *Glut1* mRNA levels in LPC028, LPC006, and PANC-1 cells after 24-h exposure to perifosine, gemcitabine, or to their combination, at IC_{50} values, as determined by qRT-PCR. **b** Representative of Glut1 membrane-bound expression in LPC006 and LPC028 cells exposed for 24 h to perifosine at IC_{50s}; **c** Representative Western blot pictures of Glut1 expression in LPC006, LPC028, and PANC-1 cells exposed for 24 h to perifosine, gemcitabine, or their combination at IC_{50s}. **d** Structure of the compound PGL13. **e** Cell growth inhibition in LPC006 cells after 72-h exposure to perifosine, gemcitabine, or to their combination, at IC_{50} values, together with DMSO or with the Glut1 inhibitor PGL13, at 30 μM. **f** Apoptosis induction by perifosine, gemcitabine, and their combination as assessed by bisbenzimide staining as described in the Methods section (insert: representative pictures of apoptotic LPC006 cells after treatment with perifosine and gemcitabine, original magnification ×40). *Columns*, mean values obtained from three independent experiments; *bars*, SEM. *Significantly different from cells treated with DMSO

features with fluorescence microscopy. In particular the LPC006 cells exposed to PGL13 with both perifosine and gemcitabine had an apoptotic index of 27%, which was similar to the apoptotic index of the LPC028 cells treated with perifosine and gemcitabine (Fig. 6f). The effect of a combined Akt inhibitor/anti-Glut1 treatment was further tested with MK-2205 and NVP-BEZ235, where it led to a −14% and −20% decrease in cell viability compared with these drugs alone (Additional file 1: Figure S11). Thus, inhibition of Glut1 promoted anti-Akt-mediated cell death, and this combined treatment shows promise for future investigation in the treatment of PDAC.

Discussion

The present study supports a role for phospho-Akt as a prognostic factor in PDAC patients, and unravels its potential role as a target for the synergistic interaction of anti-Akt agents and gemcitabine through modulation of apoptotic and invasive processes.

Several studies demonstrated that PDAC tissues have increased activation of the PI3K/Akt, as assessed with the phosphorylation of Akt, and this has been associated with higher histological tumor grade [45] and worse prognosis [46, 47]. In the present study, we further explored the clinical relevance of phospho-Akt by

screening its expression in a homogeneous cohort of 100 surgically resected PDACs. In agreement with the previous studies, even considering several clinicopathological parameters, phospho-Akt expression was the only factor correlated to differential clinical outcome. However, a systematic review and meta-analysis of prognostic tissue biomarkers for PDAC, including phospho-Akt among the 22 markers associated with limitless replicative potential eligible for examination, showed that only Ki-67 maintained statistically significant associations with outcome [48]. These discrepancies might be attributed to the different experimental procedures used, including antigen retrieval technique, antibody characteristics, and dilution, as well as observer variability in staining pattern description and cutoff point selection. Therefore, in the present study we have chosen an antibody that was previously validated in an immunohistochemical study on 102 colorectal cancer FFPE samples [49] and we have used image analysis software to calculate expression as a continuous parameter, in order to facilitate the identification of cutoff points. This method allowed the assignment of the specimens to different categories, including a subset of tissues (about 14%) characterized by extremely high expression of phospho-akt, which could clearly influence the prognostic value. Indeed this cutoff point identified a group of patients with very poor outcome, who should be treated with more aggressive, novel therapeutic approaches.

Of note, recent genomic studies showed that the PI3K/Akt signaling is among the core signaling pathways leading the intrinsic aggressiveness of PDAC, suggesting that in the *PDAC actionable genome* about 9 and 6% of the cases are Akt- and PI3K-dependent, respectively [50]. These data underline the potential importance of specific inhibitors of PI3K/Akt as novel effective therapeutics in a selected subpopulation of PDAC patients. Moreover, activation of this signaling pathway is associated with PDAC chemoresistance [13, 51], supporting the hypothesis that Akt inhibitors might also be used to overcome resistance towards conventional cytotoxic agents.

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 [52]. However, due to the unfavorable pharmaceutical properties, toxicity, and cross-over inhibition of other lipid and protein kinases, these compounds were not used in clinical studies [51].

More recently, several small molecules that inhibit the PI3K/Akt signaling entered clinical development, but more information on their activity in the preclinical setting is warranted. For instance, a recent study showed the potential inhibition of autophagy by perifosine demonstrating that this drug impairs the autophagic flux in HepG2 and U87 MG cells, which is related to defects in intracellular cholesterol transport [53]. These results might be relevant for PDAC because some research lines point at autophagy as a tumor-promoting mechanism. Although a better understanding of the complexity of autophagy is needed, the modulation of this process might therefore open new opportunities for the therapeutic use of autophagy inhibitors [54]. Further research to identify the precise mechanisms of autophagy maturation may therefore provide a new insight into the antiproliferative action of perifosine.

Our results demonstrate that perifosine is the targeted anti-PI3K/Akt antitumor agent demonstrating the most potent growth inhibitory effects in a panel of human PDAC cells characterized by distinct molecular properties. Limited published preclinical research focusing on this issue in PDAC reported similar cytotoxic activity of perifosine in PANC-1, MIA PaCa-2, and AsPC-1 cells [55]. Sensitivity to perifosine in the PDAC cells also fell within the range of IC_{50} values previously reported in PDAC cell lines and spheroids for other Akt inhibitors, such as NVP-BEZ-235 [56, 57].

Furthermore, perifosine interacted synergistically with gemcitabine 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. These results are in agreement with previous studies showing synergistic interaction of gemcitabine with perifosine in PANC-1 cells and xenografts [55] as well as enhanced apoptotic cell death after combined treatment with paclitaxel in chemoresistant ovarian cancer cells [58].

However, most previous studies were performed in ATCC cell lines, which showed similar results [59], while, to more effectively develop targeted compounds, it will be helpful to understand why these agents fail when they do. Thus, in the present study, cell growth inhibitory effects of perifosine, gemcitabine, and their combination were evaluated in several representative PDAC cells, including primary PDAC cell cultures. 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 effects only in the cells with high phospho-Akt or intermediate/high values of *Akt1* mRNA, as determined by RT-PCR. Conversely, this combination was antagonistic in the cells with low *Akt1*, and phospho-Akt1 expression. An important limitation of our findings is the use of a single-cell culture (LPC006) as a model of low phospho-Akt1. However, the results in two PDAC models (LPC028 and PANC-1) with high phospho-Akt1 levels were similar. These data suggest that the expression and activation of Akt might therefore be used to tailor perifosine therapy.

Importantly, two specific ELISA for the Akt Ser473 and the Thr308 phosphorylation showed that perifosine effectively reached and inhibited its targets in the LPC028 and PANC-1 cells, and the combination with gemcitabine additionally inhibited Akt activation in these cells. The present study demonstrated also 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 RRM1 and RRM2 in the cells with a high expression of Akt, while this effect was not statistically significant in the cells with low Akt expression. RR is a key target of gemcitabine activity and previous studies correlated the expression of its subunits to gemcitabine sensitivity in PDAC cells [60, 61]. Therefore, the synergistic interaction between perifosine and gemcitabine might be explained, at least in part, by the modulation of gemcitabine sensitivity through RRM1 and RRM2 suppressions.

However, our results suggested that the synergistic interaction of perifosine with gemcitabine is associated with other important molecular mechanisms affecting PDAC aggressiveness (Fig. 7). In agreement with previous observations showing the reduction of cell migration/invasion through Akt inhibition [16, 62], 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 [42], we demonstrated that perifosine increased the expression of E-cadherin in LPC028, CFPAC-1, and PANC-1 cells. This can at least in part explain our findings on the reduction of migration determined by perifosine. Furthermore, Toll et al. [63] showed that decreased E-cadherin was associated with poor prognosis of PDAC patients, supporting the studies on novel compound which can modulate the expression of this protein.

Since the Akt signaling pathway plays an important role in cell survival process, its blockage can result in activation of programmed cell death [15]. Thus, we

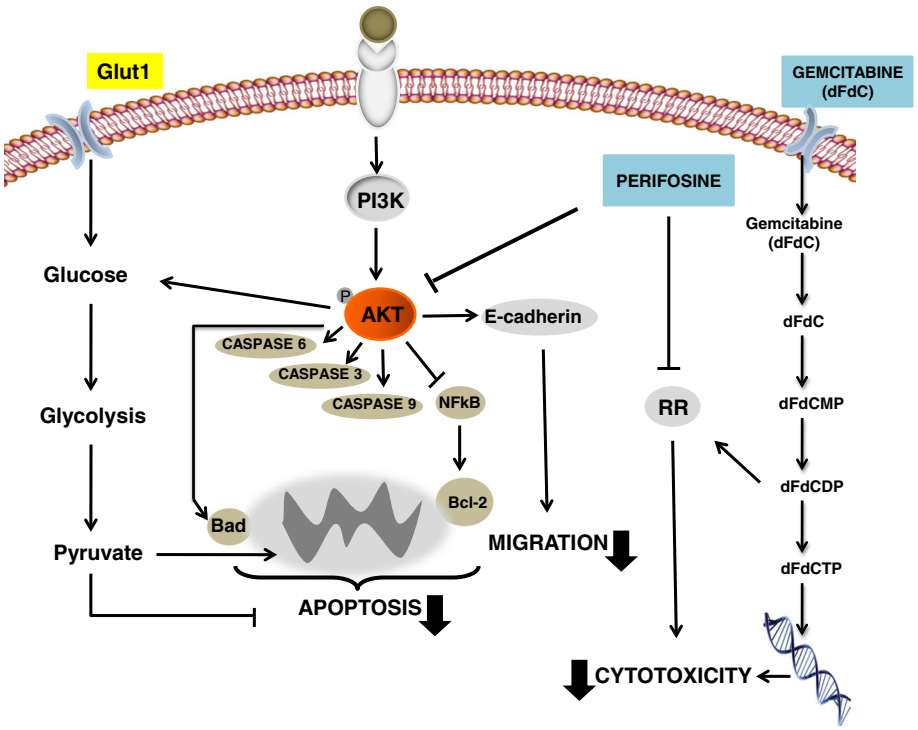

Fig. 7 Molecular mechanisms involved in the synergistic interaction of perifosine with gemcitabine. The main upstream activator of Akt is phosphatidylinositol-3 kinase (PI3K), which is activated in the response to a variety of growth stimuli through receptor tyrosine kinases and G protein-coupled receptors. 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. Moreover, Akt inhibition reduce Glut1 activity reducing glucose influx and thereby favouring apoptosis induction and sensitizing PDAc cells to treatment with cytotoxic agents

further evaluated the effect of perifosine on cell cycle perturbation and apoptosis induction. Previous findings on the effect of perifosine after 24 h treatment showed induction of G_2/M arrest, potentially favoring the activity of 6-thioguanine [64]. Our results showed that after 72 h, perifosine treatment was associated with an increase in the percentage of cells in the G_0/G_1, and 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 multiple methods, such as 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 critical factors involved in the apoptotic cascades. Previous studies showed that drug-induced Akt deactivation was associated with activation of pro-apoptotic factors, including caspase-9 and BAD, as well as with a parallel decrease in the expression of the anti-apoptotic factors Bcl-2 and NF-kB [22, 65]. Our studies showed similar results after exposure of the PDAC cells to perifosine.

Despite increasing evidence on the pivotal role of PI3K/Akt signaling in cancer, the strategies to hit PI3K/Akt/mTOR pathway have failed to demonstrate therapeutic activity in most ongoing clinical trials, and a previous phase II study testing perifosine in previously untreated patients with locally advanced, unresectable, or metastatic PDAC, was terminated as a result of unacceptable adverse events [66].

It is already known that in PDAC cells, dual PI3K-mTOR inhibition induces rapid overactivation of MAPK pathway through a PI3K-independent pathway [67], and that drug resistance may be overcome by inhibition of parallel oncogenic-dependent pathways, such as with the dual MEK and PI3K/mTOR blockade [57].

One strategy to overcome resistance consists into identifying key molecular differences in the tumors that are less likely to respond. Oncogenic *KRAS* drives metabolic reprogramming in tumor cells by increasing aerobic glycolysis, and recent studies showed that subtypes of PDAC cells with distinct metabolite levels associated with glycolysis, lipogenesis, and redox pathways, confirmed at the transcriptional level. The glycolytic and lipogenic subtypes showed striking differences in glucose and glutamine utilization, as well as mitochondrial function, and corresponded to differences in cell sensitivity to inhibitors of glycolysis, glutamine metabolism, lipid synthesis, and redox balance [68]. In the present study we demonstrated that the resistant LPC006 cells were characterized by overexpression of Glut1. Remarkably, the inhibition of Glut1 dramatically enhanced perifosine and perifosine/gemcitabine-induced cell death, suggesting a cooperativity between Akt inhibitors and Glut1 inhibition. Agents directly inhibiting Glut1 are in early phase evaluations, and a few preclinical studies have demonstrated that Glut inhibitors led to diminish tumor growth in vitro and in vivo [69]. However, the altered expression of Glut1 might also influence the sensitivity of tumor cells to chemotherapy, since a recent study showed that the knockdown of *Glut1* sensitizes head and neck cancer cells to the chemotherapy drug cisplatin [70]. To our knowledge, this is the first study showing that Glut1 inhibitors can restore the repression of aerobic glycolysis induced by PI3K/mTOR inhibitors in resistant cells, and favor their synergistic interaction with cytotoxic compounds. These results should prompt further studies to understand how PDAC cell metabolism might affect sensitivity to new anti-signaling therapies and to identify promising therapeutic targets that might be exploited by combination therapies.

Conclusions

Our data support the analysis of phospho-Akt expression as both a prognostic and a predictive biomarker, for the rational development of novel therapies targeting the Akt pathway in PDAC. In particular, we observed that phospho-Akt expression levels influence the antitumor activity of perifosine, as well as the synergistic interaction with gemcitabine, through its ability to attack key mechanisms involved in the proliferation, cell cycle control, apoptosis and migration/invasion properties. Finally, we demonstrated that inhibition of Glut1 overcame resistance to this combination treatment and might provide the basis for the development of new therapeutic approaches with Akt inhibitors in patients with PDAC.

Additional files

Additional file 1: Figure S1. PFS curves according to phospho-Akt expression in radically-resected PDACs, showing that patients with high and "very high" phospho-Akt (right panel) had a significantly worse PFS. **Figure S2.** Growth inhibitory effects after MK-2206 exposure in LPC006 (72-hours). **Figure S3.** Growth inhibitory effects after 72 hours exposure to perifosine, gemcitabine or their combination at a fixed ratio based on IC50 values in CFPAC-1 and PANC-1 cells. On the X-axis the drug concentrations for the combination are referred to gemcitabine. **Figure S4.** Phospho-Akt (serine residue-473) expression, normalized to total Akt, after 4-hour exposure, as determined by ELISA. **Figure S5. A** Phospho-Akt (serine residue-473) expression, normalized to total Akt, after 24-hour exposure. **B** Phospho-Akt (threonine residue-308) expression, normalized to total Akt, after 24-hour exposure, as determined by ELISA. **Figure S6.** Expression of gemcitabine determinants in PANC-1 cells treated with perifosine, as determined by qRT-PCR. Dashed line, values in untreated samples. **Figure S7.** Wound-healing assay in CFPAC-1 and PANC-1 exposed to perifosine, gemcitabine or their combination (IC50 values, 24 hours). **Figure S8.** Wound-healing assay in LPC006 exposed to MK-2206, gemcitabine or to their combination (IC50 values, 24 hours). **Figure S9.** Annexin-V assay in LPC028 and LPC006. **Figure S10**: Modulation of caspase-3, caspase-6/-8/ and caspase-9 in CFPAC-1 and PANC-1, as determined by a specific fluorometric assay. **Figure S11.** Cell growth inhibition in LPC006 cells after 72-hour exposure to MK-2205, NVP-BEZ235 at IC50 values, together with DMSO or with the Glut1 inhibitor PGL13, at 30 μM. *Points*, or *Columns*, mean values obtained from three independent experiments; bars, SEM. *Significantly different from controls. (PPTX 328 kb)

Additional file 2: Table S1. Effects of gemcitabine and perifosine and their combination on cell cycle distribution and on cell death (sub-G1). (DOCX 14 kb)

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Availability of data and materials
All data generated or analysed during this study are included in this published article (and its supplementary information files).

Authors' contributions
EG was the principal investigator and takes primary responsibility for the paper; UB, NF, FM, CG, BA provided patient samples, clinical data, and drugs; DM, AA, MM, AVK, RR, and CG performed the research; EG, AR, and GJP designed the research; DM and EG wrote the paper; AR, GJP, FM, and LGL edited the paper. All authors read and approved the final manuscript.

Competing interests
The authors have no conflict of interest to disclose. B. Aicher is an employee and stock option holder of Aeterna Zentaris GmbH.

Consent for publication
Consent to publish has been obtained from all the participants (or legal parent or guardian for children) to report individual patient data.

Ethics approval and consent to participate
This study was approved by the Local Ethics Committee of the University of Pisa. Date of approval: July 3, 2013 (file number 3909).
All patients have provided a written informed consent to participate to this study.

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Review

Triple negative breast cancer: shedding light onto the role of pi3k/akt/mtor pathway

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ABSTRACT

Breast cancer is one of the most widespread carcinoma and one of the main causes of cancer-related death worldwide, especially in women aged between 35 and 75 years. Among the different subtypes, triple negative breast cancer (TNBC) is characterized by the total absence of the estrogen-receptor (ER) and progesteron-receptor (PR) expression as well as the lack of human epidermal growth factor receptor 2 (HER2) overexpression or gene amplification. These biological characteristics confer to TNBC a higher aggressiveness and relapse risk along with poorer prognosis compared to other subtypes. Indeed, 5-years survival rate is still low and almost all patients die, despite any adjuvant treatment which at moment represents the heading pharmacological approach. To date, several clinical trials have been designed to investigate the potential role of some molecular markers, such as VEGF, EGFR, Src and mTOR, for targeted treatments in TNBC. In fact, many inhibitors of the PI3K/AKT/mTOR pathway, frequently de-regulated in TNBC, are acquiring a growing interest and several inhibitors are in preclinical development or already in early phase clinical trials. In this Review, we investigated the role of the PI3K/AKT/mTOR pathway in TNBC patients, by summarizing the molecular features that led to the distinction of different histotypes of TNBC. Furthermore, we provided an overview of the inhibition mechanisms of the mTOR and PI3K/AKT signaling pathways, highlighting the importance of integrating biological and clinical data for the development of mTOR inhibitors in order to implement targeted therapies for TNBC patients.

INTRODUCTION

Breast cancer (BC) is one of the most widespread carcinoma and one of the main causes of cancer-related death worldwide especially in women aged between 35 and 75 years [1]. In the last few years new molecular markers have been studied to provide new insights on BC heterogeneity but also to better understand and predict tumor behavior during treatment. It is now well established that BC can be classified into different groups according to gene expression profiles [2-5]. This new classification will certainly provide new insights into the BC biology and will probably drive treatment decisions in the near future by microarray analysis that soon will switch the clinical approach to different illnesses by giving a huge support to conventional pathology (morphology and immunohistochemistry) [6].

The different BC subgroups detected by their different gene expression profiling are below described as discussed in San Gallen Expert Consensus report:

Luminal A subgroup is characterized by estrogen/progesterone receptor (ER/PR) positivity, lower expression of Ki-67 (< 20%) and HER2 lack, accounting for the 50% of all invasive BCs; Luminal B subgroup results characterized by ER/PR positivity or variable expression of HER2 (+ or -), accounting for the 10-20% of all invasive BCs [7]; HER2 overexpression subtype is characterized by ER/PR negativity and HER2 strong positivity. This subtype accounts for 15% of all invasive breast cancer; Basal like breast cancer (BLBC) subtype exhibits an expression profile similar to that of the epithelial cell mammary tissue and includes triple negative breast cancer (TNBC) [8-10].

Among the different subgroups, TNBC presents biological characteristics that confer higher aggressiveness and relapse risk along with worse outcome in comparison to other subgroups. Several studies showed that PI3K/AKT/mTOR signaling is often altered in TNBC patients [11].

SELECTION CRITERIA

We have searched trials using Medline (PUBMED), EMBASE and COCHRAINE database, the following search strategy ("TNBC" [MESH] AND ("PI3K" [tiab] OR "mTOR" [tiab]) AND ("ER" [tiab] OR "PgR" [tiab]) and free text terms as "PI3K" [tiab], "triple negative breast cancer" [tiab] and "mTOR" [tiab].

TRIPLE NEGATIVE BREAST CANCER

TNBC is characterized by lack of ER and PR expression as well as the absence of HER2 [12, 13]. The percentage of new TNBC diagnosis is variable, but it mainly ranges between 9% and 16% with a higher frequency in young women carrying *BRCA1* gene mutation, showing a strong correlation with ethnic origin (in particular, African-American and Hispanic women) [14-17]. TNBC also shows greater size and tumor burden, and often is a more aggressive high grade tumor [18, 19].

