Cytotoxic effects exerted by pentachlorophenol by targeting nodal pro-survival signaling pathways in human pancreatic cancer cells

Susanne Schaefer, Jan N. Kreutzer, Olaf-Georg Issinger, Barbara Guerra *

Department of Biochemistry and Molecular Biology, University of Southern Denmark, Odense, Denmark

ABSTRACT

Pancreatic adenocarcinoma is one of the deadliest human solid tumors in the developed countries characterized by high resistance toward chemotherapeutic treatment. We have previously shown that silencing of the pro-survival protein kinase CK2 by RNA interference contributes to enhance the cytotoxicity of the chemotherapeutic agent 2′,2′-difluoro 2′-deoxycytidine (gemcitabine). Initial experiments showed that pentachlorophenol (PCP) inhibits CK2 and induces cell death in human pancreatic cancer cell lines. We report here evidence that exposure of this type of cells to PCP induces caspase-mediated apoptosis, inhibition of the lysosome cysteine protease cathepsin B and mitochondrial membrane depolarization. Beside cellular inhibition of CK2, the analysis of signaling pathways deregulated in pancreatic cancer cells revealed that PCP causes decreased phosphorylation levels of NF-κB/p65, suppresses its nuclear translocation and leads to activation of JNK-mediated stress response. Surprisingly, exposure to PCP results in increased phosphorylation levels of AKT at the canonical S473 and T308 activation sites supporting previous data showing that AKT phosphorylation is not predictive of tumor cell response to treatment. Taken together, our study provides novel insights into the effects induced by the exposure of pancreatic cancer cells to chlorinated aromatic compounds posing the basis for more advanced studies in vivo.

© 2014 The Authors. Published by Elsevier Ireland Ltd. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Protein kinases play an important role in the resistance of cancer cells to the cytotoxic effects of chemotherapeutic drugs. Mutations and aberrant activation of this class of enzymes is often linked to alteration of intracellular signal transduction pathways that control cell growth, differentiation, survival and motility [for a review see [1]].

Consequently, the connection between deregulated protein kinases and cancer led to the identification of small molecule compounds able to regulate the activity of this class of enzymes. In this respect, previous research focusing on the selection of compounds with a unique specificity toward individual protein kinases has shifted, in recent years, to the identification of drugs with broad specificity but high toxicity, thus, representing a therapeutic alternative to current treatment regimens.

Protein kinase CK2 is a pleiotropic and constitutively active serine/threonine kinase composed of two catalytic subunits α and/or α′ and two regulatory β-subunits. Evidence so far collected, suggests that this enzyme plays a significant role in regulating cell survival and conferring

* Corresponding author at: Department of Biochemistry and Molecular Biology, University of Southern Denmark, Campusvej 55, 5230 Odense M, Denmark. Tel.: +45 65502388; fax: +45 65502781.

E-mail address: bag@bmb.sdu.dk (B. Guerra).

resistance to apoptotic cell death [2–4]. In this respect, studies on pancreatic cancer cells, that are notoriously resistant to chemotherapeutic drugs currently employed in the clinics, revealed that down-regulation of CK2 by RNA interference significantly enhances cell death induced by gemcitabine treatment [5]. Perhaps, this effect should not come as a surprise since overexpression of CK2 has been documented in all cancer types so far investigated and associated with the aggressiveness of the tumor [2,6]. Higher than average CK2 activity offers a number of selective advantages to the tumors, hence, its inhibition or down-regulation would consequently weaken this growth advantage. In this respect, the identification of small molecule compounds able to inhibit significantly the activity of CK2 has become an important goal for the successful treatment of cancer.

Recently, the screening of small molecule compound libraries provided by the National Cancer Institute (NCI) under the Developmental Therapeutics Program (DTP), has led to the identification of C11 a two-components (i.e. PCP and DMA) cell permeable mixture able to inhibit endogenous CK2 and induce significant cell death in human pancreatic cancer cells. We have closely investigated the effects of C11 and identified pentachlorophenol (PCP) as its active component. PCP is a phenol derivative that has been extensively used as a wood preservative, insecticide and fungicide [7,8]. PCP undergoes oxidative dechlorination to form tetrachlorohydroquinone (TCHQ), a more toxic metabolite of PCP [9,10]. PCP toxicity seems to be related mainly to TCHQ-mediated uncoupling of oxidative phosphorylation and the generation of reactive oxygen species (ROS) in mitochondria [11,12]. Although it has been shown to promote tumor growth [13], studies suggesting that this compound and its derivative can induce cell death are sparse [14,15]. This work was initiated by our preliminary observations that PCP induces inhibition of CK2 in an ATP-competitive manner. The aim of this study was to contribute to the knowledge of the effects of PCP in human pancreatic cancer cells and to shed light on the intracellular signaling pathways involved in PCP-induced cytotoxicity. To the best of our knowledge, this is the first contribution on the characterization of PCP at the molecular level in this type of cells.

2. Materials and methods

2.1. Kinase assays

Protein kinase activity measurements of recombinant CK2α and CK2$\alpha_2\beta_2$ were performed in 40 μl of a reaction mixture containing varying concentrations of C11, PCP or dimethylallylamine (DMA), as indicated in the figure legends, 25 mM Tris/HCl pH 7.5, 5 mM NaCl for CK2α and 150 mM for CK2$\alpha_2\beta_2$, 18.75 mM MgCl$_2$, 0.5 mM DTT, 190 μM synthetic peptide RRRADDSDDDDD (KinaseDetect, Odense, Denmark), 125 μM ATP and 10 μCi [γ-^{32}P-ATP] (3000 Ci/mmol, Hartmann Analytic, Braunschweig, Germany). After incubation at 30 °C for 10 min, the reactions were stopped on ice and samples were spotted onto P81 cellulose paper (Whatman^{TM}, GE Healthcare, Brøndby, Denmark). Radioactivity incorporated into the

substrate target was determined by scintillation counting in a 1450 MicroBeta^2 Plate counter (PerkinElmer, Waltham, MA, USA).

2.2. Cell culture and treatments

The pancreatic ductal adenocarcinoma cell lines Panc-1 and MIA PaCa-2 (ATCC, Rockville, MD, USA) were cultured according to the manufacturer's guidelines and maintained at 37 °C in a humidified atmosphere supplemented with 5% CO$_2$. Cells were treated with C11 (NCI, Bethesda, MD, USA), pentachlorophenol (PCP, AccuStandard, New Haven, CT, USA), dimethylallylamine (DMA, Chemical point, Deisenhofen, Germany) and TNFα (R&D Systems, Abingdon, United Kingdom) as indicated in the figure legends. DMSO (Sigma–Aldrich, Schnelldorf, Germany) was used in all control experiments at a final concentration not exceeding 0.2% (v/v). Cell viability was determined by the WST-1 assay (Roche, Mannheim, Germany) in a 96-well plate. 24 h after seeding, cells were treated with various concentrations of C11, PCP and DMA, respectively, for 48 h. WST-1 reagent was added to the cells according to the manufacturer's instructions and cell viability was determined 2 h later in a VersaMax ELISA microplate reader (Molecular Devices, CA, USA).

2.3. Flow cytometry

Cell death was determined by propidium iodide staining as previously described [5,16]. In order to detect the mitochondrial membrane potential, cells were incubated with 5 μg/ml JC-1 (5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide, Invitrogen, Carlsbad, CA, USA) for 15 min at 37 °C. Cells were harvested by trypsinization, washed in PBS and re-suspended in 1 ml PBS prior to analysis by flow cytometry. For each measurement, 10,000 cells were analyzed on a FACSCalibur^{TM} flow cytometer (Becton and Dickinson, San Jose, CA, USA). Acquired data were processed by Cell Quest^{TM} Pro software (Becton and Dickinson).