TNBC patients show a higher susceptibility to develop metastases, resulting in an unfavorable clinical outcome compared to other subgroups [20-22].

Although TNBC patients initially respond to neoadjuvant treatments, only 30% of them will exhibit a survival higher than 5-years following the first diagnosis, reflecting the aggressiveness of this subtype [23, 24]. Patients with *BRCA1* mutation are often diagnosed with TNBC but not all TNBC are *BRCA1* positive. Nevertheless, it been shown that TNBC not carrying *BRCA1* mutation, behave similarly to *BRCA1*-deficient tumors, showing also similar gene expression profiles [25, 26].

The growing interest in the TNBC biology allowed to develop trials investigating new drug targeting potential biomarkers such as VEGF, EGFR, Src and mTOR [27]. Moreover, the introduction of tumor molecular features in the characterization of TNBC has led to a further subtype labeling. Indeed, 6 new TNBC subtypes have been identified [28]:

Basal-like 1 (BL1) and Basal-like 2 (BL2) subtypes: both are characterized by up-regulation of gene and cellular markers mainly implicated in cell growth. In fact, Ki-67 expression and nuclear fraction staining are higher (BL1+BL2 = 70%) if compared to other subtypes (42%). All these features combined together indicate that more efficient treatment for BL TNBCs could be that directed against the mitotic apparatus such as a taxane-based therapy [29-32]. Furthermore, BL2 shows the involvement of a different plethora of growth factors and receptors (EGF, EGFR, NGF, MET, Wnt/β-catenin, IGF1R and EPHA2).

Immunomodulatory subtype: it is characterized by immune system gene signature similar to that of medullar BC determining its better clinical outcome.

Mesenchymal and mesenchymal stem-like subtypes: both are characterized by increased expression of gene and cellular markers involved in cell motility (Rho pathway), extracellular matrix-receptor interaction and differentiation (Wnt/β-catenin, ALK, TGF-β pathways). The mesenchymal stem-like subtype shows reduced expression of proliferative genes and enrichment of genes involved in several signaling pathways, including the inositol phosphate-dependent signaling pathway, EGFR, PDGF, and ERK1/2 signaling. Moreover, notable is the contribute of the adipocytokine signaling and ABC transporter. Both subtypes exhibit gene expression pattern and chemoresistance similar to metaplastic BC [28].

Luminal androgen receptor (LAR) subtype: this TNBC subgroup is ER-negative and is characterized by high deregulation of hormone-dependent pathways. In particular, the androgen receptor pathway seems to play a pivotal role in inducing expression of specific genes of the LAR subtype [33-35].

Indeed, androgen receptor mRNA expression has been shown to be considerably increased (9-fold) with respect to the other subtypes. Furthermore, tumors here classified show the up-regulation of a plethora of downstream targets and co-activators of the androgen receptor signaling [36-38].

TARGET THERAPY IN TNBC

The major issue for targeted therapy against TNBC is the lack of specific oncogene drivers due to wide BC heterogeneity [39-41].

To date, the main approach in TNBC treatment remains the chemotherapy, in particular the administration of anthracyclines, taxanes and/or platinum compounds able to target dividing cells. Unfortunately, not all chemotherapy treated patients show a favorable outcome and is still unclear whether treatment choices should be

personalized among the different TNBC subtypes [42, 43].

Maybe a possible solution would be represented by the new proposed genetic signature tools as suggested by the recent MINDACT trial results, in the order to avoid the aggressive treatment to non-responder patients. MammaPrint genetic study allowed to identify a large group of patients which showed a good five-year progression-free survival (PFS) good though they have not received adjuvant treatment (AACR Annual Meeting 2016).

Indeed, for pre-operative treatment pCR (pathological Complete Response) would represent the best surrogate survival end-point for TNBC patients and it results doubled if platinum compounds are added to conventional therapy compared to the worse outcome achieved by TNBC patients showing residual disease [44, 45].

Given the aforementioned issues for management of TNBC patients, studies are urgently needed to improve the use of target therapies. The major difficulty is to discover actionable target because of wide heterogeneity of the disease. In fact, clinical trials on TNBCs that aim to point out a particular receptor fail to demonstrate an evident clinical benefit. One of the most important involved receptors is EGFR, that is upregulated in about 60% of TNBCs, whose trial investigating chemotherapy plus EGFR targeted agent *versus* chemotherapy alone showed a modest advantage in terms of response rate (RR) (33% vs 28%) [46]. Among the reasons why studies were not able to underline a significant clear advantage of these new proposed drugs, we should not take into account the heterogeneity of the disease that probably masks the real effect of the drug in a smaller population carrying the right target [47]. Recent studies are investigating a number of promising molecules and, thanks to some favourable hopeful results, a growing interest is developing about some specific signaling pathways such as PI3K/AKT/mTOR. [48-50].

PI3K/AKT/mTOR signaling pathway

PI3K/AKT/mTOR (PAM) represents the main signaling pathway responsible for cell proliferation, survival, metabolism and motility regulation and is often activated in BC [51-54] (Figure 1). A heterodimeric molecule belonging to the lipid kinases, phosphoinositide 3-kinase (PI3K), is the major component of this pathway. Based on structure, regulation mechanism and lipid substrate specificity, they can be categorized in three classes, but the class I PI3K is the more dysregulated in cancer [55].

PI3K signaling pathway starts following the binding of a growth factor or ligand to a variety of tyrosine kinase (TK) receptors, including HER proteins and IGF-1 receptors [56-58].

In its activated form PI3K phosphorylates phosphatidylinositol 4,5-bisphosphate (PIP2) to phosphatidylinositol 3,4,5-triphosphate (PIP3) which represents the docking site for AKT kinase. AKT activation leads to protein synthesis and cell growth by activating mTOR through TSC1/2 [59-61].

The main PI3K counteracting protein is the PTEN phosphatase, which acts by converting PIP3 to PIP2 [62]. Therefore, PIP3 results activated by PI3K and negatively controlled by PTEN [63].

Moreover, PIP3 levels seem to be also tightly modulated by another tumor suppressor, inositol polyphosphate 4-phosphatase type II (INPP4B), which dephosphorylates PIP3 to PIP2 [64].

Many research works report a higher incidence of *PTEN* and *PI3K* mutations in TNBC patients with respect to other histological subtypes [65].

A downstream component of PI3K/AKT pathway is mTOR which exists in two functionally different complexes, mTOR complex 1 (mTORC1) and mTOR complex 2 (mTORC2). mTORC1 is responsible for the activation of protein translation process by promoting mRNA translocation and is also involved in metabolism and lipid synthesis [66]. mTOR downstream substrate is S6K1 which in turn can phosphorylate estrogen determining its activation with a mechanism independent of the ligand [67-69].

On the other hand, mTOR complex 2 is involved in the organization of actin cytoskeleton and, at the same time, regulates AKT phosphorylation. The importance of mTOR complexes and their pathways is fundamental in clinics due to the ability of many drugs to target selectively mTORC1 [70]. Indeed, studies conducted on TNBC murine models highlighted the effects of the inhibitor Dactolisib in controlling the whole mTOR pathway [71]. The frequency of mTOR pathway activation is higher in TNBC compared to other subtypes and is often correlated with poor prognosis [22, 72, 73]. Moreover, the up-regulation of PAM signaling induces resistance to hormone treatment, HER2-targeted treatment and cytotoxic therapy [74].

MAMMALIAN TARGET OF RAPAMYCIN INHIBITORS

Everolimus (RAD001) is a mTOR inhibitor exhibiting vast anticancer activity in preclinical studies [75]. The combined treatment of rapamycin with paclitaxel in cell lines altered in the PI3K/AKT/mTOR signaling has been shown to increase effectiveness of treatment in TNBC [76]. This rationale has been explored in different clinical experiences. In a phase II study, Meyer and collaborators investigated the addition of everolimus 5 mg/day for 12 weeks to a short course pre-operative chemotherapy regimen containing weekly cisplatin (25

Table 1: Ongoing trials studying the role of mTOR inhibitors in TNBC

TRIAL	REGISTRATION NUMBER	INVESTIGATOR INSTITUTION
Phase Ib/II Trials of RDA001 in Triple Negative Metastatic Breast Cancer	NCT01939418	National Cancer Center, Korea
A study of Lapatinib in combination with Everolimus in patients with Advanced, Triple Negative Breast Cancer	NCT01272141	Emory University Winship Cancer Institute
Liposomal Doxorubicin, Bevacizumab and Temsirolimus (DAT) in Triple-Negative Breast Cancer (TNBC) Insensitive to Standard Neoadjuvant chemotherapy	NCT02456857	M.D. Anderson Cancer Center
Comparison of Single-Agent Carboplatin vs the Combination of Carboplatin and Everolimus for the Treatment of Advanced Triple-Negative Breast Cancer	NCT02531932	Icahn School of Medicine at Mount Sinai
Eribulin Mesylate and Everolimus in Treating Patients With Triple-Negative Metastatic Breast Cancer	NCT02120469	City of Hope Medical Center
NECTAR Everolimus Plus Cisplatin (-) Breast Cancer (NECTAR)	NCT01931163	The Methodist Hospital System
Safety and Tolerability of Everolimus in Combination With Eribulin in Triple-negative Breast Cancer	NCT02616848	Istituti Ospitalieri di Cremona
A Study of AZD2014 in Combination With Selumetinib in Patients With Advanced Cancer (TORCMEK)	NCT02583542	Queen Mary University of London

Table 2: Ongoing trials studying the role of PI3K inhibitors in TNBC

TRIAL	REGISTRATION NUMBER	INVESTIGATOR INSTITUTION
Capecitabine +BKM120 TNBC Brain Mer	NCT02000882	US Oncology Research
Phase I Study of the Oral PI3kinase Inhibitor BKM120 or BYL719 and the Oral PARP Inhibitor Olaparib in Patients With Recurrent Triple Negative Breast Cancer or High grade Serous Ovarian Cancer	NCT01623349	Dana-Farber Cancer Institute
Phosphatidylinositol 3-kinase (PI3K) Alpha iNhibition In Advanced Breast Cancer (PIKNIC)	NCT02506556	Peter MacCallum Cancer Centre, Australia

mg/m²) + paclitaxel (80 mg/m²) in patients affected by stage II / III TNBC demonstrating that no improvement was detected in the pCR after surgery and RR, following the addition of RAD001 [77]. Another phase II randomized study aimed to investigate the addition of everolimus to paclitaxel in neoadjuvant sequential regimen containing anthracyclines. Fifty women affected by stage II/III TNBC were subjected to a therapy with paclitaxel 80 mg/mq for 12 weeks or paclitaxel 80 mg/mq + everolimus 30 mg/day orally for 12 weeks followed by an FEC scheme (5-FU 500 mg/mq, epirubicin 100 mg/mq and cyclophosphamide 500 mg/mq every 3 weeks for four cycles) [78]. The addition of everolimus, although well tolerated, did not add any significant benefit in terms of 12-week-RR (48% *versus* 30% in favour of everolimus) and pCR (30% *versus* 26% in favour of everolimus) [79].

For the same principles, everolimus was tested in combination with carboplatin. In particular, Singh et *al*. enrolled 25 patients affected by metastatic TNBC who underwent to a 3-weekly chemotherapy regimen containing carboplatin AUC6 (or decreased to AUC5/4) + everolimus 5 mg/day. The treatment has shown significant hematologic toxicity especially with regimens containing carboplatin AUC6/5, but was well tolerated with AUC4,

demonstrating a clinical benefit rate > = 6 months of 28% with a total mOS of 16.6 months and mPFS of 3 months [80].

Despite TNBC is HER2- [81], RAD001 has also been tested in a regimen with anti-HER2 drugs since EGFR is overexpressed and upregulated in about 50% of TN tumors [82, 83], providing a strong rationale to investigate the association between an anti-EGFR and a mTOR inhibitor in order to overlap the resistance to anti-EGFR agents [84, 85]. Although the mTOR inhibitors paradoxically trigger the AKT pathway [86], this activation could probably serve as resistance mechanism to mTOR inhibitors thus explaining the poor performance of these drugs when used as a single agent [78, 87]. On this basis, Liu et *al*. in their experience have shown that the addition of everolimus could sensitize BC cells to anti-EGFR drugs (lapatinib) [88], demonstrating that this association may be responsible for an increased apoptosis in some TNBC cell lines and murine xenograft progression compared to the same drugs used in monotherapy [89]. The main clinical studies concerning the function of mTOR inhibitors in TNBC and currently under evaluation are reported in Table 1.

In another important work, Zhang et *al*. created a panel of seven patient-derived orthotopic xenografts from primary and metastatic neoplastic tissue having histological and immunohistochemical features matched between patient and their corresponding xenografts. Neoplasms were divided on the basis of the above characteristics in different TNBC subtypes and the authors created a response signature to mTOR inhibitors demonstrating that BLBC also possessed the highest expression rate of the genes belonging to the PI3K/AKT pathway and the highest extent of phosphorylation of 4EBP1 [90].

Despite these promising results, it is not yet known the synergistic mechanism of action between the anti-EGFR and mTOR inhibitors and, furthermore, the

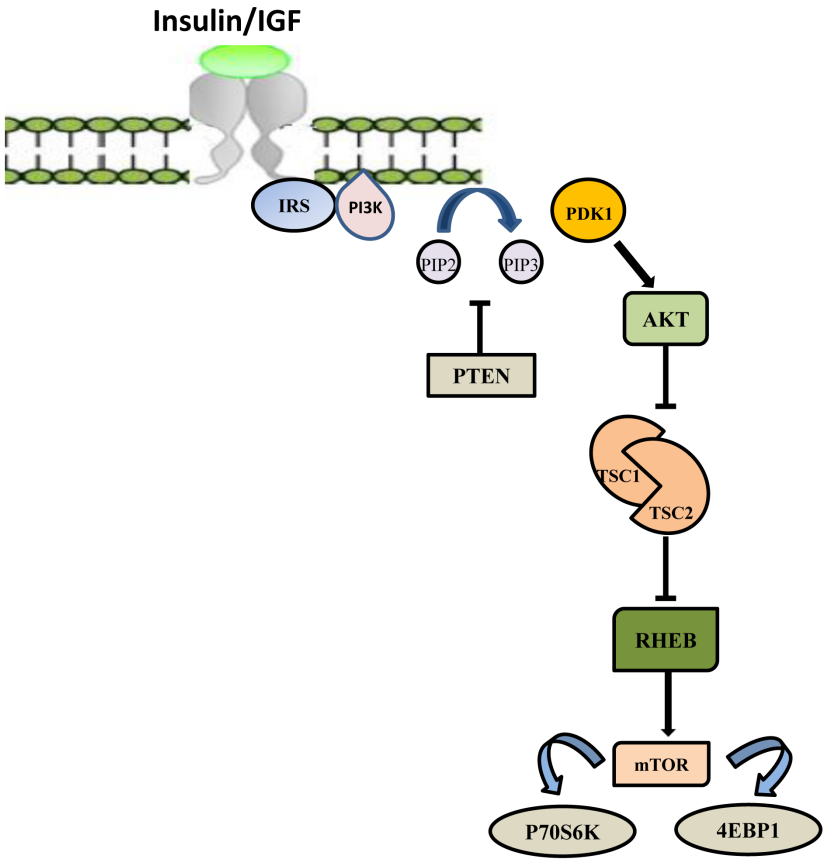

Figure 1: PI3K/AKT/mTOR signaling pathway. The PI3K signaling pathway is triggered by activation of receptor tyrosine kinase (RTK) in cell membrane. After binding to the growth factors, the intracellular domain of RTK is phosphorylated, and PI3K is activated. Activated PI3K phosphorylates PIP2 to produce PIP3. The tumor suppressor phosphatase and tensin homolog (PTEN) could negatively regulate this process via dephosphorylation of PIP3. Activated PIP3 could prompt the phosphorylation of Akt and further stimulate the Akt-mediated activation of downstream targets, including the Bcl-2 family members, Mdm2 and tuberous sclerosis complex 2 (TSC2). Activated Akt inhibits the Rheb GTPase activity of TSC1/2 complex by phosphorylating TSC2. Then, activated Rheb promotes mTOR complex 1 (mTORC1) to phosphorylate p70S6 and 4E binding protein1 (4EBP1), resulting in dysregulation of protein synthesis and cell survival.

importance of EIF4EBP1 gene is not completely clear. This topic was recently treated by Madden et *al*. that, using gefitinib (anti-EGFR) and temsirolimus (anti-mTOR) on TNBC cell lines, discovered the presence of a cross-talk mechanism between EGFR and mTOR also engaging the eukaryotic translation initiation factor 4B (eIF4B) [91]. Moreover, the action of these two molecules would seem to block phosphorylation of eIF4B, finally resulting in a growth and survival reduction in TNBC cell lines and then suggesting to investigate mTOR inhibition in association with other drugs [92, 93].

Recently, Bhola et *al*. [70] have suggested that resistance to TORC1/2 inhibitors may be exceeded via inhibition of the FGFR-mitochondrial metabolism-Notch1 axis that allows to eradicate therapy-resistant cancer stem cells in TNBC.

PI3K/AKT INHIBITORS

The PAM pathway may be targeted trough a different strategy involving the inhibition of its upstream targets such as PI3K and Akt [94]. While there are inhibitors inactivating both PI3K and mTOR, further development may be limited by issues, including increased toxicity [95]. Kalinski et *al*. have shown that subjecting patients affected by stage I/III BC (including 3 women TN) at different doses of MK-2206, an allosteric inhibitor of AKT, they experienced rash and pruritus G3, mucositis G2, fever G2 and hyperglycemia G2, leading to the trial suspension, despite two dose reductions [96].

A further setting in which the PI3K/AKT inhibitors could prove their usefulness could be in association to PARP inhibitors (PARPis) in TNBC patients who did not exhibited BRCA1/2 function loss [97]. This is because, as it is already known, PARPis result active in tumors deficient in the homologous recombination (HR) mechanisms due to alterations in the *BRCA1/2* genes [98-101], whereas their action is very negligible in non-BRCA mutant cancers [102].

Since the PI3K/AKT pathway stabilizes the function of HR, Ibrahim and collaborators have demonstrated that the use of AKT inhibitors in TNBC cell lines without BRCA1/2 alterations could cause HR function changes, and then sensitize to PARPis. In particular, the study showed that TNBC cancer cells treated with buparlisib (AKT inhibitor) were subject to a subsequent hyperactivation of ERK and MEK1, two essential component of the MAP kinase signal transduction pathway, resulting in downregulation of BRCA1 and then favoring the action of olaparib (PARPi) with subsequent reduction of cell proliferation and survival [103]. An interesting *in vitro* study showed that targeting multiple kinases such as IGF-1R, PI3K, mTORC or MEK may suppress cell proliferation and induce apoptosis in MDA-MB-231 cells, increasing also the inhibition of Akt phosphorylation [104].

A similar experience has been carried out by Kimbung et *al*. which evaluated the association between Rucaparib (PARPi) and LY294002 (PI3Ki) in BRCA1-deficient cells with the intent to improve the response to PARPis. This study showed promising results with sub-micromolar doses of both drugs, providing then a strong rationale for further research especially in TNBC [105].

The main clinical trials concerning the function of PI3K inhibitors in TNBC and currently under evaluation are reported in Table 2.

CONCLUSIONS

TNBC is a heterogeneous subtype of BC showing aggressiveness and high risk of relapse [106].

In the last years, the treatment of metastatic breast cancer has seen the development of new systemic treatments. Despite this progress, TNBC still has limited therapeutic options: cytotoxic chemotherapy is the standard of care; systemic treatment tipically has transitory efficacy and the response is early followed by disease progression.

TNBC patients exhibit, indeed, an unfavorable outcome compared to those with other subtypes.

Only recently driver mutations have been identified with encouraging results in preclinical models and have allowed to investigate new specific drugs for each different subtype of the disease.

The PI3K-AKT-mTOR pathway is an exciting target for developing new anticancer therapeutics [107]. Since several pathways may be involved, therefore, the best results are achieved by combining different molecules on various targets paying attention to toxicity. For these reasons, it will be necessary in the near future to rescue pathological tissue taken before and after therapy in order to better understand the mechanisms of drug resistance and new concepts on tumor pathogenesis. Using increasingly refined techniques, liquid biopsies could have an important role, allowing us to obtain a lot of information in a short time and in a minimally invasive manner and maintaining a high concordance rate with the primary tumor and/or metastases. Therefore, is desirable enrolling TNBC patients with different subtypes in new specific trials to ensure them the most suitable treatment.

One of the main difficulties in the field of targeted therapies is represented by the extreme heterogeneity of the disease. This condition produces a number of different cases, each constituted by the presence of a rare mutation mostly detected in cases of exceptional responders in the context of negative trials. Consequently, although the discovery of these rare mutations is leading to the approval of many new antitumor therapies, it is necessary to design new studies showing benefits, avoiding all the problems related to the extreme heterogeneity of the disease contained in other conventional trials. A possible solution could be derived from the use of the so called basket

trials that find their maximum indication where neoplasia depends on the pathway of the target and whether the therapy can effectively inhibit the action of the target itself. This would make possible to consider a marker as a probable predictor regardless of tumor histology.