2.4. Isolation of a CD24$^+$ stem-cell-like subpopulation of Panc-1

Isolation of CD24$^+$ cells was performed employing the CD24 Microbead Kit human (Miltenyi Biotec, Lund, Sweden) according to the manufacturer's instructions. Briefly, treated cells were harvested by incubation with 2 mM EDTA in PBS for 10 min at 37 °C. Cells were labeled with biotinylated anti-CD24 antibodies and subsequently incubated with anti-biotin-conjugated microbeads. Complexes were retained in a magnetic-activated Cell Sorting (MACS) column. CD24$^+$-cells were eluted after removal of the column from the magnetic field and re-seeded prior to treatment. In order to verify enrichment of stem-cell-like Panc-1, cells were stained with antibodies directed against two surface markers of stem cells, i.e. FITC-conjugated anti-CD24 antibody and APC-conjugated anti-CD44 antibody, respectively, both at 1:30 dilution for 30 min at 4 °C (Miltenyi Biotec) prior to FACS analysis.

2.5. Isolation of mitochondria

Cells were harvested by trypsinization and washed with ice-cold PBS. Mitochondria were isolated by using the Mitochondria Isolation Kit–human (Miltenyi Biotec) according to the manufacturer's instructions. Briefly, cell lysates were incubated with anti-TOM22 (Translocase of outer membrane 22 kDa subunit homolog)-microbeads. Labeled mitochondria were loaded on a MACS column and subsequently eluted after removal of the column from the magnetic field.

2.6. Immunofluorescence staining

For immunofluorescence analysis, cells were grown on cover slides and treated as indicated in the figure legends. For detection of NF-κB/p65, cells were fixed and permeabilized as previously described [17,18]. Cells were incubated with rabbit monoclonal anti-NF-κB/p65 (Cell Signaling Technology, Danvers, MA, USA) overnight at 4 °C. Incubation with the primary antibody was followed by labeling with biotinylated swine anti-rabbit immunoglobulins for 1 h at room temperature and FITC-conjugated streptavidin (all from Dako, Glostrup, Denmark) for 30 min at room temperature. Cells were counterstained with Dapi (4′6-diamidino-2-phenylindole, Sigma–Aldrich) for 5 min at room temperature.

For the analysis of the mitochondrial membrane potential, cells were incubated with JC-1 as described above, washed twice with growth medium and immediately analyzed under a fluorescence microscope.

Cells were analyzed on a Leica DMRBE microscope equipped with a DFC 420C camera and Leica Application Suite V 3.3.0 software (Leica Microsystems, Wetzlar, Germany) at 400× magnification.

2.7. Preparation of whole cell extracts, Western blot analysis and antibodies

Whole cell lysates and immunoblotting were as previously described [16]. The following antibodies were employed: mouse monoclonal anti-ATP5B, rabbit monoclonal anti-JNK, mouse monoclonal anti-Cdc37, rabbit polyclonal anti-p38MAPK (all from Santa Cruz Biotechnology, Santa Cruz, CA, USA); mouse monoclonal anti-caspase 8, mouse monoclonal anti-caspase 9, mouse monoclonal anti-phospho-p38MAPK (T180/Y182), mouse monoclonal anti-phospho-AKT (S473), rabbit polyclonal anti-phospho-AKT (T308), mouse monoclonal anti-cytochrome c, rabbit polyclonal anti-p44/42 MAPK (ERK1/2), rabbit polyclonal anti-phospho-GSK3β (S9), rabbit monoclonal anti-caspase 3, rabbit monoclonal anti-phospho-p44/42 MAPK (ERK1/2, [T202/Y204]), rabbit monoclonal anti-NF-κB/p65, rabbit monoclonal anti-phospho-NF-κB/p65 [(S536), all from Cell Signaling Technology]; mouse monoclonal anti-GSK3β, anti-PARP and anti-AKT1 (BD Biosciences, CA, USA); mouse monoclonal anti-β-actin (Sigma–Aldrich); mouse monoclonal anti-CK2α/α′ (1AD9) and mouse monoclonal anti-CK2β (6D5) (both from KinaseDetect); rabbit polyclonal anti-phospho-JNK [(T183/Y185), Invitrogen, Carlsbad, CA, USA]. Rabbit polyclonal anti-phospho-Cdc37 (S13) was kindly provided by Dr. Miyata, Kyoto University, Japan. Secondary antibodies goat-anti-rabbit and goat-anti-mouse, coupled to alkaline phosphatase, were purchased from Jackson ImmunoResearch, Newmarket, United Kingdom. Protein–antibody complexes were visualized by a chemiluminescent detection system using CDP-Star (Applied Biosystems, Foster City, CA, USA) substrate according to the manufacturer's instructions.