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CONFLICTS OF INTEREST

The authors declare no conflict of interest.

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REVIEW

Dietary restriction: could it be considered as speed bump on tumor progression road?

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Abstract Dietary restrictions, including fasting (or long-term starvation), calorie restriction (CR), and short-term starvation (STS), are considered a strong rationale that may protect against various diseases, including age-related diseases and cancer. Among dietary approaches, STS, in which food is not consumed during designed fasting periods but is typically not restricted during designated feeding periods, seems to be more suitable, because other dietary regimens involving prolonged fasting periods could worsen the health conditions of cancer patients, being they already naturally prone to weight loss. Until now, the limited amount of available data does not point to a single gene, pathway, or molecular mechanism underlying the benefits to the different dietary approaches. It is well known that the healthy effect is mediated in part by the reduction of nutrient-related pathways. The calorie restriction and starvation (long- and short-term) also suppress the inflammatory response reducing the expression, for example, of IL-10 and TNF-α, mitigating pro-inflammatory gene expression and increasing anti-inflammatory gene expression. The dietary restriction may regulate both genes involved in cellular proliferation and factors associated to apoptosis in normal and cancer cells. Finally, dietary restriction is an important tool that may influence the response to chemotherapy in preclinical models. However, further data are needed to correlate dietary approaches with chemotherapeutic treatments in human models. The aim of this review is to discuss the effects of various dietary approaches on the cancer progression and therapy response, mainly in preclinical models, describing some signaling pathways involved in these processes.

Keywords Cancer cells · Cellular stress response · Chemotherapy · Diet · Fasting · Short-term starvation

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Introduction

"Let food be thy medicine and medicine be thy food." This sentence of Hippocrates explains excellently the importance that feeding assumes in the establishment of a healthy life. In particular, various dietary approaches, such as calorie restriction and long- and short-term starvations, seem to be involved in the protection from aging and age-related diseases.

Fasting (or long-term starvation) is commonly defined as a prolonged deprivation of nutrients from a system that can be represented by cells in culture (normal or immortalized) or by multicellular organisms (from fruit flies to humans). In vitro serum deprivation, for example, is often considered as a routine procedure to reduce basal cellular activity [1] and to make the population homogeneous of proliferating cells that, in this condition, can enter in the quiescent G_0/G_1 phase [2]. Its utility includes the metabolic research in which the establishment of serum starvation-based protocols submits the physiological response to a hormonal change [3]. Moreover, other researchers have used serum starvation as a system to analyze molecular mechanisms involved in cellular stress response [4], apoptosis, and autophagy [5, 6]. That is why serum starvation has also been referred to as "environmental stress" [7] and "apoptotic trigger" [8]. Recently, starvation is assuming a

part of protagonist. In particular, it has been spreading the idea that fasting cycles can prevent the onset of age-related diseases [9] and to improve the response to certain therapies like the oncological ones [10].

Like fasting, the reduction in calorie intake or calorie restriction (CR), without malnutrition, has been indicated as a strong non-genetic intervention that, simultaneously, can protect against various diseases, like diabetes and cancer, and that can increase the life-span in mammalians [11]. CR can consist of feeding once daily or thrice weekly over a range of restricted food amounts [12]. Also, it seems to cause a large number of benefits by reducing cardiovascular risk factors [13], improving insulin sensitivity [14], enhancing mitochondrial function (also inducing the generation of new mitochondria) [15], increasing cellular quality control through autophagy [16, 17], and reducing oxidative damage in both DNA and RNA [18].

The above considerations demonstrate that fasting or CR could be applied to protect patients from toxic side effects of chemotherapy. However, in vivo preclinical studies showed that several months may be necessary for people under CR to reach a protected state and, accordingly, these two types of approaches are not feasible for patients already prone to weight loss because of cancer itself or chemotherapy [19].

In this regard, another dietary approach that could generate similar biological changes, such as fasting and calorie restriction, seems to be more suitable. Short-term starvation (STS or intermittent fasting) is considered as a limited exposure to several nutrients, in which food is not consumed during designed fasting periods but is typically not restricted during designated feeding periods [11]. Other authors indicated it as the period of time in which animals lose weight after initiation of food restriction but prior to rebound or weight maintenance [20]. Accumulating evidence suggests that STS can produce, like CR, similar beneficial biological effects, including modulation of reactive oxygen species (ROS) and inflammatory cytokines, and also antimutagenic, antibacterial, and anticarcinogenic effects [21, 22]. Moreover, it has been shown that STS may be advantageous even after certain injuries. For example, in rats, a day of fasting can promote recovery following moderate but not severe damage in a controlled cortical impact injury model [23].

This manuscript proposes to review literature data for a better understanding of the correlation between the effects of the dietary restriction and response to anticancer treatment.

Dietary restriction-induced molecular changes

Until now, the restricted available data do not point to a single gene, pathway, or molecular mechanism underlying the benefits of the different dietary approaches. It is well known that the salutary effect is mediated in part by the negative regulation of nutrient-signaling pathways, including, in particular, the growth-promoting insulin-like growth factor 1 (IGF-1) receptor and its downstream effectors, such as extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (MAPK), and phosphoinositide 3-kinase (PI3K), which are known to regulate several detoxification enzymes [24] (Fig. 1).

The IGF-1 pathway influences both animal life-span and oxidative stress sensitivity. IGF-1 receptor-deficient mice showed a great resistance to oxidative stress [25]. The Forkhead box protein O1 (FOXO1), a downstream target of IGF-1/AKT signaling, is able to enter the nucleus, in the absence or reduction of IGF-1/AKT signaling, and to modulate genes involved in oxidative stress resistance, metabolism, and longevity [26–28].

Circulating IGF-1, in collaborations with other hormones and growth factors, plays an important function in the regulation of cell differentiation and proliferation and body size and also exerts a tumorigenic effect on a variety of tumors by promoting proliferation and inhibiting apoptosis [29, 30].

Consistent with the effect of calorie restriction and starvation (short- and long-term) in reducing growth factor signaling, fibroblasts isolated from mice deficient in the GH/IGF-1 pathway are resistant to oxidative stress, UV, heat, and genotoxins [31]. On the other hand, exposure of murine hepatocytes to IGF-1 reduces the levels of superoxide dismutases and catalase activity [32].

Moreover, it has been shown that a fasting period of 24 h does not alter the hepatic expression of many ABC family members but induces the expression of Abca1 (involved in cholesterol transport) and Abcg8 (involved in sterol transport) [33].

Dietary restrictions (DRs) also suppress the inflammatory response. Restricted rats have exhibited reduced mRNA expression levels of inflammatory cytokines and chemokines (IL-1β, TNF-α, and MCP-1) in various tissues such as the liver, kidney, and spleen [34]. Coherently, 4 weeks of DR decrease signs of illness (fever, cachexia, etc.), mitigating pro-inflammatory gene expression (for example, COX-2 and leptin) and increasing anti-inflammatory gene expression (e.g., SOCS3 and IL-10) [20].

Some researchers have submitted to serum starvation primary human myotubes, rat L6 myotubes, and human embryonic kidney cells for 24 h and assessed phosphorylation changes in the energy-sensing AMP-activated protein kinase (AMPK) and its downstream targets, including acetyl-CoA carboxylase (ACC), ERK1/2, and mammalian target of rapamycin (mTOR) pathway. Additionally, the phosphorylation of AMPK, ACC, and ERK1/2 reacted to serum starvation dynamically in a time-dependent way. Changes induced in the mTOR pathway after serum starvation can be associated with growth factor deprivation [3].

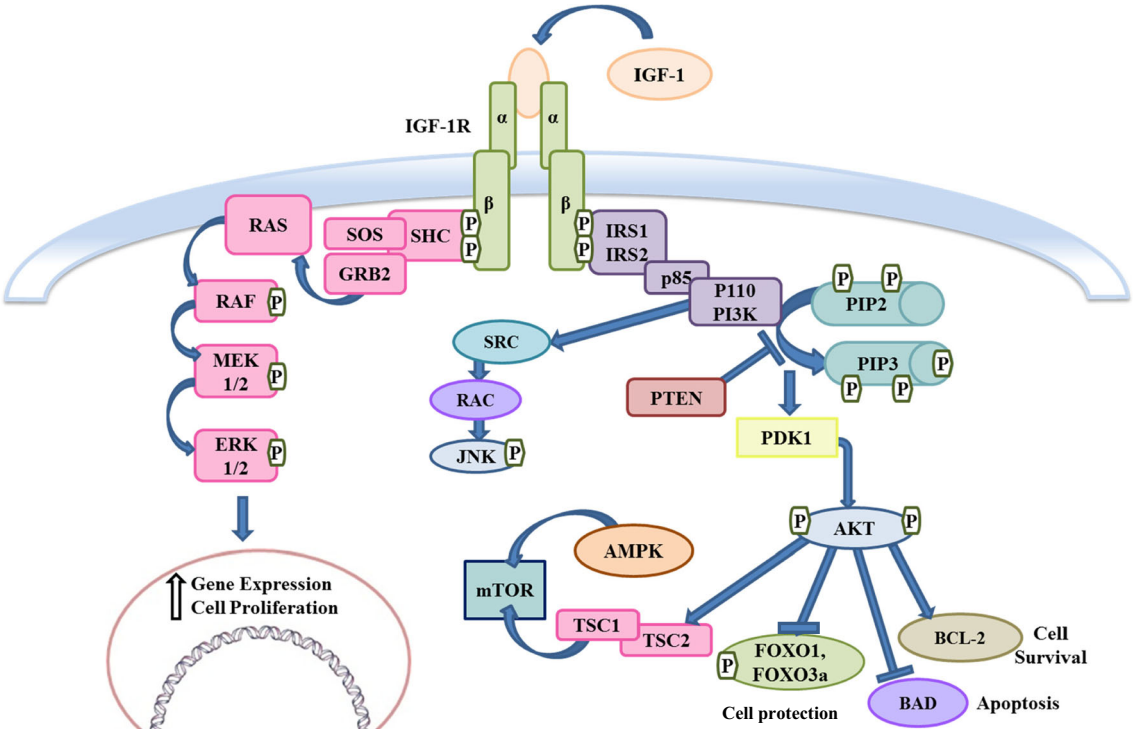

Fig. 1 Insulin-like growth factor-1 (IGF-1) signaling pathway. *IGF-1* insulin-like growth factor-1; *IGF-1R* insulin-like growth factor-1 receptor; *SHC* Src homology 2 domain-containing adaptor protein B; *SOS* Son of Sevenless; *GRB2* growth factor receptor-binding protein 2; *RAS* rat sarcoma viral oncogene homolog; *RAF* RAF-1 proto-oncogene, serine/threonine kinase; *MEK1/2* mitogen-activated protein kinases 1/2; *ERK1/2* extracellular signal-regulated protein kinases 1/2; *IRS1* insulin receptor substrate 1; *IRS2* insulin receptor substrate 2; *p85* phosphatidylinositol 3-kinase 85-kDa regulatory subunit alpha; *P110 PI3K* phosphatidylinositol 3-kinase, catalytic, 110-kDa, alpha; *PIP2* phosphatidylinositol 4,5-bisphosphate; *PIP3* phosphatidylinositol 3,4,5-trisphosphate; *PTEN* phosphatase and tensin homolog; *PDK1* pyruvate dehydrogenase kinase, isozyme 1; *AKT* V-Akt murine thymoma viral oncogene homolog 1; *BCL-2* B cell CLL/lymphoma 2; *BAD* BCL2-associated agonist of cell death; *FOXO1* Forkhead box O1; *FOXO3a* Forkhead box 3a; *TSC1* tuberous sclerosis 1; *TSC2* tuberous sclerosis 2; *mTOR* mammalian target of rapamycin (serine/threonine kinase); *AMPK* AMP-activated protein kinase; *SRC* SRC proto-oncogene, non-receptor tyrosine kinase; *RAC* Ras-related C3 botulinum toxin substrate; *JNK* c-Jun N-terminal kinase

Different dietary approaches may regulate genes involved in cellular proliferation, such as the insulin signaling adaptor (Irs2) and mitogenic hormone prolactin receptor (Prlr), in both normal and cancer cells [19]. It has been demonstrated that the PI3K pathway is important in determining the sensitivity of tumors to dietary restrictions. In particular, mutations that make PI3K constitutively active affect the response of cancer cells to dietary restrictions [35]. Instead, studies showed that STS had a greater effect in reducing glucose compared to calorie restriction diets, even when the CR caused an equivalent weight loss. This can be explained by the fact that STS and calorie restriction diets have distinct physiological responses. For example, short-term fasting induced a 70 % decrease in blood glucose that occurred within 60 h, while a 90 % CR diets caused only a 40 % glucose reduction after 96 h [21, 36].

Previous findings have clarified that serum starvation activates in cancer cells the ATM/Chk2/p53 pathway that sensitizes them to chemotherapy with cisplatin, probably due to a temporary loss of the coordination between cell proliferation driven by oncogenic mutations and the growth stimulated by growth factors [37].

Dong et al. [38] demonstrated, through in vitro studies using A549 non-small cell lung cancer cell lines, that serum starvation increases E-cadherin expression. E-cadherin is involved in the pathobiology of several types of cancer: the loss or dysfunction of E-cadherin is associated with increased lung cancer cell proliferation and invasiveness [39].

Braun et al. [40] observed that starvation induces the increase of p21 levels in MCF7 cell lines independently from p53 and this mitigates the efficacy of the Puma/Bax-dependent apoptotic signal (also induced by starvation). In addition, the anti-apoptotic function exerted by p21 in starved cells occurs upstream of mitochondrial permeabilization at the level of the Puma interactions with Bcl-x. Therefore, p21 protects cells against starvation-induced apoptosis. Furthermore, an important step for cellular apoptosis is the phosphorylation of H2AX (a variant of the histone H2A family) that is mediated by MAPK family proteins, such as JNK and p38. Several data showed that serum starvation induces strongly the phosphorylation of p38 and H2AX at Ser139 in a time-dependent

manner. These results indicate that H2AX phosphorylation is regulated by p38 during serum starvation. In addition, serum starvation increased the level of activated caspase-3, resulting in apoptosis stimulation [41].

Nutrient starvation puts cells in alarm and induces them to autophagy, a conserved self-eating process in which intracellular membrane engulf a part of cytoplasmic organelles for lysosomal degradation. Autophagy is a way by which cells under starvation condition transfer nutrients from unnecessary to essential processes [42]. The stimulation of autophagy by starvation needs the activation of poly(ADP-ribose) polymerase (PARP)-1, a nuclear enzyme switched on by DNA damage. During starvation, ROS that could induce the activation of PARP-1 are produced [43]. PARP-1 seems to be involved in the hydrolysis of ADP ribose: its lack, in fact, does not reduce ATP levels as much as when it is present [44]. As result, the permanence of AMPK in an inactive state and the consequent absence of the signal for mTOR inactivation were detected, leading to impaired autophagy. An interesting in vivo study suggested that PARP-1 regulates autophagy and makes available a link between the PARP-1 function and overall cellular response to nutrient starvation. Then, starvation, ROS production, and DNA damage cause PARP-1 activation that is required for starvation-induced autophagy [43]. It is well known that ROS-induced DNA damage consists of different lesions, including single-strand breaks (SSBs), double-strand breaks (DSBs), and oxidized DNA nucleotides, the most common of which is 8-oxo-7,8-dihydroguanine (8-oxoG). The latter type of damage requires the intervention of the base excision repair (BER) system, involving the repair enzyme 8-oxoguanine DNA glycosylase (OGG1). It has been observed that nutrient availability affects BER causing the loss of OGG1 both in vitro and in vivo. In particular, the induction of autophagy showed no consequence on OGG1 expression without starvation [45].

Dietary restriction and cancer

Tumor cells are exposed to numerous cellular stresses, such as oncogene-induced genotoxic [46], oxidative [47], and metabolic [48] stresses to which normal cells are not subjected. That is why tumor cells are more dependent on stress by supporting pathways for survival than normal cells.

Malignant cells undergo a series of genetic and epigenetic alterations that allow them to be both self-sufficient for growth and non-sensitive to anti-growth stimulation. Cancer cells express oncogenes that can continue to relay the effect of growth factor, while healthy cells are unable to grow in the absence of stimulation. This *independence* could be explained by the autocrine production of growth factors or by mutations that make constitutively active intracellular signal transduction proteins or membrane receptors. For example, factors overproduced by cancer cells are platelet-derived growth factor (PDGF), vascular endothelial growth factor (VEGF), and IGF-1 [49]. Probably, the most important contribution regards alterations in genes coding for components of the Ras/Raf/MAPK and PTEN/PI3K/AKT pathways. In fact, mutated Ras alleles can render the cell proliferation independent from extracellular signals found in approximately a quarter of all cancers [50] and, in particular, in half of colon cancers [51].

In vitro serum starvation and in vivo short-term starvation reduce the levels of growth factors in normal cells [52, 53]. Furthermore, in the same cells, the depletion of paracrine communication signal of growth factors reduces the activity of proliferation signaling pathways and also the basal cellular metabolism. Consequently, cells enter in a proliferative/quiescent status [3]. Unlike healthy cells, it has been suggested that cancer cells cannot rapidly adapt to fasting and thus STS determines a differential stress resistance. Malignant cells, in fact, struggle to adapt to the loss of external growth factors by regulating autonomous growth stimulation and reprogramming their metabolism, thereby maintaining continuous proliferation [49]. This fundamental difference between healthy and unhealthy cells may explain why starvation condition protects normal cells without interfering with the killing of malignant cells [21, 54] (Fig. 2).

In vitro and in vivo studies demonstrated that fasting alone can modulate cell proliferation and apoptosis in different types of tumors by determining an increased cytotoxicity when combined with chemotherapy, the main treatment for tumor injuries. Several chemotherapy drugs harm DNA and cause cell death in part by promoting oxidative damage that involves not only tumor cells but also normal tissues (especially with high-dose chemotherapy) [55]. This increase in ROS may act synergically with fasting and chemotherapy [19]. It seems that the different types of dietary restriction selectively protect normal cells from chemotherapy toxicity, sensitizing simultaneously tumors to therapy, and STS is much more effective than calorie restriction or long-term fasting [21].

STS is able to sensitize various cancer types to chemotherapeutic agents. Some authors, in fact, showed that growth factors and glucose restriction slowed proliferation and increased cell death in 3 different murine cancer cell lines, while 24 h of fasting before and after drug treatment sensitized 15 of 17 cancer cell lines. They also evaluated the effect of fasting in a model of human metastatic cancer by applying five cycles of fasting for 48 h in immunosuppressed nude mice xenografted subcutaneously with human neuroblastoma cells and comparing the results with

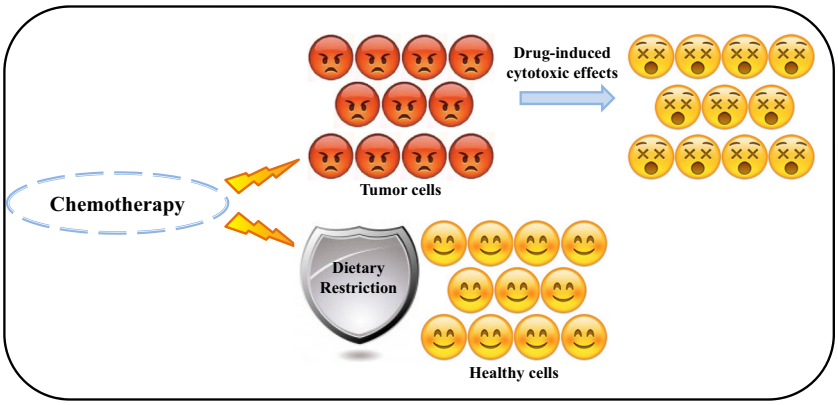

Fig. 2 Effects of dietary restrictions on effectiveness of chemotherapy in cancer cells

normally fed mice. After 34 days, fasting cycles and cyclophosphamide treatment reduced tumor size to less than a half of the one achieved in the treatment with cyclophosphamide or fasting alone [19].

STS followed by chemotherapy is not only safe and feasible but also helps to attenuate the side effects related to chemotherapeutic drugs. Some studies have shown that STS induces cardioprotective effects against the injury caused by doxorubicin [56]. Moreover, Raffaghello et al. [54] studied the effects of high-dose etoposide, a chemotherapeutic drug with a non-specific toxicity profile, in murine models undergone to fasting for 48–60 h, before the administration of a high-dose drug, and in a control group fed ad libitum. The latter group, after the treatment, showed many of the signs associated with the toxicity of etoposide, including the reduced mobility and the ruffled and posture alterations, while mice submitted to fasting showed no obvious signs of stress or pain. The beneficial effect of fasting was confirmed by comparing the mortality rate of mice in the two groups (43 % of the animals in the control group), and only one case in the fasting group died from acute toxicity by etoposide. A second group of mice was starved for 60 h and then treated with a dose of etoposide four or five times higher than the maximum dose recommended for humans. While non-starved mice died or showed toxicity, starved mice only lost the 40 % of their weight, which was regained after 1 week of re-feeding.