2.8. Cathepsin B activity assay

The measurement of cathepsin B activity was carried out with the cathepsin B activity fluorometric assay kit (BioVision, San Francisco, CA, USA). In brief, cells were collected by scraping, washed with cold PBS and lysed with lysis buffer. 100 μg whole cell lysate was employed for the determination of enzyme activity in the presence of amino-4-trifluoromethylcoumarin (AFC) conjugated to the cathepsin B sequence target Ac-RR (Ac-RR-AFC, 200 μM final concentration in the assay). Fluorescence emission was measured with a fluorometer (SPEX Fluorolog F2C, NJ, USA) employing a 400 nm excitation filter and a 505 nm emission filter. Acquired data were processed by DataMax software (Jobin Yvon™, NJ, USA).

2.9. Statistical analysis

All experiments were carried out at least three times and with triple measurements, if not otherwise stated. Standard deviation values (S.D.) are indicated in the diagrams as error bars. Statistical significance of results was calculated with the Student's *t*-test (two-tailed, same variance). Statistical significance is indicated in the figure legends by *P* values calculated between two sets of data.

3. Results

3.1. PCP is a newly identified ATP-competitive inhibitor of CK2

A preliminary chemoluminescence-based screening of a small molecule compound library in search of novel protein kinase CK2 inhibitors, led to the identification of C11, a mixture of two individual compounds; i.e. pentachlorophenol (PCP) and 2-methyl-n-(2-methyl-2-propenyl)-2-propen-1-amine (dimethylallylamine, DMA) in a 1:1 molar ratio. C11, PCP and DMA (Fig. 1a) were initially assayed for their ability to inhibit the kinase activity of CK2. As shown in Fig. 1b and summarized in Table 1, C11 inhibited both the CK2α subunit (IC$_{50}$ 4.96 μM) and CK2 holoenzyme (IC$_{50}$ 4.64 μM) in the low micromolar range. In the case of PCP, the IC$_{50}$ was 3.73 μM and 1.99 μM for CK2α and CK2 holoenzyme, respectively. DMA did