There is also an evidence of the benefits of the STS before chemotherapy in humans. Ten volunteers affected by different types of tumors were starved for 48–140 h before chemotherapy and 56 h after the treatment. All patients showed decreased chemotherapy-induced side effects [54]. A period of fasting after chemotherapy treatment could be relevant, because the combination of re-feeding and drug-induced DNA damage can increase the growth of aberrant foci in the liver, colon, and rectum. This can be explained by the hypothesis that prolonged fasting induces cell death and atrophy in the organs and thus cell proliferation stimulated by re-feeding can lead to a DNA damage, if high levels of toxins are present [57, 58].

Effects of dietary restrictions on the main tumors

During disease, the interactions between tumor and stroma cells are modified, producing a microenvironment that favors the primary tumor growth (remodeling, invasion, and angiogenesis) and its spreading (metastasis). Tumor microenvironment is heterogeneous and, in addition to the tumor cells, includes macrophages, fibroblasts, adipocytes, and endothelial cells that appear different from those of normal tissues [59]. Among these, adipocytes suffer the influence of dietary restrictions and physical exercise. This condition implies that dietary restrictions could be an effective method to favorably change the tumor microenvironment and its activities [60]. Adipocytes localized near the tumor mass are called "cancer-associated adipocytes" and show a reduction of adipose markers, such as HSL, APN, and resistin, and an increased expression of inflammatory cytokines (e.g., IL-6 and IL-1β) [61]. Adipocytes play an important role in cancer progression and may contribute to carcinogenesis and tumor invasiveness, since they are a source of pro-inflammatory cytokines, such as IL-6 and TNF-α, ROS, and matrix metalloproteases [62–65]. Moreover, adipocytes are able to secrete adipokines that increase angiogenesis, fibrosis, and inflammation by recruiting macrophages and endothelial cells inside the cancer microenvironment in a way mediated by NF-KB [66]. Dietary restriction can modulate the function of the tumor microenvironment by altering the size of adipocytes [63] and, consequently, reducing adipokine secretion, such as IL-6 [67]. Furthermore, dietary approaches are often associated with an increased life-span in mammalian and a better response to chemotherapy in various types of cancer. It is well known, in fact, that the bioavailability of several anticancer oral drugs changes because of food exposure. Chemotherapy

drugs are not the only cytotoxic strategy used for the treatment of cancer. Evidences suggest that caloric restriction might also be associated with radiation therapy (RT). Champ et al. [68] reported that CR can modulate pathways involved in resistance to treatment (such as IGF-1) and make cancer cells more sensible to cytotoxicity induced by radiotherapy.

The standard chemotherapy can enhance cellular metabolism by creating a tumor microenvironment rich in glucose and glutamine. Recently, as an alternative to standard treatment, metabolic therapies aimed to target metabolic alterations present in all tumor cells and which give a benefit to normal cells have been developed. For example, among the metabolic therapies that improved the prognosis of patients with glioblastoma multiforme and brain cancer, there is the calorie-restricted ketogenic diet (KD-R), which shows anti-angiogenic, anti-inflammatory, and anti-apoptotic effects [69].

Below we discuss some data in the literature concerning the effects of dietary restrictions on the main tumors.

Breast cancer

Breast cancer is the most common cancer and represents the first cause of the cancer death in women worldwide [70]. A case report has shown the effect of a fasting period on Caucasian women affected by breast cancer. The first case was that of a 51-year-old woman (stage IIA) who has received adjuvant chemotherapy (docetaxel and cyclophosphamide) and has been subjected to fasting 140 h before and 40 h after treatment. During fasting-chemotherapy cycle, the patient showed no side effects in contrast to when she has undergone chemotherapy alone. Even the complete blood count (CBC) appeared best if the treatment was preceded by a period of fasting. Another case was that of a 53-year-old woman (stage IIA, HER2+) who has been exposed to four chemotherapy cycles (with docetaxel and cyclophosphamide) and fasting for 64 h before and 24 h post chemotherapy. Negligible side effects were observed, including only mild weakness and short-term memory impairment. The third case was represented by a 48-year-old woman treated with four cycles of doxorubicin and cyclophosphamide followed by paclitaxel and trastuzumab. During treatment, the patient was subjected to fasting for 60 h prior and 5 h post drug administration, showing no side effects associated with these drugs. The last reported case was that of a 78-year-old woman (HER2+) submitted to mastectomy and to six cycles of chemotherapy with carboplatin, docetaxel, and trastuzumab. The patient adopted fasting periods of variable lengths during therapy and, despite this, has not been reported severe side effects [71]. Therefore, it becomes apparent that a short period of fasting in association with chemotherapy treatment improved patient's tolerability, reducing side effects even if underlying molecular mechanisms are unclear. Factors related to the DNA repair systems or apoptosis may be involved [72]. One of these is REV1, a DNA polymerase involved in DNA repair, whose role is to control cell metabolic fate. In a recent work, REV1 was indicated as a binding partner of p53 and, consequently, as a regulator of its activity. Under fasting conditions, SUMO2/3 (small ubiquitin-like modifier) induces changes in REV1 by increasing the apoptotic effects of p53 on breast cancer cells. The regulation of REV1 could be used as a non-toxic strategy to increase p53-mediated cell death and thus to improve the effectiveness of treatment [73].

Ovarian cancer

Ovarian cancer is the third most common gynecological cancer worldwide [74]. In vitro studies suggested that serum starvation reduces cell proliferation by inducing G1 arrest in human ovarian cancer cells (SK-OV-3), through the suppression of Skp2-dependent CDK2 activity and Skp2-independent CDK4 activity. Skp2 is an ubiquitin ligase that positively regulates cell cycle by inducing degradation of p27, resulting in CDK2-induced progression from G1 to S phase [75].

A clinical study reported the case of a 44-year-old Caucasian woman affected by ovarian cancer who, after a series of drug resistances, has been treated with antineoplastic drugs in combination with a fasting period of 62 h prior and 24 h after treatment. STS has been shown to improve the CBC [71].

Colorectal cancer

Colorectal cancer (CRC) is the third most common cancer in men and the second in women worldwide [74]. Among the initial modifications causing the CRC onset, there is the suppression of the serotonergic system that seems to be related to a high-fat diet [76]. Moreover, this type of diet enhances the formation of preneoplastic lesions and consequently promotes CRC tumorigenesis [77]. However, it has been shown that, during the advanced stages, the disease can induce a reduction of serum total cholesterol levels [78]. Kaska et al. [79] had evaluated the effects of a preoperative fasting period in CRC patients, observing no clinical benefit. Conversely, intake of nutrients seems to be a protective factor against surgical trauma. However, results about the beneficial effects of dietary restrictions are conflicting. Indeed, some authors reported that nutritional deficiencies promote tumorigenesis because the lack of certain nutrients increases chemically induced carcinogenesis in preclinical models [80]. Furthermore, another study evaluated the combined influence of a carcinogen (1,2-dimethylhydrazine or DMH) and food deprivation regimen in murine models. Food deprivation has been shown to induce an increase of the lipid peroxidation

processes in liver and colon tissues, suppressing the serotonergic system in the colon [81].

Lung cancer

Lung cancer represents the most common cause of cancer death worldwide [82]. The therapy of choice for patients with locally advanced or metastatic non-small cell lung cancer (with or without epidermal growth factor receptor (EGFR) mutation) involves the use of erlotinib (an EGFR tyrosine kinase inhibitor). Currently, its recommended dose is 150 mg daily, either 1 h before or 2 h after a meal. Katsuya and colleagues [83] observed that these two types of fasting determine a different drug absorption, resulting in increased therapeutic effectiveness when drug administration occurs 2 h after meal. Also, the authors found no major differences in terms of toxicity or bioavailability between the two conditions [83]. An interesting clinic case is represented by a 61-year-old Caucasian woman with poorly differentiated non-small cell lung cancer. During chemotherapy cycles, the patient experienced numerous side effects that have been attenuated after the implementation of fasting 48 h before and 24 h after chemotherapy [71].

Prostate cancer

Prostate cancer is the second most common cancer in men and the fifth leading cause of cancer death [74]. A high-fat diet and a sedentary lifestyle can be considered risk factors for the onset of prostate cancer by changing the amount of hormones and growth factors in the serum. An interesting study has recruited men with prostate cancer randomly divided into two groups: one group fed with a low-fat diet and the other without restrictions. After 4 weeks, the serum was collected from the patients and cultured with LNCaP prostate cancer cells. The serum of both groups was not different in terms of serum prostate-specific antigen (PSA), sex hormones, insulin, IGF-1 and IGF-2, and insulin-like growth factor-binding proteins, but serum triglyceride and linoleic acid (omega-6) levels were decreased in the low-fat diet group (in which omega-3 levels were increased). It was observed that LNCaP cells in contact with the serum of patients with low-fat diet grew very slowly. Thus, a reduction in serum fatty acid levels may induce a decrease in human LNCaP cell growth [84]. A case report described the effect of a fasting period on the response to an antineoplastic treatment in two Caucasian men affected by prostate cancer. The first case was that of a 74-year-old man with stage II prostate adenocarcinoma and submitted to prostatectomy. After therapy failure and severe side effects, the patient was submitted to 60 h of fasting prior and 24 h post chemotherapy. As result, a reduction of side effects and PSA levels was observed compared to previous cycles without STS. Similar results were obtained in a 66-year-old man with prostate adenocarcinoma subjected to fasting for 60–66 h prior and 8–24 h after chemotherapy [71].

The beneficial effect of STS may be also observed when the natural compound Guttiferone F (a prenylated benzophenone derivative) is used. Guttiferone has been shown to inhibit cell proliferation in prostate cancer cell lines (LNCaP and PC3) submitted to serum starvation. In this case, the compound leads to DNA fragmentation; causes the entry of cells in the G1 phase; stimulates mitochondria-dependent apoptosis, by regulating the Bcl-2 family proteins; attenuates the androgen receptor expression and ERK1/2 phosphorylation; but activates JNK and calcium ion flow. The authors hypothesized that the combination of dietary approaches and Guttiferone F could increase the antitumor effect without causing cytotoxicity in vivo [85].

In vivo studies investigated the effects of intermittent calorie restriction (ICR) on mice xenografted with prostate cancer cells or transgenic adenocarcinoma mouse prostate (TRAMP) models. Bonorden et al. [86] observed that ICR (with a CR of 50 % lower than the classic feed) delays the onset of cancer in TRAMP mice. If future studies will confirm this data, STS could become an interesting strategy for prostate cancer treatment.

Conclusions

Since dietary approaches represent an easy tool in delaying aging, healing from damages, and preventing the onset of age-related diseases, they may become efficient allies for cooperating in the treatment of tumors, even though STS seems to be more tolerated by sick patients. It is essential to understand each change or hidden feature induced by the processes described above to reap the benefits related to fasting.

The currently available data have associated the various dietary approaches to numerous biological processes (from cellular proliferation and vitality to inflammatory response) and to the response to the chemotherapy. Recent studies have reported that dietary restriction induces molecular changes in genes, including IGF-1 and its receptor, as well as downstream effectors. The modification regards also inflammatory cytokines and chemokines, such as IL-1β and TNF-α, and elements associated to apoptosis (Bax/Bcl-x). However, additional insights are needed. Moreover, it might be interesting to get more results in humans in order to ascertain the effect of fasting and create standard protocols that are able to correlate dietary approaches with chemotherapeutic treatments.

Compliance with ethical standards

Conflicts of interest None

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REVIEW

A headlight on liquid biopsies: a challenging tool for breast cancer management

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Abstract Breast cancer is the most frequent carcinoma and second most common cause of cancer-related mortality in postmenopausal women. The acquisition of somatic mutations represents the main mechanism through which cancer cells overcome physiological cellular signaling pathways (e.g., PI3K/Akt/mTOR, PTEN, TP53). To date, diagnosis and metastasis monitoring is mainly carried out through tissue biopsy and/or re-biopsy, a very invasive procedure limited only to certain locations and not always feasible in clinical practice. In order to improve disease monitoring over time and to avoid painful procedure such as tissue biopsy, liquid biopsy may represent a new precious tool. Indeed, it represents a basin of "new generation" biomarkers that are spread into the bloodstream from both primary and metastatic sites. Moreover, elevated concentrations of circulating tumor DNA (ctDNA) as well as circulating tumor cells (CTCs) have been found in blood plasma of patients with various tumor types. Nowadays, several new approaches have been introduced for the detection and characterization of CTCs and ctDNA, allowing a real-time monitoring of tumor evolution. This review is focused on the clinical relevance of liquid biopsy in breast cancer and will provide an update concerning CTCs and ctDNA utility as a tool for breast cancer patient monitoring during the course of disease.

Keywords Liquid biopsy · Breast cancer · Circulating tumor cells · CTCs · Circulating tumor DNA · ctDNA

Introduction

Breast cancer is the most common carcinoma and one of the leading causes of cancer-related deaths in women aged between 35 and 75 years [1, 2]. Although breast cancer seems to be most often sporadic, about 5–10 % of new cases are hereditary. Indeed, about half of these would be associated with germline mutations occurring on codifying DNA sequences which determine a high risk of developing the disease during life and for this reason called "susceptibility genes" [3, 4].

The two major identified susceptibility genes are the tumor suppressor genes BRCA1 and BRCA2 involved in DNA double strand break repair trough the homologous recombination (HR) pathway. Germline mutations in these genes cause genetic instability that enhance the development of additional mutations in other pivotal genes involved in cell cycle control and many others cellular processes [4–7]. Gene expression profiling studies, performed by microarray analysis, highlighted the molecular heterogeneity of breast cancers, allowing the identification of different molecular subgroups with similar characteristics. Thus, based on the gene expression profiles, they were classified into four distinct subtypes: luminal A, luminal B, human epithelial growth factor receptor 2 (HER2) overexpressing, and basal-like [8]. Furthermore, immunohistochemical characterization of the aforementioned subtypes on the basis of ER, PR, and HER2 expression is shown in Table 1. Current ESMO guidelines on primary breast cancer management consider this classification and implement it with Ki-67 levels. The cutoff to distinguish high from low levels of ki-67 is by 30 % [9]. Histologically, breast

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Table 1 Immunohistochemical profile of breast cancer subtypes

Luminal A	Luminal B	HER2+/ER−	Basal like
• ER+	• ER+	• ER−	• ER−
• and/or PR+	• and/or PR+	• PR−	• PR−
• HER2−	• HER2+	• HER2+	• HER2−

ER estrogen receptor, *PR* progesterone receptor, *HER2* receptor type 2 for EGF

cancer can be divided into in situ and invasive carcinoma, both of which can be further subclassified into ductal and lobular carcinomas [10]. Triple negative breast cancer (TNBC) represents 15–20 % of all breast cancers. This unfavorable cancer histotype lacks in the expression of estrogen, progesterone, and human epidermal growth factor receptor-2 receptors. Moreover, it usually presents at young age (age <50 years), advanced stage at first diagnosis, unfavorable outcome, grade 3, high proliferative index, and higher risk of metastases [11, 12].

Even though breast cancer represents the second leading cause of cancer-related death among women, a significant proportion of patients can be treated by surgery only or surgery and adjuvant systemic therapies. Despite the improvements in disease management, mortality is still high due to the emergence of distant metastasis. With the introduction of new approaches, as well as next-generation sequencing (NGS), it starts to be clear that clonal evolution occurs, among a cell population, within the primary tumor and it is mainly due to intra-tumor heterogeneity [13]. Furthermore, anticancer therapies may also cause a selective acquisition of new genetic alterations.

Another relevant drawback in luminal breast cancer is that the metastatic event can appear years after first diagnosis. This might be referred as "tumor dormancy," it means that cancer cells may colonize earlier distant sites but stay dormant until they do not accumulate enough genetic and/or epigenetic modifications that allow their awakening from "dormancy" [14]. Two different possible scenarios can be forecasted for explaining the metastatic event. According to the first scenario, tumor cells leave the primary site to colonize distant organs only if they acquire a totally competence to generate a metastatic development. In the second case, "almost normal epithelial cells" leave preneoplastic lesions earlier and evolve simultaneously with the primary tumor cell population [15].

Currently in the clinical practice, it is very difficult to find a reliable tool to follow tumor evolution over time, and this lack is probably the main reason of treatment failure whether administered at wrong time points during the course of disease. Liquid biopsy may represent the solution to the aforementioned problems. Recent findings demonstrate that circulating tumor cells (CTCs) and circulating tumor DNA (ctDNA) are detectable in blood samples even at early stage disease. Moreover, technological improvements in CTCs and ctDNA isolation, detection, and analysis are paving the way for their introduction in the current clinical practice. Moreover, new insight into the nature and severity of breast cancers could arise from small extracellular vesicles. Among the extracellular bodies, the interest of the scientific community in exosomes biology is daily growing. Indeed, exosomes are small vesicles (diameter 40–100 nm) involved in mediating a cross-talk between the extracellular matrix and the cell acting as a shuttle of many compounds as nucleic acids and proteins [16, 17]. Indeed, these could represent a new intriguing diagnostic marker because of their possible involvement in a multitude of cellular processes as well as the event of tumor growth, metastasis, and not less notable, drug resistance as effect of anticancer drugs removal from the breast cancer cells [17, 18].

Few data have been available yet about the assessment of serum microRNAs in breast cancer as it has been carried out in other malignancies. The development of microRNA panels in serum would add relevant information for breast cancer classification along with messenger RNA (mRNA) expression levels in tissue and ctDNA panels in serum [19–22].

The review is focused on the clinical relevance of liquid biopsy in breast cancer. We will provide an update concerning CTCs and ctDNA utility as a tool for breast cancer patients monitoring during the course of disease.

An overview on main signaling pathways involved in breast cancer

Mutation gain in somatic cells is the pivotal mechanism through which cancer cells alter normal signaling pathways [23], in addition to other modifications such as epigenetic variations which are primarily influenced by the local microenvironment and germline genetic variations.

Cancer whole-genome studies highlighted a still growing number of involved somatic mutations, most of which have not yet a known biological function. The accumulation of mutations within a cancerous and/or precancerous cell population is a dynamic process not fully understood, and until now, several pathways have been identified as responsible for the process of mammary carcinogenesis.

The evaluation of estrogen, progesterone, and epidermal growth factor type 2 receptors (ER, PgR, and HER2, respectively) is widely used in clinical practice in order to obtain prognostic and predictive information [24]. Also, several other markers, such as B-cell lymphoma 2, androgen receptor, EGF, phosphatase and tensin homolog (PTEN), and phosphatidylinositol 3-kinase (PI3K), may have prognostic value in breast cancer. Several studies have reported that mutations in PI3K are associated with lower recurrence and mortality

rates [25, 26]. PI3K/Akt/mTOR pathway is often altered in patients with breast cancer. This pathway controls several biological activities within the cell, and its activation is one of the fundamental downstream molecular events following tyrosine kinase growth factor receptor activation [27]. The first event is the activation of PI3K, which, when not inhibited by PTEN, phosphorylates and activates AKT localizing it in the plasma membrane [28]. AKT can have a number of downstream effects by activating different substrates such as the mammalian target of rapamycin (mTOR) whose substrate is S6 Kinase 1 (p70S6K) [29–31] (Fig. 1).