not exert any inhibitory effect on both enzymes indicating that PCP is the active component in the C11 mixture. Further, a kinetic analysis was performed with two different PCP concentrations (i.e. 1 μM and 10 μM, respectively) in order to address the mechanism by which CK2 is inhibited by the aforementioned compounds. As shown in Fig. 1c and Table 1, K_m values increased concomitantly to increasing concentrations of PCP, moreover, the double reciprocal
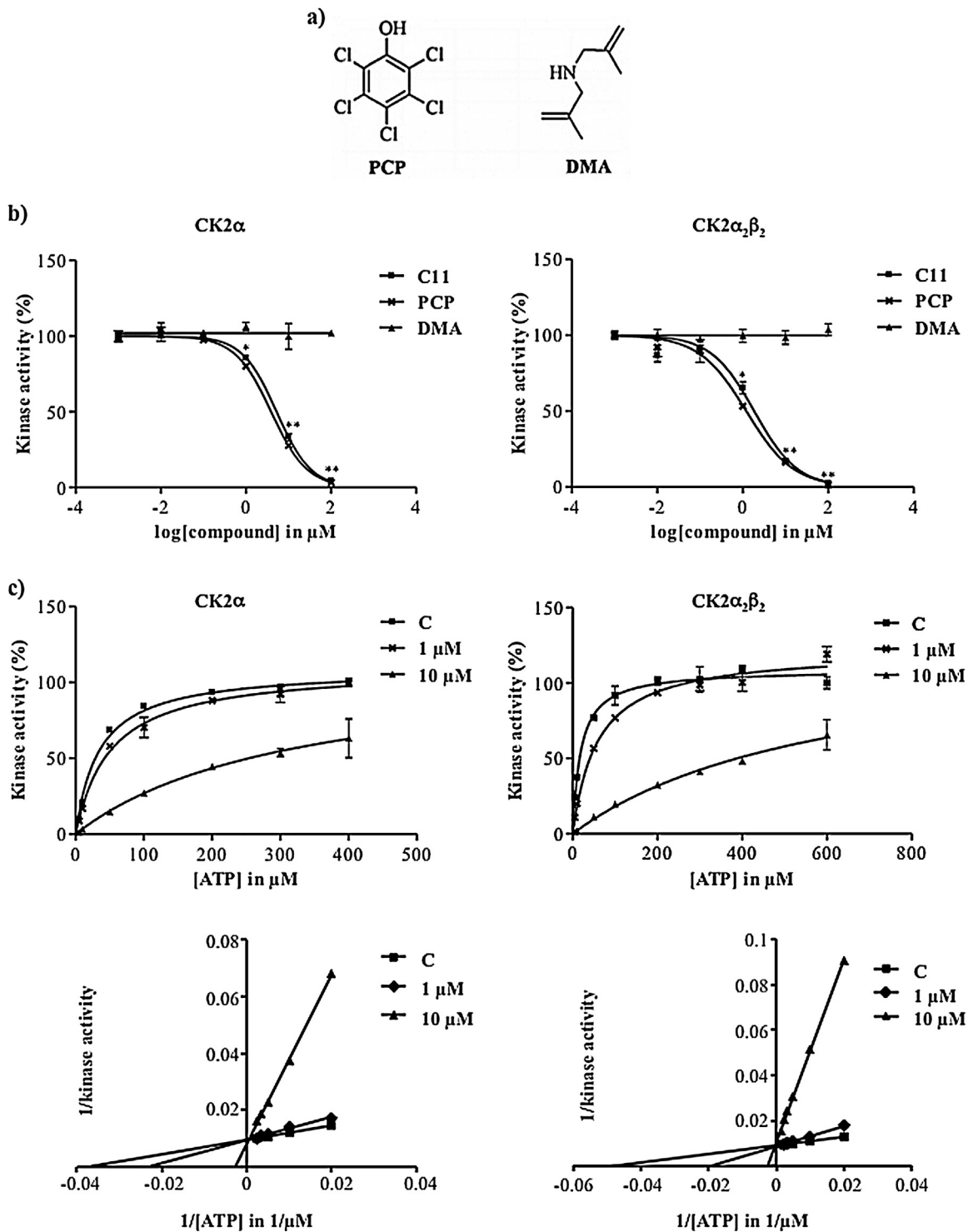
Fig. 1. PCP is the active component of C11 exerting ATP site-directed inhibition of CK2. (a) Chemical structures of PCP and DMA present in C11 in a 1:1 molar ratio. (b) The IC_{50} values for the CK2α subunit and the CK2 holoenzyme were determined by a dose-response curve for each individual compound/mixture, respectively. Inhibition is expressed in percentage relative to the activity of the enzyme measured in the absence of compound/mixture (i.e. control experiment). Results are mean values ± standard deviation (S.D.) from assays run in triplicates. $^{*}p < 0.005$, $^{**}p < 0.001$ denote statistical significant differences for measurements performed with C11 and PCP with respect to control values. (c) Kinetic analysis of enzyme inhibition by PCP. Upper bar-graphs refer to Michaelis–Menten kinetics while lower bar-graphs to Lineweaver–Burk plots. Enzyme activity was determined at various ATP concentrations in the absence or presence of two fixed concentrations of PCP, i.e. 1 μM and 10 μM, respectively. Results are mean values ± S.D. from experiments run in triplicates.

Table 1

Determination of IC$_{50}$ (μM), V_{max} and K_m values for the inhibition of CK2α and CK2α$_2$β$_2$ by C11, PCP and DMA, respectively. Data show V_{max} and K_m values ± S.D. of non-linear (i.e. Michaelis–Menten) regression of the obtained values calculated by GraphPad Prism. The results shown represent means of at least three independent experiments performed in triplicates.

	IC$_{50}$ C11 (μM) ± S.D.		IC$_{50}$ PCP (μM) ± S.D.		IC$_{50}$ DMA (μM)
CK2α	4.96 ± 0.32		3.73 ± 0.09		–
CK2α$_2$β$_2$	4.64 ± 0.39		1.99 ± 0.13		–

PCP (μM)	CK2α		CK2α$_2$β$_2$	
	V_{max} (U/ng)[a] ± S.D.	K_m (μM)[a] ± S.D.	V_{max} (U/ng)[a] ± S.D.	K_m (μM)[a] ± S.D.
0	6.7 ± 0.02	47.9 ± 0.73	5.7 ± 0.07	18.7 ± 1.9
1	6.4 ± 0.07	54.0 ± 1.18	6.1 ± 0.17	48.4 ± 3.29
10	6.9 ± 0.14	352.2 ± 4.75	5.0 ± 0.14	384.3 ± 26.55

1 U = pmol/min.

[a] Non-linear regression (Michaelis–Menten) values.

plots indicated that the inhibition is consistent with an ATP-competitive binding of the enzyme.

3.2. PCP reduces metabolic activity and induces cell death in human pancreatic cancer cells

We performed a WST-1 viability assay with two human pancreatic cancer cell lines, i.e. Panc-1 and MIA PaCa-2, for studying the effects of treatment with C11 and its individual components. As shown in Fig. 2a, incubation of cells with increasing concentrations of C11 or PCP for 48 h resulted in progressive cytotoxicity in both cell lines. Similar effects, albeit less pronounced, were obtained when PCP and DMA where combined in a 1:1 ratio. Incubation of cells with DMA alone did not result in reduced metabolic activity indicating that PCP is the component within C11 responsible for the reduced metabolic activity of the cells.

A subpopulation of cells within the Panc-1 cell line exhibits features of cancer stem cells (CSCs) such as extensive self-renewal, proliferation, tumorigenesis and high chemoresistance [19,20]. Thus, we asked the question whether PCP induced cytotoxic effects also in this fraction of cells by performing a WST-1 assay. Panc-1 cells were enriched in CSCs expressing the cell surface markers CD44 and CD24. The percentage of CD44$^+$/CD24$^+$ cells was determined by flow cytometry showing an increase from 4.35% to 23.33% (Fig. 2b). Panc-1 cells as well as a population of depleted and enriched CSCs were left untreated or exposed to C11 and PCP, respectively, for 48 h. Data reported in Fig. 2c show that PCP is toxic to all cell populations in a dose dependent manner and independently of the stem-like properties of the cells. Next, flow cytometry analysis was performed to measure the percentage of cells in sub-G1 indicative of cell death, in response to C11 and PCP treatment, respectively, (Fig. 3a). Cells were left untreated or incubated with 100 μM C11, PCP and DMA for the indicated times, respectively. In Panc-1 cells, 72 h incubation with C11 and PCP resulted in 23% and 29% of hypodiploid cells (fraction of cells in sub-G1), respectively. In the case of MIA PaCa-2 cells, the percentage varied from 16% (C11 treatment) to 25% (PCP treatment). Control cells or cells incubated with DMA for 72 h showed similar amounts of cells in sub-G1 that was equal to or below 10%.

3.3. The mechanism of cell death involves the extrinsic and intrinsic pathways of caspase activation and inhibition of cathepsin B activity

In order to gain insight into the type of cell death induced by PCP, we investigated cleavage of PARP as a measure of early-stage apoptosis and the cleavage of the major members of the extrinsic and intrinsic pathways of caspase activation. Panc-1 and MIA PaCa-2 cells were incubated with C11 and PCP at 100 μM concentration for 48 h, respectively. As shown in Fig. 3b, cell death activation appears to occur through the extrinsic caspase pathways as Western blot analysis revealed cleavage of caspase-8, -3 and PARP as compared to untreated cells. In the case of caspase-9, we observed a decreased intensity of full-length caspase-9 band in MIA PaCa-2 cells treated with PCP with respect to control cells indicating activation of the intrinsic apoptotic pathway. However, in the case of Panc-1 cells, there was no significant difference in the caspase-9 band intensity between control and PCP-treated cells suggesting activation of the sole extrinsic apoptotic pathway.