PI3K mutations are more frequent in node-negative (not spread to nearby lymph nodes), ER-positive, and Her2- negative patients, and are generally related to a favorable clinical outcome. Several studies have reported that mutations in PI3K are associated with lower recurrence and mortality rates [25, 26]. PI3K mutations are very high recurrent genetic alterations in breast cancers with a rate of 20–40 % in these cancers [32]. The PI3K/AKT/mTOR pathway plays a central role in regulating cell proliferation, growth, apoptosis, and motility. The PI3K enzyme is a heterodimer composed of a regulatory (p85) and a catalytic subunit (p110), and it is activated after the dimerization and auto-phosphorylation of tyrosine kinase receptors, such as HER2. Following its activation, PI3K is able to phosphorylate phosphatydilinositol 4,5-trisphosphate (PIP2) in phosphatydilinositol 3,4,5-trisphosphate (PIP3) that is the main actor for the activation of PKB/AKT [33, 34]. Under physiological conditions, the PIP3 levels are tightly regulated by specific phosphatases, such as PTEN. Alteration in the PI3K/AKT pathway, due to loss or gain of expression as well as genetic changes of its members, is a pivotal event toward a malignant transformation, as already well described in literature. Several studies have demonstrated that the gene encoding the PI3K catalytic subunit p110a (PI3KCA) is mutated in different cancers [35]. Activating mutations in the p110α catalytic subunit of PI3K has been also described in 9–45 % of breast cancers [32]. The preferred mutational "hot spots" for PI3KCA are predominantly exons 9 and exon 20; mutations of these exons determine the following amino acid substitutions: H1047R, E545K, and E542K. This mutations account for 70–80 % of PI3KCA alterations in breast cancer [36]. These hot spot mutations are known to

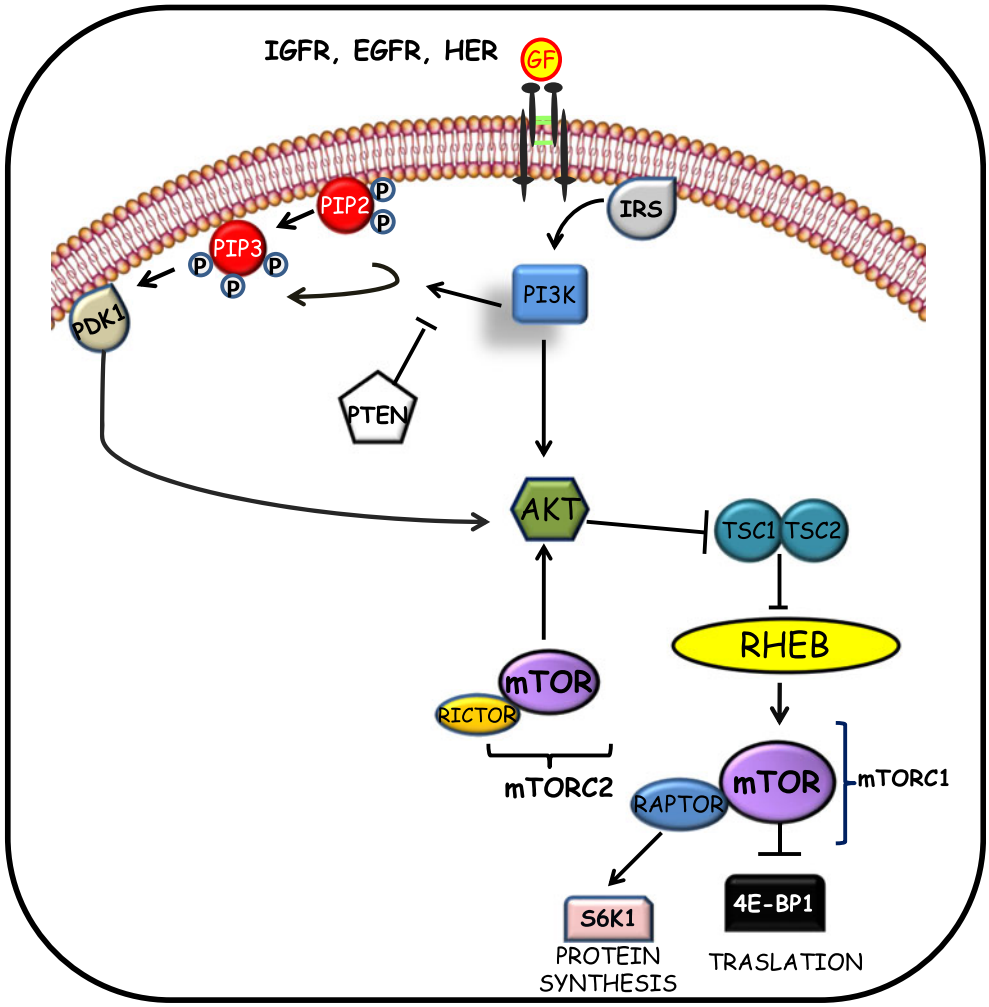

Fig. 1 Activation mechanism of PI3K/Akt/mTOR signaling pathway

modulate the activity of the PI3K in the signaling pathway regulating cell growth, motility, and other important cellular functions [36, 37].

Also, PTEN and AKT are frequently altered in breast cancer. As widely highlighted in many forms of infiltrating breast cancers, PTEN genetic alteration and in particular its loss of expression yield a probability of incidence quantified in 4–35 %, while mutations or variations in AKT expression levels give a percentage of 6 and 15–41 %, respectively. Taken together, these genetic aberrations seem to play a pivotal role in the deregulation of PI3K/AKT pathway, and consequently, this possibly leads to disease progression and therapy resistance [38].

Other relevant mutations in breast carcinogenesis are those in TP53 gene. Indeed, 83 % of basal-like tumors showed TP53 mutations compared to 15 % in the luminal, normal-like, and HER2-positive tumors [39]. TP53 is a tumor suppressor gene encoding a 393 amino-acid nuclear phospho-protein whose role is to arrest propagation of genetically aberrant cells [40]. TP53 mutations also seem to play a role in the epithelial-mesenchymal transition (EMT) through the inhibition of epithelial markers such as E-cadherin and the transcriptional activation of genes associated with a mesenchymal phenotype such as Twist, ZEB-1, and ZEB-2 [41]. These molecular events encourage cells to acquire a cancer stem cell-like phenotype and to promote tumor growth and metastatic spread. TP53 expression is also tightly correlated with Ki67 expression and is negatively associated with histological grade, tumor size, and co-expression of vascular endothelial growth factor (VEGF), epidermal growth factor receptor (EGFR), and type II topoisomerase (TOPO II). In addition to the aforementioned genetic alterations, also point mutations, as well as gene amplification in the ESR1 gene, even though at a low-frequency, seem to play a role in metastatic breast cancer [32, 42]. Some of these identified mutations seem to lead to ERα transcriptional activity in a hormone-free manner, promoting resistance to hormonal treatments [43, 44].

Liquid biopsy

Diagnosis and metastasis monitoring is mostly carried out through tissue biopsy and re-biopsy, a very invasive technique, limited, and not easily acceptable by the patients.

In order to obtain an efficient and painless management of the disease over time, liquid biopsies may represent a precious basin of "new generation" biomarkers. These biomarkers are released into the bloodstream from both primary and metastatic sites, and thus, they can provide intriguing information on tumor evolution. Indeed, elevated concentrations of circulating nucleic acids have been found in blood plasma or serum of patients with various tumor types and were often associated with unfavorable outcome in some clinical studies. These findings could improve the actual approach to breast cancer patients and might in the near future help clinicians in modifying the treatment choices according to the tumor evolution.

Circulating tumor DNA: a general overview

Cell-free DNA (cfDNA) was firstly reported in 1948 by Mandel and Metais in the blood of healthy individuals [45]. Cell-free DNA is a double-stranded nucleic acid with low molecular weight than genomic DNA that circulate in the bloodstream in short (between 70 and 200 base pairs in length) or long fragments up to 21 kb. To date, the source of cfDNA is still not completely understood, while a big contribute seems to derive essentially from nucleated blood cells. In cancer patients, a considerable proportion of cfDNA is thought to origin from tumor cells although a proportion of fragments is represented by wild-type DNA as well as in the plasma of healthy controls. Given that somatic mutation occurs exclusively in tumor cells, the identification of a mutation in cfDNA can define the portion of circulating tumor DNA (ctDNA) [46]. The possible reason for the high presence of nucleic acids into the blood of cancer patients could be explained as a part of the cell death process (apoptosis and necrosis) in cancer cells. Within the physiological clearance process of an apoptotic and/or necrotic cell, macrophages take a pivotal role in engulfing dead cells and in realizing the digested DNA into the tissue environment [47]. Another hypothesis is that ctDNA arises from the lysis of circulating cancer cells or micro metastases, already into the bloodstream, shed by tumor. The amount of ctDNA that derives from tumor cells is also affected by specific tumor characteristics, i.e., the size and the stage of the tumor as well as all the physiological filtering events triggered into the blood and lymphatic circulation [48].

Circulating tumor DNA levels are considerably lower in earlier stage disease and consequently more difficult to detect than in advanced or metastatic disease [49].

Targeted therapies have deeply changed the approaches to treatment of cancer over the past 10 years. Indeed, almost all tumors acquire resistance to systemic treatment as a result of clonal evolution and selection of resistant cells within the tumor mass. This "clonal process" is tightly dependent from the heterogeneous characteristic of a tumor. Although genotyping is the most reliable method applied for classifying tumors for future clinical decisions, tumor tissues provide only a snapshot and are often difficult to obtain. Therefore, methods are needed for a rapid, cost-effective (less-expensive) and noninvasive identification of biomarkers at various time points during the course of disease. Since ctDNA is a potential surrogate for the entire tumor genome, the use of ctDNA as a liquid biopsy may represent a valuable tool for obtaining the genetic follow-up data that are urgently needed.

Some quantitative study reported differences in terms of circulating DNA amount between breast cancer and healthy individuals [50]. Furthermore, the levels of ctDNA in cancer patients would represent a stable parameter whose fluctuations during the course of the disease may be correlated with clinical outcome. Indeed, it has been demonstrated that high levels of ctDNA correlate with tumor size, lymph node involvement, histopathological grade, and clinical staging [51].

The presence of specific mutations helps to differentiate ctDNA from normal cfDNA. These somatic mutations, commonly single base pair substitutions, are present only in the genomes of cancer cells and are never present in normal cell DNA within the same individual. These features confer elevated specific properties to ctDNA as a biomarker. One of the most common somatic alterations in breast cancer are the mutation in PI3K gene that occurs in 40 % of all breast cancers. This high mutational frequency renders PI3K a highly interesting circulating biomarker. In a recent study [52], the PI3K mutations were detected in ctDNA of 13/46 (28 %) patients with metastatic breast cancer, but no trace of the mutation was found in patients with resectable breast cancer. These results suggested that the detection of ctDNA mutations is lower in the early stage disease compared to more advanced stage and demonstrated the feasibility of detection of PI3K mutation in plasma [53].

A more recent study highlighted the utility of ctDNA in the clinical management of breast cancer patients. The study of Dawson et al., conducted on 30 women with metastatic disease under chemotherapy, compared the radiographic measurement of tumors with the molecular analysis of ctDNA as well as CA 15–3 and circulating tumor cells. With respect to CA15-3 and CTCs which showed not high detectability within the sample, ctDNA was identified in almost all 30 patients (29/30) in whom mutations of TP53 and PI3K were already characterized in tumor biopsy specimens. This assay proved the high sensitivity of ctDNA for detecting presence of metastatic disease and study tumor burden when compared to the aforementioned biomarkers. Furthermore, the researchers found that ctDNA levels are often associated with both treatment efficacy and survival. In fact, patient with longer survival showed lower ctDNA levels if compared with patients who showed early disease progression and shorter survival [54, 55].

Technical approaches for ctDNA

Nowadays, circulating tumor DNA represent a very high challenging and innovative analyte. Because of its very low concentration and degree of fragmentation in liquid samples, it needs very sensitive detection methods. Moreover, it has been demonstrated that plasma represents a better ctDNA source than serum, although the amount of ctDNA in serum can be 2–24 times higher than in plasma [56]. Indeed, during the ctDNA isolation process, it exists a high risk of cell contamination, and for this reason, it has been recommended the use of plasma as a source for the analysis of tumor-specific DNA. Moreover, another benefit deriving from plasma ctDNA is the lower concentration of background wild-type DNA [57]. One of the main issues in ctDNA analysis is the assay specificity and sensitivity. In fact, a major drawback of the ctDNA assays is the low frequency of some mutations that occur in tumors and the risk of interference by wild-type sequences [58]. There are now many methods for assessing ctDNA including the following:

- BEAMing a technology which combines emulsion PCR with magnetic beads and flow cytometry for the highly sensitive detection and quantification of tumor DNA molecules
- Next-generation sequencing approaches
- Digital PCR platforms

Each technique is able to detect mutant alleles with a sensitivity of at least 2 %. Unfortunately, costs and feasibility of developing the aforementioned methods in the clinic practice show several technical issues. However, the use of these strategies would allow and help clinicians in monitoring the disease progression by quantifying the number of ctDNA copies per milliliter of plasma (copies/mL) [59].

Circulating tumor DNA: applications

The application of new sensitive methods for very rare circulating mutation is urgently needed. In fact, the feasibility and potential utility of ctDNA for detection of PI3K mutations have been already deeply highlighted in patients with metastatic breast cancer [53, 60].

Recently, the detection of tumor DNA from tissue specimens and paired plasma samples has been reported. Moreover, the possibility to find mutations in ctDNA at early stage breast cancer can acquire high value as a marker for "minimal residual disease" (MRD) in those patients subjected to curative surgery. In particular, a recent work published by the group of Beaver et al. [61] investigated PI3K exon 20 and exon 9 mutational status through droplet digital PCR (ddPCR) in both tissue and paired plasma samples. The ddPCR is a new, highly sensitive, and cost-effective method that allows the analysis of rare mutation in a background of thousands of wild-type molecules. The aim of this study was to provide a proof-of-concept on the potential role of ctDNA as a marker for MRD. Nowadays, breast surgery represents the elective treatment for localized breast cancer patients. Unfortunately, to date, we do not have any marker to recognize patients definitively cured from those who may still have residual disease and thus may benefit from adjuvant treatment [46]. Beaver et al. [61] have analyzed 29 presurgery plasma

samples and compared the results obtained with paired post-surgery samples with the aim to highlight differences in PI3K fraction abundance among samples before and after resection. They showed that PI3K (exons 9 and 20) mutation levels in plasma differ among pre- and post-surgery withdrawal. These results, although preliminary, are very intriguing and could lead to a more personalized breast cancer management [61].

The feasibility of ctDNA analysis have been, not so far, investigated by Higgins et al., by using a BEAMing approach. This analysis was conducted within a double, retrospective and prospective, cohort of metastatic breast cancer patients. In the retrospective study, the aim was to verify BEAMing feasibility for the detection of PI3KCA mutations in plasma that was present in paired tumor tissue specimens. The method resulted reliable in identifying the same mutations observed in FFPE-derived tissue samples with a concordance of 100 %. In the prospective analysis, a cohort of 51 metastatic breast cancers was studied for PIK3CA mutations both by sequencing and BEAMing in tumor tissue and paired ctDNA. Also for this cohort, a high concordance rate was highlighted [62].

Another interesting molecular marker is TP53 mutation that can be evaluated from ctDNA as well as PI3K mutations. In particular, Madic et al. have demonstrated that TP53 mutations in plasma may help in the management of triple-negative breast cancer patients [63]. Mutations within TP53 sequence were analyzed by using next-generation sequencing platforms. TAm-Seq-based Illumina and 454 sequencing successfully allowed TP53 mutation detection in 84 % of tumor tissue specimens. The double NGS approach confirmed the same genetic alteration also in the paired plasma in 81 % of cases, demonstrating the robustness of NGS as a method to detect TP53 mutations [63].

CTCs: a general overview

Circulating tumor cells (CTCs) were first observed in the nineteenth century [64, 65]. Generally, CTCs have peculiar features including a visible nucleus and cytoplasm, and are mainly characterized for the expression of cytokeratin and no CD45 expression [66].

Since CTCs do not circulate in healthy patients and have been detected in patients with almost all cancer types, they acquire high relevance for studying the biology of the early metastatic disease and for diagnosis of metastatic patients [67].

Circulating tumor cells show a very low frequency in bloodstream [68]. Indeed, the number of CTCs would provide real-time information on the clinical behavior of many tumors, and they could additionally predict clinical outcome in metastatic patients [69, 70] as they seem to play a crucial role in mediating metastatic spread [71].

In fact, metastasis is a multistep process starting with the detachment of high malignant potential cells from the primary tumor site that could flow in bloodstream or the lymphatic system, and reach new distant sites. The final step for a circulating tumor cell is to extravasate, implant, and then proliferate generating a macroscopic, clinically detectable neoplastic growths [72].

Since CTCs seem to play crucial roles not only in metastasis but also in resistance to drug administration, it is fundamental to understand their clinical role during the early stages of the disease [73, 74].

Indeed, CTCs can predict early recurrence as well as decreased overall survival in chemo-naive patients with nonmetastatic breast cancer. These results confirm that the assessment of CTC might provide important prognostic information in these patients [75].

In a work by Franken et al. [76], a correlation study between stage and CTCs number was reported. The CTCs number evaluation was carried out using three cohorts of patients in stages I, II, and III, respectively. The detection of ≥1 CTCs in bloodstream was 16 % for breast cancer patients at stage I, 18 % for stage II, and 31 % for stage III. Therefore, CTC number positively correlates to disease stage, and patients with at least one CTC detected showed higher risk of recurrence than patients with no CTC detected. Furthermore, CTC evaluation before surgery plays a pivotal role in predicting disease-free survival (DFS) [76].

Furthermore, a massive presence correlates more strongly with poor prognosis than single CTCs in metastatic breast cancer patients [77]. On the basis of the different degree of epithelial and/or mesenchymal marker expression, CTCs can be divided different subpopulations [78, 79]. These subpopulations may also acquire general cancer stem-like properties allowing them to act as not differentiated cells by regulating several biological processes as quiescence, self-renewal, asymmetric division, drug resistance, resistance to radiation, and abilities in surviving within a foreign microenvironment, resulting then in metastasis. In fact, these stem-like breast cells commonly show a CD44+/CD24− phenotype [80] or the expression of aldehyde dehydrogenase 1 (ALDH1) [81].

Technical approaches for CTCs

CTCs are high malignant cells that circulate in bloodstream at extremely low frequency. CTC number is even lower in nonmetastatic disease [82, 83]. Current technical approaches for detecting CTCs can be mainly classified in enrichment and detection steps on the basis of specific capture properties (Fig. 2). Through enrichment methods, CTCs are detected for their physical properties such as cell size, density, and positive or negative immunoselection. In fact, CTCs are 20–30 μm in diameter, while blood cells

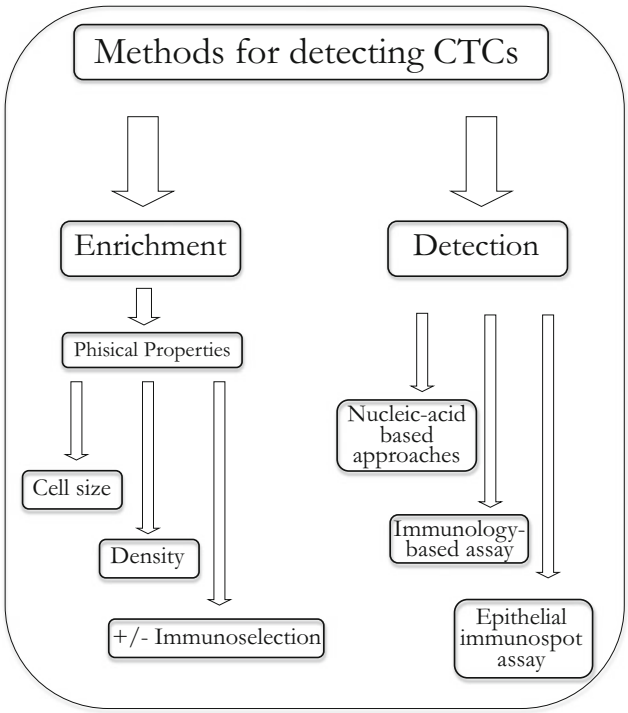

Fig. 2 Main technical approaches for CTCs isolation and enrichment

measure 8–12 μm [84]. Indeed, in order to discriminate cells for their size, several filtration methods have been developed [85]. Mononuclear cells and CTCs can also be discriminated from other cells on the basis of their density by performing a ficoll-gradient enrichment [86]. However, the most commonly used techniques for CTCs enrichment are the immuno-based approaches. The only FDA- approved automatized immunomagnetic enrichment is the CellSearch system. It relies on the ability of magnetic ferrofluids coupled to anti-EpCAM antibodies to recognize and bind only epithelial tumor cells expressing EpCAM, indirectly discriminating them from blood cells. Anti-EpCAM antibodies are added to 7.5 mL of peripheral whole blood [51]. EpCAM and members of the CKs family (CK8, CK18, and CK19) have been identified as useful markers for positive discrimination in patients with carcinoma. In particular, positive markers for breast cancer enrichment are considered EpCAM/CK, HER-2, Mammag lobin, and MUC-1 for their high expression degree in cancer cells [87]. On the contrary, negative discrimination can be carried out by using antigens as CD45, expressed in leukocytes, and CD61, expressed in megakaryocytes and platelets [88].

On the other hand, methods of CTC detection are divided into the following:

1. Nucleic-acid-based approaches: it relies on specific DNA or mRNA markers useful to identify and discriminate CTCs as epithelial-specific genes (*CK* and *EpCAM*), organ-specific markers (*CEA*, *PSA* [89], *mammaglobin* [90], and *MUC-1* [91]) and tumor-specific markers such as the *EGFR* and *HER-2* genes which are normally absent in circulation [92].
2. Immunology-based assay: it represents an effective method for CTCs detection and isolation. It uses labeled antibodies against epithelial or tumor-associated antigens along with automated digital microscopy or flow cytometry to identify and quantify CTCs.
3. Epithelial immunospot (EPISPOT) assay: it allows the detection of tumor-specific proteins.