Cathepsins are a family of lysosomal proteases stored in lysosomes as inactive precursors known for their ability to initiate apoptotic cell death independent of caspases [21,22]. However, of the cysteine proteases, cathepsin B has been often implicated in the invasive and malignant progression of several types of tumors including pancreas, making this enzyme a relevant marker to cancer [23–25]. Hence, we addressed the question whether cathepsin B is involved in the cell death mechanisms of pancreatic cancer cells. As shown in Fig. 4, cells were incubated with 100 μM C11 and 100 μM PCP for 48 h, respectively. The activity of cathepsin B from whole cell extracts was measured by a fluorescence-based assay. The assay revealed a decrease of more than 50% in enzyme activity in both cell types suggesting that PCP-mediated inhibition of cathepsin B activity contributes to induce cell death in the investigated cell lines. Treatment of cells with 150 μM temozolomide (TMZ) served as a positive control indicating activation of cathepsin B. A negative control experiment was performed in parallel in the presence of CB inhibitor.

Next, cells were analyzed for the release of cytochrome c from isolated mitochondria. As shown in Fig. 5a, detection of cytochrome c content in MIA PaCa-2 cells revealed
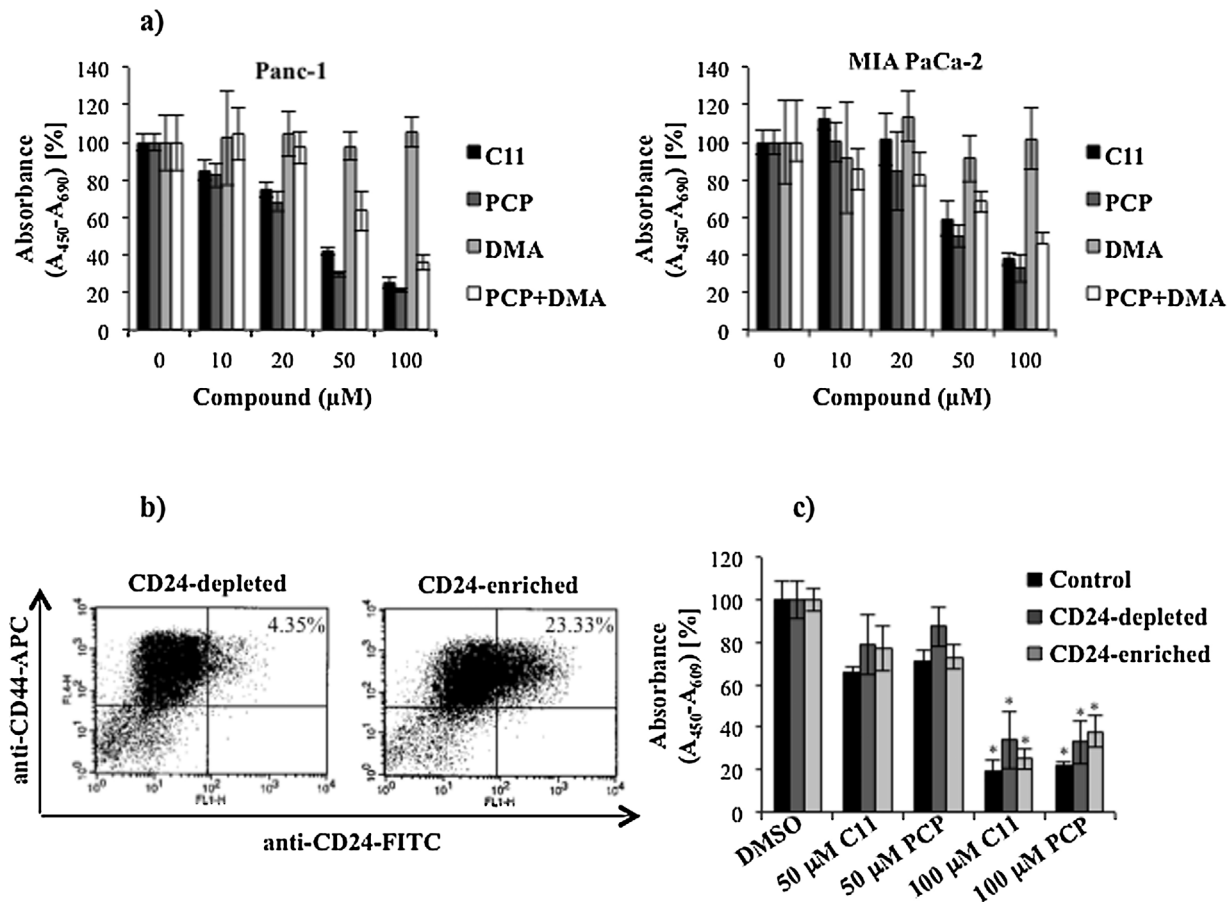
Fig. 2. Cytotoxic effects of PCP on human pancreatic cancer cells. (a) Panc-1 and MIA Paca-2 cells were incubated with increasing concentrations of C11, PCP, DMA for 48 h, respectively. PCP+DMA refers to the two individual compounds added to the cells in a 1:1 molar ratio. Cells incubated with solvent DMSO ($0\,\mu$M) were used as a control. Cell viability was determined by the WST-1 assay as described in Section 2. Viability was determined as a difference in absorbance measured at 450 nm and 690 nm (reference) wavelengths, respectively. Values were expressed in percentage relative to control assay. (b) Flow cytometry analysis of CD44 and CD24 expression in Panc-1 cells after enrichment. Two phenotypic subpopulations were collected. CD24-depleted cells and CD24-enriched cells. Percentage refers to the amount of $CD44^{+}/CD24^{+}$ cells in whole $CD44^{+}$-cell population. (c) CD24-depleted and CD24-enriched cell populations were treated with C11 or PCP for 48 h. Cytotoxicity was determined as described above. Control refers to the cell population before the enrichment procedure. Experiments were repeated three times obtaining similar results. Data from a representative experiment (mean values ± S.D. of eight replicates) are shown.