Circulating tumor cells: applications

CTCs represent a reliable therapeutic indicator for many forms of cancer as indicated in recent evidences that suggested a strong correlation between CTC number and outcome with respect to PFS and OS [93, 94]. Many clinical trials have been specifically drawn to demonstrate that CTC identification and characterization may be used for improving the management of breast cancer patients as well for patients' stratification. Indeed, metastatic breast cancer patients showing a ≥5 CTCs number after 3–5 weeks from systemic and hormone- therapies correlate with shorter PFS than patients with less than five CTCs. Moreover, this strong correlation has been also demonstrated between CTCs counts and radiographic disease progression in patients treated with chemotherapies and hormone therapies [95]. Therefore, CTC isolation could be used in addition to standard methods for monitoring disease status in metastatic breast cancer [96]. A few studies suggest that CTC number may have a potential prognostic role in early-stage patients. Indeed, CTCs are detectable in 20–40 % of patients with early-stage breast cancer according to the aforementioned PCR-based assays and almost 10 % of early-stage patients according to the CellSearch system [93, 94, 97]. With respect to the main genetic alteration involved in breast carcinogenesis, Fernandez et al. [98] showed the feasibility of using CTCs for TP53 mutation detection as a noninvasive method. In particular, CTCs from two triple negative breast cancer patients were enriched using CellSearch system and single cell selected by DEPArray™. Distinct CTC populations were found, some of which harboring the same TP53 mutation (R110 delG), and also confirmed in the paired tumor samples, while some other showed either a different TP53 mutation (TP53 R110 delC) or the wild-type allele. These results indicate that CTCs could represent a noninvasive source of cancer cells for the determination of disease progression and the identification of new potential therapeutic targets [98].

Conclusions

In the last few years, we have experienced an increased interest of genetic aberration role in carcinogenesis. In fact, the use of molecular biomarkers in the next future would probably help clinicians in evolving their actual approach to patients' management for different forms of cancer (i.e., *c-KIT* and *PDGFRA* in GIST, *EGFR* in lung cancer, *BRAF* in melanoma, *RAS* in colorectal cancer, *PTEN* and *TP53* in breast cancer). Moreover, the introduction in clinical practice of many new generation approaches is day-by-day increasing the spectrum of gene mutations whose involvement in cancer would drive oncologist to new personalized treatments delivering. Despite this wide range of new acquired knowledge, mortality rate is still high. This is mainly caused by the development of metastasis due to cancer cell detachment from the primary tumors. It is now widely demonstrated that metastasis and primary tumors are biologically different. Therefore, these heterogeneity characteristics give to the metastatic disease the reason for considering it as a world apart to be still completely discovered. Thus, characterizing new lesions through a re-biopsy appears to be fundamental. Unfortunately, re-biopsy is not always indicated due to many reasons, first of all the extreme invasiveness that limits the procedure only to certain locations. In order to overcome these issues, the possibility of using liquid biopsies plays a primary role. CTCs and ctDNA have been well characterized as useful new and noninvasive biomarkers that can be used routinely because of their ease of access within many body fluids. Circulating tumor DNA is a double-stranded nucleic acid with low molecular weight that has been detected in the plasma and serum of cancer patients as well as in that of healthy controls. In cancer patients a considerable proportion of plasma DNA seems to originate from tumor cells, although another amount seem to derive from circulating cancer cells lysis as well as micro metastases. Moreover, ctDNA amount strongly correlates with tumor stage and size. The use of ctDNA as a liquid biopsy may help to obtain the genetic follow-up data that are urgently needed. Regarding CTCs analysis, at present, their role can be mainly limited to prognostic purposes, but there is an increasing interest in the development of new techniques for their molecular characterization. CTCs are cells that escape from the primary tumors and migrate through the circulation until they colonize a new district. It is well-known from recent clinical studies that their count is proportional to a poorer prognosis in various tumor types. Following this consideration, the growing interest in the last few years by the scientific community to liquid biopsies gives hope for their routinely application in clinic practice. Even if the road seems to be long and winding, new efforts and investments are needed to bridge the gap still existing.

Compliance with ethical guidelines

Conflicts of interest None

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Review Article

Stabilizing versus Destabilizing the Microtubules: A Double-Edge Sword for an Effective Cancer Treatment Option?

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Microtubules are dynamic and structural cellular components involved in several cell functions, including cell shape, motility, and intracellular trafficking. In proliferating cells, they are essential components in the division process through the formation of the mitotic spindle. As a result of these functions, tubulin and microtubules are targets for anticancer agents. Microtubule-targeting agents can be divided into two groups: microtubule-stabilizing, and microtubule-destabilizing agents. The former bind to the tubulin polymer and stabilize microtubules, while the latter bind to the tubulin dimers and destabilize microtubules. Alteration of tubulin-microtubule equilibrium determines the disruption of the mitotic spindle, halting the cell cycle at the metaphase-anaphase transition and, eventually, resulting in cell death. Clinical application of earlier microtubule inhibitors, however, unfortunately showed several limits, such as neurological and bone marrow toxicity and the emergence of drug-resistant tumor cells. Here we review several natural and synthetic microtubule-targeting agents, which showed antitumor activity and increased efficacy in comparison to traditional drugs in various preclinical and clinical studies. Cryptophycins, combretastatins, ombrabulin, soblidotin, D-24851, epothilones and discodermolide were used in clinical trials. Some of them showed antiangiogenic and antivascular activity and others showed the ability to overcome multidrug resistance, supporting their possible use in chemotherapy.

1. Introduction

Microtubules are dynamic and structural cellular components, typically formed by 13 protofilaments, which constitute the wall of a tube; each of the protofilaments consists of a head-to-tail arrangement of α/β tubulin heterodimers [1]. They are involved in several cell functions, including cell shape, motility, and intracellular trafficking. In proliferating cells, they are one of the essential components in the division process through the formation of the mitotic spindle. This event can take place because of the dynamic nature of microtubules through polymerization and depolymerization cycles [2]. As a result of these functions, tubulin and microtubules are targets for anticancer agents [3, 4]. Microtubule-targeting agents can be divided into two groups: microtubule-stabilizing and microtubule-destabilizing agents. The former bind to the tubulin polymer and stabilize microtubules, while the latter bind to the tubulin dimers and destabilize microtubules [5, 6].

Despite these differences, alteration of tubulin-microtubule equilibrium leads to the same final result: it disrupts the mitotic spindle, halting the cell cycle at the metaphase-anaphase transition and eventually resulting in cell death [7] (Figure 1).

Clinical application, however, has unfortunately shown several limits, such as a high level of neurological and bone marrow toxicity and the emergence of drug-resistant tumor cells due to the overproduction of P-glycoprotein (Pgp),

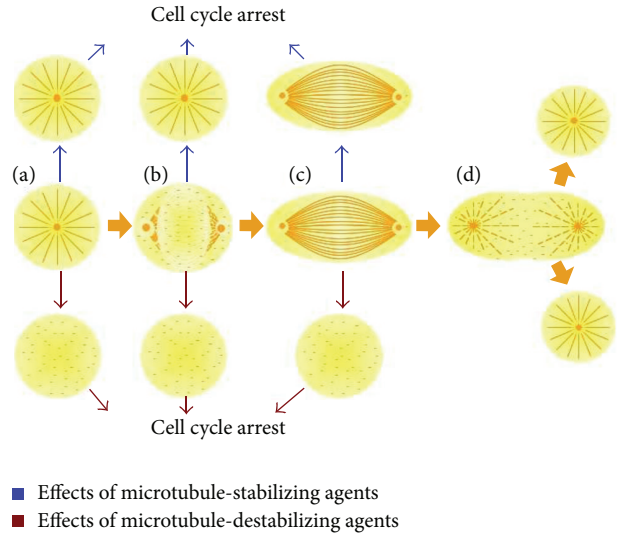

Figure 1: The dynamic nature of cytoskeleton is due to cycles of microtubule catastrophes. (a) Model structure of assembled cytoskeleton. The variety of shapes and sizes of the microtubule cytoskeleton is as great as the number of different cell types. In interphase, microtubules are long and stable because there are almost no catastrophes. (b) In mitosis, catastrophes are relatively frequent, resulting in highly dynamic microtubules that reach a steady-state length after a few minutes of growth (c). (d) After the segregation of chromatids, a new cycle of depolymerization and polymerization begins, resulting in a new stable microtubule cytoskeleton in daughter's cells (d). Blue and red arrows indicate effects of stabilizing and destabilizing agents, all resulting in cell cycle arrest.

an ATP-binding cassette (ABC) transmembrane transporter [8], the overexpression of different beta-tubulin isotypes, including βIII-tubulin [9, 10], or tubulin mutations [11].

Several natural and synthetic microtubule-targeting agents, exhibiting antitumor activity and increased efficacy in comparison to traditional drugs in various preclinical and clinical studies, have been discovered and their mechanisms have been elucidated [12, 13]. Apart from the well-known antimitotic function, for some of these drugs antiangiogenic and antivascular activity were demonstrated; for others the ability to overcome multidrug resistance was found. Many of these new generation microtubule-targeting agents are still under evaluation for clinical use. Some of them showed good tolerability and antitumor activity in particular cancers.

This review provides an overview of those microtubule-targeting drugs which are to date under clinical evaluation. A particular attention will be paid to the translation of preclinical data into the design of clinical trials.

2. Microtubule-Destabilizing Agents

Colchicine and Vinca alkaloids are two of the first microtubule-destabilizing agents to be discovered. These two compounds depolymerize microtubules by interacting with various β-tubulin sites. In particular, Vinca alkaloids interact with tubulin at specific binding sites which differ from those of other agents, including colchicine or taxanes, interfering with microtubule dynamics, blocking polymerization at the end of the mitotic spindle, and leading to metaphase arrest. Thanks to their peculiar mechanism of action, Vinca alkaloids have been widely used in anticancer therapy, usually in combination with other chemotherapeutic agents which do not have cross-resistance with them. First-generation Vinca alkaloids such as vinblastine have been included in the treatment protocol of both Hodgkin and non-Hodgkin lymphomas and testicular carcinoma, while vincristine has been approved for several years in the treatment of hematological tumors such as acute leukemia and multiple myeloma but also of rare tumors such as rhabdomyosarcoma and neuroblastoma. However, vincristine treatment was associated with a severe neurotoxicity, while the suppression of the bone marrow was more frequently reported during vinblastine therapy [14]. Second-generation semisynthetic Vinca alkaloids, vinorelbine and vindesine, have shown a broader spectrum of antitumor activity *in vitro*, along with a decreased neurotoxicity. Vinorelbine was approved as single agent and in combination therapy for the treatment of both hematological and solid tumors, including lung cancer, breast cancer, and gynecological tumors [15]. Recently, another synthetic Vinca alkaloid, vinflunine, has been approved in Europe for the second-line treatment of metastatic urothelial carcinoma. It is the first fluorinated microtubule inhibitor, which was associated with a higher antitumor activity than other Vinca alkaloids, showing also an excellent safety profile [16].

In order to overcome the clinical limits of these agents, in the last years attention has been focused on natural and synthetic compounds with a different structure but which act in a similar way [7, 17] (Table 1).

2.1. Cryptophycins. Cryptophycins are synthetic derivatives of macrocyclic depsipeptides, isolated by *Nostoc sp.* [18]. They block cell division and prevent the correct formation of the mitotic spindle, by inhibiting tubulin polymerization, probably at the binding site of the *Vinca alkaloids* [19]. In particular, C-52 and C-55 induce apoptosis by means of Bcl-2 hyperphosphorylation and inactivation [20–22] (Figure 2). These compounds are able to induce this phosphorylation at a greater extent than other microtubule inhibitors [23]. The first form discovered was epoxide cryptophycin 1, which showed antitumoral activity both in preclinical *in vitro* (colon, breast, ovarian, lung, and nasopharyngeal carcinomas) and *in vivo* (lung, breast, and prostate tumors) models. This has led to isolation and synthesis of cryptophycin analogs, divided into epoxides, chlorohydrins, and glycinate chlorohydrins [24] (Figure 3).

Cryptophycin 8 is the first C-1 analog synthesized in order to improve its antitumoral efficacy by means of conversion of the epoxide group into chlorohydrin. Its activity has been shown both in murine and human tumors. Although it is not as powerful as C-1, it is more soluble in water and has a stronger therapeutic effect. Nevertheless, it is still too unstable in solution to be considered clinically relevant [25].

Table 1: Microtubule-destabilizing agents.

Chemical lead	Properties and effects	Clinical trial/status	References
Cryptophycins	Apoptosis induction. Synergistic with chemotherapy and radiation.	Phase II clinical trials in platinum-resistant ovarian cancer and in NSCLC (C-52) but withdrawn due to peripheral neuropathy.	[26, 28, 31, 32, 36]
Combretastatin A-4-P	Antivascular and antiangiogenic activity. Synergistic with radiation, hyperthermia, chemotherapy, and immunoradiotherapy.	Phases II and III clinical trials in advanced solid tumors (lung and thyroid cancer) and in combination with carboplatin.	[63, 64, 66–70, 72, 73]
Combretastatin A-1-P	Antivascular and antitumoral activity superior to CA-4-P. Synergistic with chemotherapy.	Phase I clinical trials in solid tumors and in acute myelogenous leukaemia and myelodysplastic syndromes.	[78, 79]
Ombrabulin	Antivascular and antitumoral activity superior to CA-4-P. Synergistic with chemotherapy.	Phase I clinical trials as a single agent or in combination; phase III clinical trial in advanced soft-tissue sarcoma.	[86]
Soblidotin	Apoptosis induction. Antivascular activity. Antitumoral activity in tumors resistant to vincristine, docetaxel, and paclitaxel.	Phase II clinical trials in advanced solid tumors (soft-tissue sarcoma, NSCLC).	[99–105]
D-24851	Curative at nontoxic doses in rat tumor. No neurotoxic effects. Oral applicability. Activity versus MDR cell lines.	Phase I/II clinical trials in advanced solid tumors.	[140, 141]
Pseudolaric acid B	Antiangiogenic activity. No neurotoxic effects in tested animal. Activity versus MDR cell lines.	Preclinical phase.	[148, 149]
Embellistatin	Antiangiogenic activity.	Preclinical phase.	[150]

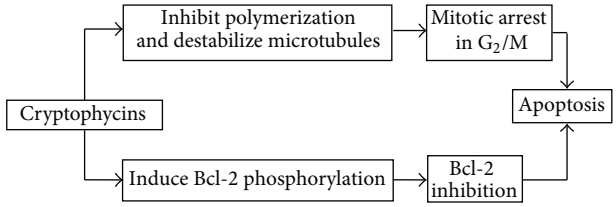

Figure 2: Mechanism of action of cryptophycins.

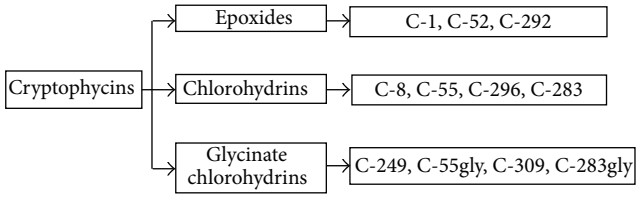

Figure 3: Classification of cryptophycins.

2.1.1. Cryptophycins 52 and 55. Cryptophycin 52 (LY355703) is a synthetic epoxide, used in phase II clinical trials, which presents a cytotoxic effect 400 times stronger than paclitaxel and *Vinca* alkaloids [26, 27]. It shows *in vitro* antitubulin, antimitotic, and cytotoxic activity which is dose-dependent against tumor cells. Furthermore, its activity has been evaluated both in murine tumor models and in human tumor xenografts [23]. C-52 resulted to be also effective against multidrug-resistant tumors [26, 28, 29].

Paclitaxel and the Vinca alkaloids are sensitive to the multidrug resistance (MDR) transporters P-glycoprotein (P-gp, MDR-1) and/or MDR-associated protein (MRP-1). Cryptophycin 52 was tested for its sensitivity to multidrug resistance in several paired cell lines in which a sensitive parental line was matched with a multidrug-resistant derivative line. Compared to other antimitotic agents (paclitaxel, vinblastine, and vincristine), the potency of cryptophycin 52 was shown to be minimally affected in multidrug-resistant cells compared to their sensitive parental lines [30]. Cryptophycin 52 fragment A analogues was synthesized to improve the potency and the aqueous solubility of the molecule allowing for the modification of its formulation. However, the same functional groups that rendered these analogues more potent and more water soluble also contributed to making them better substrates of the Pgp efflux pump. It is an unacceptable feature in the development of a clinically relevant antitumor agent [29].

Preclinical toxicological studies on animals (rats and dogs) have shown that above a certain concentration level C-52 causes secondary effects such as neutropenia and gastrointestinal problems but not neurotoxicity. These studies have allowed evaluating the optimum phase II dosage and tracing the plasma pharmacokinetic profile [26]. Furthermore, phase I clinical trials identified 1.5 mg/m^2 as a well-tolerated dose level of C-52. It was delivered as a 2-hour i.v. infusion on day 1 and day 8 repeated every 3 weeks [31]. This schedule was employed in a phase II study to determine the activity of C-52 in non-small cell lung cancer (NSCLC) patients

previously treated with platinum-based chemotherapy and to characterize its toxicity profile. A good rate of disease stabilization and an unacceptable toxicity was found in this setting [32]. Also, a multicenter trial was performed to evaluate the same schedule of the drug in patients with platinum-resistant advanced ovarian cancer. A considerable clinical benefit without serious adverse events was achieved [28]. Afterwards, these phase II clinical trials were terminated due to significant neurological toxicity [12].

Cryptophycin 55, a C-52 chlorohydrin, shows higher cytotoxic activity and therapeutic efficacy than its epoxide precursor, but its low stability in solution has delayed its clinical application [33]. This problem has been overcome, however, by means of the synthesis of glycinate esters (C-55gly, C-283gly, and C-309) which show not only an *in vivo* activity similar to their precursors but also a high level of stability [34].

Treatment with C-52 and C-55 combined with other chemotherapy agents has produced synergic effects without increased toxicity, bringing about a greater survival rate in ovarian carcinoma murine models [23, 28]. The use of human tumor xenografts has made it possible to evaluate C-52 and C-55 activity combined with cisplatin, carboplatin, and oxaliplatin in different tumors. C-52 showed a synergic effect only when associated with cisplatin, whereas C-55 showed increased activity with all the platinum compounds [35]. *In vivo* antitumoral activity of C-52 and C-55 has been assessed in combination with radiotherapy (2γ) or with 5-FU in tumor xenografts, showing an increased effect. Pharmacokinetic analyses performed in murine models have demonstrated that C-52 concentration in the tumor increased after administration and remained high for 24 hours. The mean life of C-55 was the longest in the liver, intermediate in tumor tissue, and less in plasma. After C-55 administration, the mean life of C-52 was the longest in tumor tissue, less in plasma, and even less in the liver, suggesting almost total conversion of C-55 into C-52 in the tumor. The greater C-52 accumulation in tumor tissue depends on the bioconversion of C-55 in C-52 and different binding affinities towards different tissue proteins. The use of C-55 to deliver C-52 increased the retention of C-52 in tumor tissue and reduced its presence in all studied normal tissues. Furthermore, extracellular acid pH of the tumor increased C-55 stability, whereas intracellular basic pH encouraged bioconversion by stimulating its pharmacological activity [36].

The obtained results indicated that C-52 and C-55 fulfilled all the criteria required by ideal chemotherapy agents, since they showed an action mechanism against a specific target and considerable activity against drug-resistant cells. However, the lack of response observed in some tumors and peripheral neuropathy have been limiting factors in the development of these agents leading to termination of their study.

2.1.2. Second-Generation Cryptophycins. C-309, C-249, and C-283 are second-generation candidates for clinical use. The first two are glycinate esters, synthesized in order to provide a higher chemical stability and more solubility in water. C-309 is a derivative of C-296 which has proved to have more therapeutic activity than C-55, C-283, C-249, and C-296; it is able to bring about a complete or partial regression of murine tumors at lower doses than those of other glycinate analogs. C-249 derives from C-8 and is active against MDR tumors. Moreover, it has the advantage of being easier to synthesize.

These second-generation analogs have proved to be up to 1000 times as active as those of the first clinical candidates (C-52) but with the same or even less toxicity [34].

2.2. Combretastatins. The combretastatins, isolated from *Combretum caffrum*, are molecules structurally related to colchicine which have been extensively developed since the late 1990s as vascular-disrupting agents (VDAs) [37]. The vascular-disrupting effect of these compounds is present well below the maximum tolerated dose, with a wide therapeutic window [38]. A number of combretastatins are currently in clinical trials: combretastatins A4- and A1-phosphate, verubulin, crolibulin, plinabulin, and ombrabulin [12].

2.2.1. CA-4-P. Combretastatin A-4 interacts with tubulin at the colchicine binding site but not in the same pseudoirreversible manner. It is used as a combretastatin A-4 3-O-phosphate (CA-4-P), a prodrug which is soluble in water and transformed into its active form by endogenous phosphatases [39]. It showed cytotoxicity in tumor cell lines and in human endothelial cells, HUVEC, which are sensitive to the drug only if they are actively proliferating, suggesting a potential use as an antiangiogenic agent [38]. By interfering with microtubule polymerization and with mitotic spindle assembly, CA-4-P induces G2/M arrest, thus bringing about cell death by either mitotic catastrophe or apoptosis [38, 40, 41].

Recent computational studies, using fluorescence spectroscopy, identified a potential binding site on γ-tubulin for both CA-4-P and colchicines [42]. Since high levels of γ-tubulin have been reported in poorly differentiated and aggressive brain tumors, such as human glioblastoma and medulloblastoma [43, 44] and lung [45] and breast cancer [46], the discovery of a potential site interaction on this molecule would offer the possibility of targeting inhibition with a new class of chemotherapeutic agents. However, the experimental validation of such interesting observation is underway.

CA-4-P (also known as Zybrestat or fosbretabulin) shows a potent *in vivo* antivascular activity since it causes a rapid and widespread reduction of the tumoral blood flow and an increased vascular resistance, effects which are extremely reduced in the normal tissues [47]. At a dose of 1/5–1/10 of the maximum tolerated dose (MTD), the central area of the tumor undergoes hemorrhagic necrosis, while a thin peripheral ring of live cells remains [38, 48, 49]. On the contrary, colchicine and other drugs act only at approximately MTD [50]. This constitutes an important advantage for the therapeutic application of CA-4-P. An immediate effect of CA-4-P treatment is an increased vascular permeability, which is important for the reduction of blood flow through vascular collapse, and an increased viscosity consequent to fluid loss from the vasculature. However, endothelial barrier function alterations and increased vascular permeability

might contribute to hastening tumor cell extravasation, causing progression to stages of greater malignancy, with heightened invasiveness and, in some cases, increased distant metastasis. It is no coincidence that susceptibility of tumors to CA-4-P showed a positive correlation with tumor vascular permeability [51]. Experiments conducted on HUVEC cells have shown that the CA-4-P-inducted microtubule depolymerization triggers off the actin reorganization through Rho activation and MLC (Myosin Light Chain) phosphorylation, thus causing rounding and retraction of cells and membrane blebbing. These events are associated with increased permeability, while the morphological cell change might contribute to determining the effects observed *in vivo* by means of vascular constriction [52, 53]. Furthermore, since CA-4-P interferes with the formation of stress fibers, it inhibits the VE-cadherin/β-catenin complex, thus leading to the destabilization of cell-cell junctions and increasing endothelial permeability [54].

The *ex vivo* perfusion of animal tumors highlights a lower increase in vascular resistance to that found *in vivo*, suggesting that the blood might also contribute towards the antivascular action of the drug [48]. It has been demonstrated that CA-4-P induces increased expression of endothelial CAM, responsible for the observed neutrophil recruitment which *in vivo* probably contributes both to vascular damage and to tumor cell death [55].

Apart from being an antivascular agent, CA-4-P inhibits the formation of new blood vessels, both *in vitro* and *in vivo*, presumably through inactivation of the VE-cadherin/β-catenin complex and Akt, all proteins required for cell adhesion, survival, and proliferation during neoangiogenesis. The same study has shown that smooth muscle cells, which are resistant to the drug, interfere with its antiangiogenic activity *in vitro*, suggesting that they may confer resistance to the endothelium by stabilizing cell-cell junctions [54]. CA-4-P selectivity towards the neoplastic tissue might therefore depend on the immaturity of tumor vessels, together with the proliferative status of tumor endothelial cells. Moreover, CA-4-P reduces *in vitro* HIF-1 expression (Hypoxia Inducible Factor-1) under hypoxia mainly in endothelial cells compared to that in cancer cell lines, suggesting a further possible mechanism of action for the drug [56] (Figure 4).

However, the effects of CA-4-P on tumor growth are not particularly significant, probably because of the persistent presence of vital peripheral cells [50], although the administration of several doses compared to the same total dose of the drug does increase its antitumoral effect [57, 58]. Furthermore, CA-4-P activity is directly proportional to tumor size [49]. This aspect, together with its capacity to act on the tumor core, differentiates this drug from more common therapeutic approaches, which target the peripheral tumor area. These complementary properties, together with the limited action of CA-4-P as a single agent, have led to experimentation involving combined treatments. It has been demonstrated that CA-4-P increases the response to radiotherapy and hyperthermia in treated tumors [57, 59] and, what is more, leads to a 90% increase in the retention of the anti-CEA antibody marked with I^{131} in the tumor, which is eradicated in 83% of the cases [60]. Similarly, CA-4-P increases the effect of chemotherapy drugs such as cisplatin, vinblastine, 5-fluorouracil, and irinotecan [57, 61].

The overall *in vivo* results obtained with CA-4-P have led to its introduction in phase I clinical trials [12, 62–66]. A phase I trial was performed to determine the MTD, safety, and pharmacokinetic profile of CA-4-P. This study showed absence of traditional cytotoxic side effects, with a toxicity profile which seems consistent with a "vascularly active" drug [67, 68]. The effects on tumor blood flow were assessed using dynamic contrast-enhanced magnetic resonance imaging (DCE-MRI) techniques. Dosages < or = 60 mg/m^2, as a 10 min infusion at 3-week intervals, define the upper boundary of the MTD. Similar effects were seen in other phase I clinical trials using a weekly and daily schedule [69, 70]. Afterwards, a further phase I trial investigated the combination of CA-4-P with carboplatin. A greater thrombocytopenia was observed as a consequence of altered carboplatin pharmacokinetics [63].

In order to improve its efficiency and reduce its side effects, a specific therapeutic system has been realized, based on the use of liposomes containing CA-4-P, carrying superficial RGD-peptides able to bind with the $\alpha_v\beta 3$ integrins overexpressed on proliferating tumor endothelium. *In vitro* tests have demonstrated the specificity and stability of the system, essential properties for its *in vivo* application [71]. To date, phase II/III clinical trials in lung and thyroid cancer are currently being evaluated [12]. These studies showed that CA-4-P with or without carboplatin and paclitaxel combination therapy was well tolerated in thyroid cancer patients, although it did not meet statistical significance in OS improvement [72]. Instead, preliminary data suggests survival benefits and increased responses without significant additional toxicity in NSCLC patients treated with CA-4-P in combination with carboplatin, paclitaxel, and bevacizumab compared to patients treated with carboplatin, paclitaxel, and bevacizumab only [73].

2.2.2. CA-1-P. Combretastatin A1 phosphate (also known as Oxi4503), a CA-1 water-soluble prodrug, shows a powerful antivascular activity. When used in murine and human tumor xenografts at much lower doses than those required by CA-4-P, CA-1-P brings about a drastic reduction of blood flow, with resulting necrosis [74]. CA-1-P causes an increase in vessel permeability, in VEGF production and apoptosis induction in endothelial cells [75]. At high doses it is more easily tolerated than CA-4-P and shows a much higher antitumoral activity, leading to complete regression of human tumors even at extremely low doses [74]. Excellent results have been obtained with combined treatments involving several chemotherapy agents [76].

In vitro pharmacokinetic studies have suggested that CA-1-P is transformed into a more reactive metabolite than CA-4-P, which is responsible for most of the antitumoral activity; this has formed the basis for further clinical developments of the drug as an antivascular and antitumor agent [77]. The drug has completed the phase I evaluation as a potential anticancer drug at three different centres in the United Kingdom, and it was studied in other phase I clinical trials [78, 79].

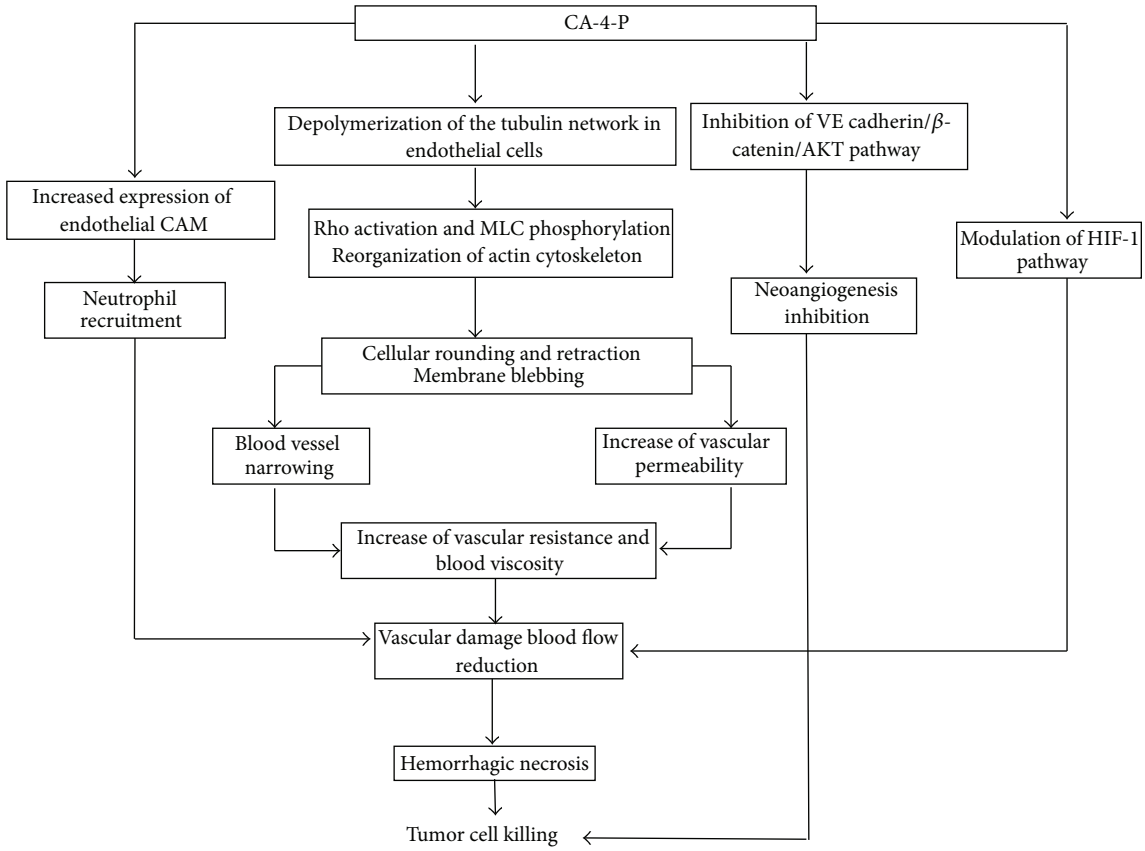

Figure 4: Combretastatin A-4-P: mechanisms of action at tumor level.

Recently, a new series of combretastatin derivatives have been synthesized and evaluated in seven cancer cell lines, exhibiting good anticancer activity [80, 81].

2.2.3. Ombrabulin. Ombrabulin (also known as AC-7700) is a serinamide hydrochloride, synthetic derivative of CA-4-P, which inhibits growth in a large number of drug-resistant animal tumors and carcinogen-induced tumors [39, 82]. Differently from CA-4-P, it does not act directly on the tumor vessels but instead causes constriction of the arterioles, resulting in complete downstream arrest of the blood flow and tumor growth [83]. These effects are obtained at doses half of MTD and 100 times less than that of CA-4-P [84]. Finally, the combination of AC-770 with cisplatin increases the effect of both drugs in murine tumors, with curative effects, and in human tumor xenografts [85]. In 2002, AC-7700 was introduced into phase I clinical trials in the United States and in Europe (AVE8062, *Aventis Pharma*). Recently, ombrabulin in combination with cisplatin was used in a phase III clinical trial for patients with advanced soft-tissue sarcomas after failure of anthracycline and ifosfamide chemotherapy, significantly improving progression-free survival. However, this improvement was not clinically relevant, despite being statistically significant [86].

2.3. Dolastatins. Dolastatins are pseudopeptides isolated from the sea hare *Dolabella auricularia* [50]. Dolastatins 10 and 15 showed antiproliferative activity. These agents induce apoptosis through interaction with tubulin [87]. Dolastatin 10 is a natural peptide able to interfere in microtubule assembly by means of the noncompetitive binding to the Vinca alkaloid site [88]. A phase II trial investigated dolastatin 10 in NSCLC patients. A low response rate was observed, even though a good tolerability was achieved. Myelosuppression was confirmed as the only noteworthy toxicity [89].

Other phase II clinical trials of dolastatin 10 were carried out in patients with metastatic melanoma, advanced colorectal and breast cancers, recurrent platinum-sensitive ovarian carcinoma, and hormone-refractory metastatic prostate adenocarcinoma [90–94]. These studies confirmed the same results previously obtained in terms of tumor response and toxicity. No activity was found in advanced pancreaticobiliary cancers and metastatic soft-tissue sarcomas [95, 96]. For this reason, it was suggested to not pursue the clinical development of this drug in further studies, not only because of its side effects [97] but also because of the low mean survival rate of the treated patients [95].

2.3.1. Soblidotin. Soblidotin (TZT-1027) is a synthetic analog of dolastatin 10 which inhibits the growth of several tumoral cell lines and induces caspase-3-dependent apoptosis. It shows *in vivo* antivascular effects in tumoral models overexpressing VEGF and in murine colon tumors, with an increase in vascular permeability, vessel closure, and

widespread hemorrhage. Soblidotin also shows antitumoral activity in vincristine-, docetaxel-, and paclitaxel-resistant tumors, which makes it a potential chemotherapy drug for use in tumors which do not respond to other microtubule inhibitors [98].

The first two European phase I clinical trials identified a recommended dose of soblidotin between 2.4 and 2.7 mg/m^2 for a 3-weekly administration with neutropenia, fatigue, and a reversible peripheral neuropathy as the DLT. Moreover neurological side effects seemed to correlate with previous exposure to other neurotoxic agents such as platinum compounds. No correlation was found with body surface area suggesting possible use of flat dose regimen for next trials [99, 100]. In a Japanese phase I clinical trial MTD of 1.5 mg/m^2 administered on days 1 and 8 in 3-week courses was found [101]. A combination of this drug with carboplatin was also tested. The recommended dose was 1.5 mg/m^2 for soblidotin and AUC 5 for carboplatin and no pharmacokinetics interaction was observed [102]. In NSCLC patients a phase I trial indicated a recommended dose of 4.8 mg/m^2, administered every 3-4 weeks as recommended dose [103].

Phase II clinical investigations suggested activity in advanced or metastatic soft-tissue sarcomas with prior treatment with anthracycline-based chemotherapy. This study confirmed tolerability profile, but objective response was demonstrated in none of the patients [104]. Another phase II trial showed no anticancer activity for soblidotin in NSCLC patients previously treated with platinum-based chemotherapy [105].

2.3.2. Dolastatin 15. Dolastatin 15 is very similar to dolastatin 10. It was demonstrated by chromatography that the binding domain is the same as Vinca alkaloids and antimicrotubule peptides. The site of the binding is not a well-defined locus but a series of overlapping domains [106]. This drug showed an effect on growth and differentiation in leukaemia cell lines [107], induction of apoptosis through Bcl-2 phosphorylation in small cell lung cancer cell lines [108], and G2/M cell cycle arrest in human myeloma cell lines [109]. Romidepsin (Istodax), a dolastatin 15 analog, which also possesses activity as a histone deacetylase inhibitor, was found to be active in cutaneous T-cell lymphoma with a 34% objective response rate and for this it was approved in 2009 [110, 111]. Other two analogues of dolastatin 15 are used in clinical trials: cemadotin and tasidotin.

2.3.3. Cemadotin. Cemadotin (LU103793) exerts its effect by inhibition of microtubule polymerization [112]. This drug is not able to inhibit the binding of vinblastine to tubulin and it can suppress microtubule growth without a significant microtubule depolymerization [113]. This agent was first evaluated in three phase I clinical trials for advanced refractory solid tumors with different schedules. Daily 5-day every 3 weeks schedule identified a recommended dose of 2.5 mg/m^2 per day. It was associated with neutropenia, peripheral edema, and liver function test abnormalities as DLTs. This dose showed lack of prohibitive cardiovascular effects. Acceptable general toxicity profile has allowed prompting phase II trials [114]. Meanwhile, cemadotin was studied for 24-hour intravenous (i.v.) continuous infusion every three weeks. 15 mg/m^2 was the recommended dose for this schedule. Hypertension was highlighted as the DLT, even if its nature remained unclear [115]. Even 5-day continuous intravenous (CIV) infusion was investigated. MTD was 12.5 mg/m^2. There were moderate nonhematologic toxicities and no evidence of the cardiovascular toxicity [116]. Pharmacokinetic analysis in these phase I trials suggested that cardiovascular toxicity may be associated with the magnitude of the peak blood levels of cemadotin or its metabolites, whereas myelotoxicity depends on the duration of time that blood levels exceed a threshold concentration.

The first phase II clinical trial which used this drug at 2.5 mg/m^2 daily 5-day schedule repeated every three weeks obtained clinical activity with durable response in chemotherapy-naïve patients with metastatic melanoma. Toxicity profile previously determined for this schedule was confirmed [117]. In contrast, no activity was observed with the same schedule in metastatic breast cancer patients previously treated with two lines of chemotherapy and in untreated non-small cell cancer patients [118, 119].

2.3.4. Tasidotin. Tasidotin (ILX651) is a third-generation dolastatin 15 analogue that is metabolically stable through its resistance to hydrolysis [120]. It demonstrated *in vitro* cell cycle arrest in the G2 and M phases and inhibition of tubulin polymerization similar to cemadotin and the Vinca alkaloids. It can inhibit the extent of microtubule assembly even at low concentrations [121]. *In vitro* study with MCF7/GFP breast cancer cells and *in vivo* pharmacokinetic analysis through LOX tumors xenografts proposed that tasidotin is converted in tasidotin C-carboxylate, a functionally active intracellular metabolite, 10 to 30 times more potent [122]. Capability of inducing apoptosis was observed in Ewing's sarcoma, rhabdomyosarcoma, synovial sarcoma, and osteosarcoma cell lines. Preclinical xenograft models of pediatric sarcomas showed antitumor activity [123].

Like cemadotin the schedule indicated for clinical use is daily administration for 5 days every 3 weeks. The recommended dose for investigation in phase II trial was 27.3 mg/m^2/day. The toxicity profile was favourable and antitumor activity was found in melanoma patients [124]. The other two schedules were evaluated in phase I trial: 34.4 mg/m^2 d1,3,5 q3 wk and 46.8 mg/m^2 d1,8,15 q4 wk [125, 126]. Tolerability was similar with these schedules.

2.4. Rhizoxin. Rhizoxin (NSC 332598) is a macrolide antitumor antibiotic extracted from a pathogenic fungus, *Rhizopus chinensis*. It is known for its antifungal activity, but it is also studied for cytotoxic activity in a variety of human tumor cell lines, including melanoma, leukaemia, sarcoma, and some human tumor xenografts of melanoma, lung, and breast cancer [127]. The drug can bind to tubulin and inhibits microtubule assembly, blocking the cell cycle at the G2-M phase [128]. It is a more potent cytotoxic compound than vincristine *in vitro*, and, in addition, it showed activity in vincristine-resistant cells [129].

A recommended dose of 2.0 mg/m^2 administered by i.v. bolus injection at 3-week intervals was identified through phase I trial because of its good tolerability with mucositis and neutropenia as the main toxicities [130]. Minimal or absent antitumor activity was found in phase II studies for patients with various advanced solid tumors [131–134]. A pharmacological study demonstrated the rapid and variable elimination of rhizoxin. These data could explain the low levels of systemic toxicity and the little response rates [135]. For this reason, alternative dosage and schedule were studied in phase I trial. A 72-hour continuous i.v. infusion indicated the dose of 1.2 mg/m^2/72 hours as the MTD. The toxicity profile was similar to that obtained with brief infusion, but yet no antitumor responses were found [136].

2.5. D-24851. D-24851 (N-(pyridin-4-yl)-[1-(4-chlorbenzyl)-indol-3-yl]-glyoxyl-amid) is a synthetic compound which has been selected by a cell-based screening assay by ASTA Medica AG, Germany. This drug destabilizes microtubules by interacting with a binding site that does not overlap with those of known microtubule-destabilizing agents like vincristine or colchicine [137, 138].

D-24851 (also known as indibulin) induces Bcl-2 and Bax-mediated apoptosis in both p53^{wt} and p53$^{-/-}$ cell lines [137, 139]. It produces *in vivo* curative effects in rat sarcomas at nontoxic doses, is suitable for oral use, does not give rise to neurotoxic effects at curative doses, unlike vincristine and paclitaxel, and is effective in MDR tumor cells, so that it is an excellent candidate as a chemotherapy agent [137]. In 2004, an LC/MS/MS (liquid chromatography/tandem mass spectrometry) system was proposed for quantitative analysis of D-24851 in human plasma and urine in phase I clinical trials. Indibulin was used in phase I/II clinical trials of patients with advanced solid tumors (metastatic breast cancer) [27, 140, 141]. In a phase I clinical trial indibulin was studied for oral administration once daily for 14 days every 3 weeks in patients with various solid tumors. Pharmacokinetic analysis showed a better tolerability under feeding condition. The recommended dose identified for further studies was 60 mg daily for 14 days. Dose-limiting toxicities were nausea and vomiting, which seemed to be related to solvent lactic acid [141].

Furthermore, the effects of two N-heterocyclic indolyl glyoxylamides derivatives of D-24851, BPR0C259, and BPR0C123 were investigated in NSCLC cells. The obtained results showed that these compounds can suppress the cell proliferation, by inducing p53-independent apoptosis and G2/M phase arrest, and potentially increase radiosensitivity of human lung cancer cells in a p53-independent manner [142].

2.6. Pseudolaric Acid B. Pseudolaric acid B (PAB) is a diterpene isolated from *Pseudolarix kaempferi Gordon* which is able to selectively inhibit the growth of actively proliferating cancer cells. It induces apoptosis through the intrinsic pathway, involving JNK/SAPK and p53. Nevertheless, its cytotoxic effects were found also in p53$^{-/-}$ cell lines, which is interesting for its therapeutic use [143, 144].

It interacts with a different binding site on tubulin compared with those of colchicine and vinblastine [143] and, both *in vitro* and *in vivo,* inhibits endothelial cells proliferation and VEGF-dependent formation of blood vessels. In fact, PAB antagonizes VEGF-mediated antiapoptotic activity by inhibiting the phosphorylation/activation of KDR, the VEGF receptor implicated in mediating this effect [145]. Furthermore, at nontoxic doses, PAB inhibits VEGF secretion from tumor cells by reducing its HIF-1-dependent transcription. PAB, in fact, acts by accelerating the proteasome-mediated degradation of HIF-1α, by means of a mechanism so far unknown [146]. PAB also induces endothelial cell retraction, intercellular gap formation, and actin stress fiber formation, effects which can be attributed to disruption of tubulin cytoskeleton and which contribute to its antiangiogenic action [147]. Moreover, PAB circumvents P-glycoprotein overexpression-induced drug resistance and the doses used are well tolerated and nontoxic and have not proved lethal on tested animals [143]. PAB showed significant inhibitory effect and an additive inhibitory effect in combination with adriamycin on the growth of gastric cancer *in vivo* [148, 149].

2.7. Embellistatin. Embellistatin is a ketone isolated from *Embellisia chlamydospora* which inhibits microtubule polymerization and shows a strong antiangiogenic activity. It inhibits *in vitro* bovine endothelial cells (BAEC) proliferation through p53 and p21 activation, thus inhibiting bFGF-induced formation of vessels. This antiangiogenic activity has been confirmed *in vivo* on murine models. Similar effects have been found in human tumor cell lines, suggesting that it could be suitable for use in the development of new anticancer drugs [150].

2.8. CI-980. CI-980 (mivobulin) acts at the colchicine binding site and it appears to have significantly less vesicant activity than vinblastine [151]. It is a mitotic inhibitor with *in vivo* and *in vitro* activity against murine multidrug-resistant sublines. Its interactions with microtubules *in vitro* are similar to other drugs, but cellular microtubule and mitotic inhibition is more potent [152]. The uptake of CI-980 is not temperature or energy dependent, and its passive diffusion is followed by a significant but largely reversible binding to intracellular or membrane components [153].

Mivobulin was tested in a phase I trial using 24-hour infusion repeated every 3 weeks. MTD was 14.4 mg/m^2. The main toxicities were neutropenia, dose-dependent but not dose-limiting, and early and reversible neurotoxicity characterized by dizziness, headache, loss of coordination, loss of consciousness, nervousness, and other symptoms. Tumor responses and tumor marker reductions were observed in a colon cancer patient and two ovarian cancer patients, respectively [154]. The same toxicity profile was confirmed in other studies [155, 156]. A continuous 72-hour infusion of MTD 4.5 mg/m^2/day every 21 days was associated with reduced neurotoxicity but dose-limiting neutropenia [157]. For this reason, it was used in phase II clinical trials. A similar tolerability profile was found. CI-980 seems inactive in metastatic colorectal carcinoma, advanced soft-tissue sarcomas, treated

and untreated melanoma, hormone-refractory prostate cancer, and malignant gliomas [158, 159]. Minimal activity was observed in platinum-refractory advanced epithelial ovarian carcinoma [160].

2.9. T138067. T138067 is a synthetic compound which irreversibly disrupts microtubule assembly by a selective and covalent binding to beta1, beta2, and beta4 isotypes of beta-tubulin at a conserved cysteine residue (Cys-239). Its action results in cell cycle arrest at G2/M and induction of apoptosis. It exhibits cytotoxic activity in tumor cell lines resistant to various antimicrotubule agents (vinblastine, paclitaxel, etc.) and in multidrug-resistant human tumor xenografts [161]. The covalent interaction of T138067 with β-tubulin may be proposed as a new way to overcome MDR. *In vivo* studies showed that this drug can cross the blood-brain barrier in mice, suggesting a possible use for brain tumors [162].

Phase I trials of T138067 were conducted by using a 3-hour infusion of drug given weekly or every 21 days with a recommended dose of 330 mg/m^2 per week. DLTs were neutropenia and neurological effects, consisting of encephalopathy, headache, hearing loss, and ataxia [163, 164]. This weekly dosage was used in two phase II clinical trials for patients with malignant glioma and metastatic colorectal cancer previously treated with irinotecan and 5-fluorouracil, respectively. The good toxicity profile was confirmed in both studies. No clinical activity in terms of antitumor responses was observed in both cases [165, 166].

2.9.1. T900607. T900607 is similar to T138067 for the kind of binding to tubulin in Cys-239 residue, but it is distinguished for a reduced ability to cross the blood-brain barrier.

A phase I trial indicated a recommended dose of 130 mg/m^2 delivered in i.v. infusion over 60 minutes on a 21-day cycle. No objective responses were observed but stable disease was reported in 7/20. Cardiac toxicity is the main drug-related side effect with this schedule. A different schedule consisting of weekly administration of T900607 identified MTD of 100 mg/m^2. This schedule was used in a phase II clinical trial for untreated patients with unresectable hepatocellular carcinoma. It showed good tolerability and moderate activity in some of these patients [167].

2.10. ABT-751. ABT-751, also known as E7010, is a sulfonamide able to impair microtubule formation and inhibit cell growth. Its binding characteristics seem to be different from that of colchicine and Vinca alkaloids. This agent has antiproliferative effects in many tumor cell lines which are drug-resistant due to the P-glycoprotein overexpression [168]. It showed a broad spectrum of activity against a variety of tumors in mice and human tumor xenografts, when administered orally [169]. Beta3 isotype is the preferential binding target. ABT-751-resistant cells were characterized by decreased expression of this tubulin isotype [170]. A warning derived from an *in vivo* study, which shed light on a possible testicular toxicity related to this drug administration in mice. It consisted of loss of seminiferous epithelial cells due to apoptosis of meiotic spermatocytes [171]. This drug selectively reduces tumor blood flow through tumor necrosis, regardless of a direct cytotoxic effect on tumor cells. Negligible is the effect on normal vascular function [172].

In a phase I clinical trial ABT-751 was administered as oral single or 5-day doses. The recommended dose for phase II trials is identified at 320 mg/m^2 for single dose and 200 mg/m^2/day for 5-day repeated dose. Peripheral neuropathy and intestinal paralysis were the DLTs. Gastrointestinal toxicity was dose-dependent but hematological toxicity was not dose-dependent [173]. Pharmacokinetic analysis of this study suggested that activity of ABT-751 may be time-dependent. For this reason a new schedule using a divided dose in order to maintain the blood level of ABT-751 has been formulated. The recommended dose in hematologic malignancies is 175 mg/m^2/day orally for 21 days every 4 weeks [174]. In a phase I trial for a pediatric population of patients with solid tumors ABT-751 was administered orally once daily for 21 days, repeated every 28 days. The MTD obtained for this schedule was 100 mg/m^2/day. DLTs included fatigue, sensory neuropathy, transient hypertension, neutropenia, thrombocytopenia, nausea, vomiting, dehydration, abdominal pain, and constipation [175]. In a phase II clinical trial, 21-day every 28 days schedule at the dose of 200 mg daily was studied in taxane-refractory NSCLC patients. Toxicity was acceptable. Median time to tumor progression and overall survival was 2.1 and 8.4 months, respectively. The objective response rate was 2.9% [176]. The combination of this agent with other cytotoxic drugs was proposed for future clinical studies. A phase IB study investigated clinical antitumor activity of ABT-751 in combination with docetaxel in patients with castration-resistant prostate cancer. Based on the cumulative safety analysis, the recommended phase II dose of ABT-751 is 200 mg daily with docetaxel 60 mg/m^2 for this patient population [177]. Further phases I and II clinical trials were carried out to evaluate activity of ABT-751 in combination with other drugs in advanced or metastatic NSCLC patients [178, 179]. ABT-751 showed adverse effects, although it has the advantage of being orally bioavailable.

3. Microtubule-Stabilizing Agents

Unlike the microtubule-destabilizing agents, there are other compounds that enhance microtubule polymerization. One of the most important classes of microtubule-stabilizing chemotherapy agents is that of taxanes, which target the cytoskeleton and spindle apparatus of tumor cells by binding to the microtubules, thereby disrupting key cellular mechanisms, including mitosis. The first microtubule-stabilizing agent used in anticancer chemotherapy [180] was paclitaxel. Thanks to their peculiar mechanism of action, taxanes are among the most effective chemotherapeutic agents used in the treatment of multiple solid tumors, such as breast, ovarian, lung, and prostate cancers. However, the occurrence of resistance limits treatment options and creates a major challenge for clinicians. Several potential mechanisms of resistance to these drugs have been identified, occurring at different pharmacodynamics levels. Besides the well-known overexpression of Pgp, an ABC transmembrane transporter

Table 2: Microtube-stabilizing agents.

Chemical lead	Properties and effects	Clinical trial/status	References
Epothilones	Elevated water solubility, activity versus MDR cell lines, and chemical malleability.	Phase II/III clinical trials in taxane-sensitive solid tumors (breast, lung, and prostate).	[195, 196]
Ixabepilone	Epothilone B analog, superior metabolic stability, and activity versus MDR cell lines.	Approved in 2007 for metastatic breast cancer; several ongoing trials in solid tumors.	[194, 197]
Laulimalide	Activity versus MDR cell lines and angiogenic activity, synergistic with docetaxel.	Preclinical phase.	[199, 201]
Dictyostatin	Activity against MDR cell lines, synergistic with taxol.	Preclinical phase.	[205, 206]

which pumps the drugs out of the tumor cells [8], the alterated expression of specific beta-tubulin isotypes, seems to play an important role. Most notably, the increased expression of βIII-tubulin isotype has been associated with resistance to taxanes in several cancers, including ovarian, breast, and lung cancer [9, 181, 182]. It was originally correlated to the qualitative or quantitative modifications of the microtubule complex, which represents the target of such agents, definitively reducing the drug binding affinity [27]. However, the aberrant expression of βIII-tubulin can also interfere with microtubule dynamics, increasing the dynamic instability and counteracting the stabilizing effect of taxanes, with consequences for drug sensitivity/resistance [183]. Recent studies have suggested βIII-tubulin as a prosurvival factor adaptively expressed by cancer cells exposed to microenvironmental stressors, such as hypoxia or deficient nutrient supply [184, 185]. The activation of the βIII-tubulin-dependent pathway in partnership with GTPases, such as guanylate-binding protein 1 (GBP1), is associated with the incorporation of PIM1 into the cytoskeleton of tumor cells, conferring a survival advantage in a hostile microenvironment and ultimately leading to the development of drug resistance [186]. Finally, a multitude of alterations involving the apoptotic signaling pathways downstream the microtubule complex, as well as aberrant expression of microRNA, have been also found in resistant tumors. A better understanding of the mechanism underlying the occurrence of acquired resistance has led to the development of a new class of microtubule-stabilizing agents, including epothilones, discodermolide, sarcodictyins, eleutherobin, and laulimalide, which are more readily modifiable, with different structures but a similar mechanism of action [187] (Table 2). Epothilones, discodermolide, eleutherobins, and sarcodictyins compete with paclitaxel for binding to microtubules and bind at or near the taxane site, whereas laulimalide seems to bind to unique sites on microtubules (Figure 5). Recently, a novel generation of paclitaxel derivatives have been designed, targeting a specific intermediate binding site in the microtubule with differential affinity, depending on the β-tubulin isotype expressed in the tumor. Since βIII-tubulin is overexpressed in the majority of aggressive, resistant tumors, the design of a βIII-tubulin targeted agent was expected to enhance the drug activity, reducing common toxicities. However, none of the new molecules tested in breast cancer cell lines was superior to the currently used taxanes [188].

3.1. Epothilones. Among several classes of microtubule-targeting chemotherapy agents that may maintain activity despite clinical resistance to taxanes, there are the epothilones which have been isolated from the soil bacterium *Solangium cellulosum* and have been studied most extensively in the clinical setting [189]. They induce the formation of an aberrant mitotic spindle, mitotic arrest, and apoptosis [190]. Their greater solubility in water and their activity in MDR cells have made them an alternative to paclitaxel in anticancer treatment [191, 192]. Moreover, their simple structure makes it easy to produce synthetic analogs during the clinical experimentation phase [190]. There are 4 classes of natural epothilones (A, B, C, and D). By means of the selection of resistant or taxane-dependent cells, it has been observed that tubulin β1 plays an important role in epothilone B functionality [193].

Ixabepilone (Ixempra) is a semisynthetic analog of epothilone B, selected because of its greater metabolic stability and its simple preparation. It is more powerful *in vitro* than paclitaxel and also presents cytotoxicity against MDR cells. It causes regression of MDR tumors and is more effective than paclitaxel in a wide spectrum of pediatric tumors [194]. Ixabepilone is currently the only approved epothilone derivative and the most clinically advanced (phases II and III clinical trials), showing efficacy in several patient subgroups and in various stages of breast cancer. This analog is used for the treatment of locally advanced or metastatic breast cancer as monotherapy after failure of a taxane, an anthracycline, and capecitabine, or in combination with capecitabine after failure of a taxane and an anthracycline [195].

A great number of phase II clinical studies of epothilones in cancer treatment have been reported, and significant activity in taxane-sensitive tumor types (such as breast, lung, and prostate cancers) has been observed [12, 17]. Response rates in taxane-refractory metastatic breast cancer are relatively modest, but ixabepilone and patupilone have shown promising efficacy in hormone-refractory metastatic prostate cancer and in taxane-refractory ovarian cancer [196, 197].

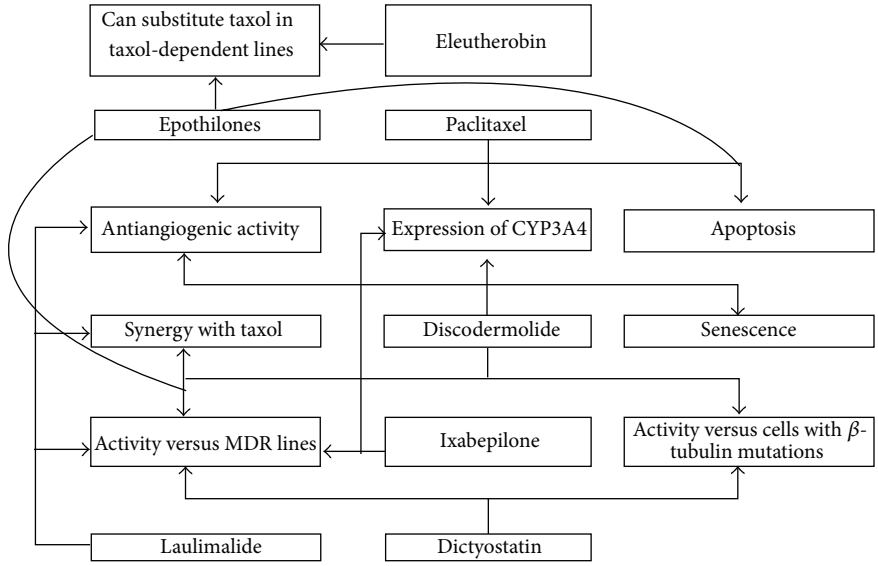

Figure 5: Similarities and differences between mechanisms of action and activity of microtubule-stabilizing agents.

3.2. Laulimalide. Laulimalide is a macrolide isolated from marine sponge (*Cacospongia mycofijiensis*) which inhibits cell proliferation, promoting assembly of the microtubules and stabilizing them in a taxol-like way, but at a different binding site located on two adjacent β-tubulin units between tubulin protofilaments of a microtubule [198–200]. This agent is also active in MDR cells which overexpress glycoprotein-P. When administered below cytotoxic doses, the drug prevents blood vessel formation and the VEGF-induced endothelial cell migration [201]. Docetaxel and laulimalide possess a synergic effect in these two processes, whereas they have antagonistic effects towards cell proliferation.

Used at low doses, laulimalide inhibits events downstream of VEGFR-2 activation, such as FAK and paxillin phosphorylation, VEGFR-2/FAK/Hsp90 interaction, and integrin activation. Compared with docetaxel, laulimalide has less effect on the VEGF-induced VEGFR-2/integrin $\alpha5\beta1$ interaction and is more effective with regard to phosphorylated paxillin levels. Furthermore, it inhibits RhoA/integrin $\alpha5\beta1$ association, suggesting that synergic effects of the two drugs might be explained by two different modalities of action.

The low quantities of the drug found in nature, together with its instability caused by its transformation into isolaulimalide, have led to the synthesis of the drug itself and of several analogs. The removal of a electrophilic and/or nucleophilic group, which prevents the substitution process, leads to major functional stability of the drug [202]. In preclinical phase, laulimalide so far showed poor efficacy and systematic toxicity [12]. The macrolide peloruside A shared many of the same properties of laulimalide, including its binding site and synergistic effects with the taxanes [203].

3.3. Dictyostatin. Dictyostatin is a macrolactone produced from sponges which induces *in vitro* tubulin assembly in the same way of paclitaxel but more rapidly. Like discodermolide, this drug possesses an antiproliferative action against paclitaxel-resistant human tumor cells as a result of β-tubulin mutations [204]. Dictyostatin inhibits the binding of discodermolide with microtubules and both drugs are able to inhibit the binding of epothilone B and paclitaxel with microtubules [204]. Several discodermolide/dictyostatin hybrids have been designed and have been found to maintain antiproliferative activities against several taxane-resistant cell lines [205, 206].

3.4. Eleutherobin. Eleutherobin is a glycosylate diterpene isolated from *Eleutherobia sp.* [207], which inhibits cell proliferation stabilizing microtubules. It binds at a site overlapping that of paclitaxel [208]. There is another group of cytotoxic agents, called sarcodictyins, which are structurally and functionally correlated to eleutherobins but not so toxic [209]. A form of cytotoxic diterpene, known as (Z)-sarcodictyine A, has been isolated from *Bellonia albiflora*; this exhibits a high level of toxicity towards human HeLa cells of the cervix [210]. Eleutherobin and the sarcodictyins have not been pursued clinically likely due to their susceptibility to Pgp-mediated transport [211].

4. Clinical Implications

In the last few years, a great amount of efforts has been put into the identification of new microtubule-targeting agents for use in anticancer therapy [212]. These last generation agents are also active in MDR tumors, which are resistant to the traditional antitubulin drugs used in chemotherapy, such as Vinca alkaloids and taxanes. Furthermore, these compounds have shown significant antivascular and antiangiogenic activity, leading to the possibility of using them both as alternatives to or in combination with preexistent

drugs, as already indicated in several published studies [213]. A lot of clinical trials were conducted to study microtubule-targeting agents. In particular, epothilones are in advanced phases of clinical development [214, 215]. In cancer therapy, microtubule-targeting agents can target angiogenesis, cell migration, and intracellular trafficking to prevent tumor growth and induce cancer cell apoptosis. These new agents, which impair or enhance tubulin polymerization, can be classified in two groups: natural and synthetic drugs. The natural compounds are derived from different species of uni- and multicellular organisms. To improve their pharmacodynamic and pharmacokinetic features some of these compounds are transformed in semisynthetic molecules. Other agents are produced by a totally synthetic procedure. The great diversity of natural and synthetic compounds capable of interacting with microtubules represents an important source for developing of novel potential anticancer agents. However, the effectiveness of these agents in cancer therapy has been impaired by various side effects and drug resistance. Phase I trials have allowed identifying more tolerable schedules with the most frequent toxicities represented by neutropenia and neurological, cardiovascular, and gastrointestinal effects. The main way of delivery is the i.v. infusion. Oral assumption was studied for the synthetic compounds D-24851 and ABT-751. The most evident efficacy was observed for rhizoxin, above all in NSCLC. For the other agents only minor or no responses were obtained. The identification of new schedules or the transformation in more potent analogues should allow overcoming these hurdles in their clinical advancement.

5. Conclusions

Data obtained up till now have allowed introducing some of these microtubule-targeting drugs into the clinical experimentation phase, whereas others, still in their preclinical phase, represent excellent candidates for a future use in cancer treatment, thus opening new roads towards the development of new, individual, and efficient therapeutic approaches.

Conflict of Interests

The authors declare no conflict of interests.

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