that treatment with C11 and PCP leads to a decreased protein band signal with respect to control experiment, suggesting release of cytochrome c into the cytosol and, hence, caspase-mediated activation of apoptotic cell death. $100\,\mu$M PCP was the most effective concentration. However, a clear decrease of cytochrome c content was not observed in Panc-1 cells as compared to control experiment represented by cells incubated with DMSO.

A hallmark of apoptosis is the loss of mitochondrial membrane potential [$\Delta\Psi_m$, [26,27]]. The release of cytochrome c from the mitochondrial intermembrane space into the cytosol has been shown to occur prior the loss of $\Delta\Psi_m$, locating cytochrome c release as an upstream event. However, some studies indicated that release of cytochrome c results from the opening of the mitochondrial permeability transition pore suggesting that loss of $\Delta\Psi_m$ is an earlier event in the activation of death pathways. Therefore, we analyzed the mitochondrial $\Delta\Psi_m$ using the lipophilic cationic dye JC-1, a

sensitive marker for mitochondria potential that emits green fluorescence when present at low concentration (i.e. monomeric form) and orange fluorescence when it accumulates in the mitochondria as aggregates. As shown in Fig. 5b, incubation of cells with $100\,\mu$M C11 or $100\,\mu$M PCP for 24 h led to a significant loss of orange fluorescence emission with respect to control experiments in both cell lines indicating severe loss of $\Delta\Psi_m$. Quantification of orange fluorescence emission by flow cytometry confirmed results obtained by fluorescence microscopy (Fig. 5c). Taken together, these data indicate that activation of cell death by PCP treatment results in mitochondrial depolarization in both cell lines while release of cytochrome c occurs solely in MIA PaCa-2 cells but not in Panc-1 cells. This suggests that the type of caspase-dependent activation of cell death following PCP treatment is cell type-specific and that mitochondrial depolarization and cytochrome c release are two events that occur independently from each other.
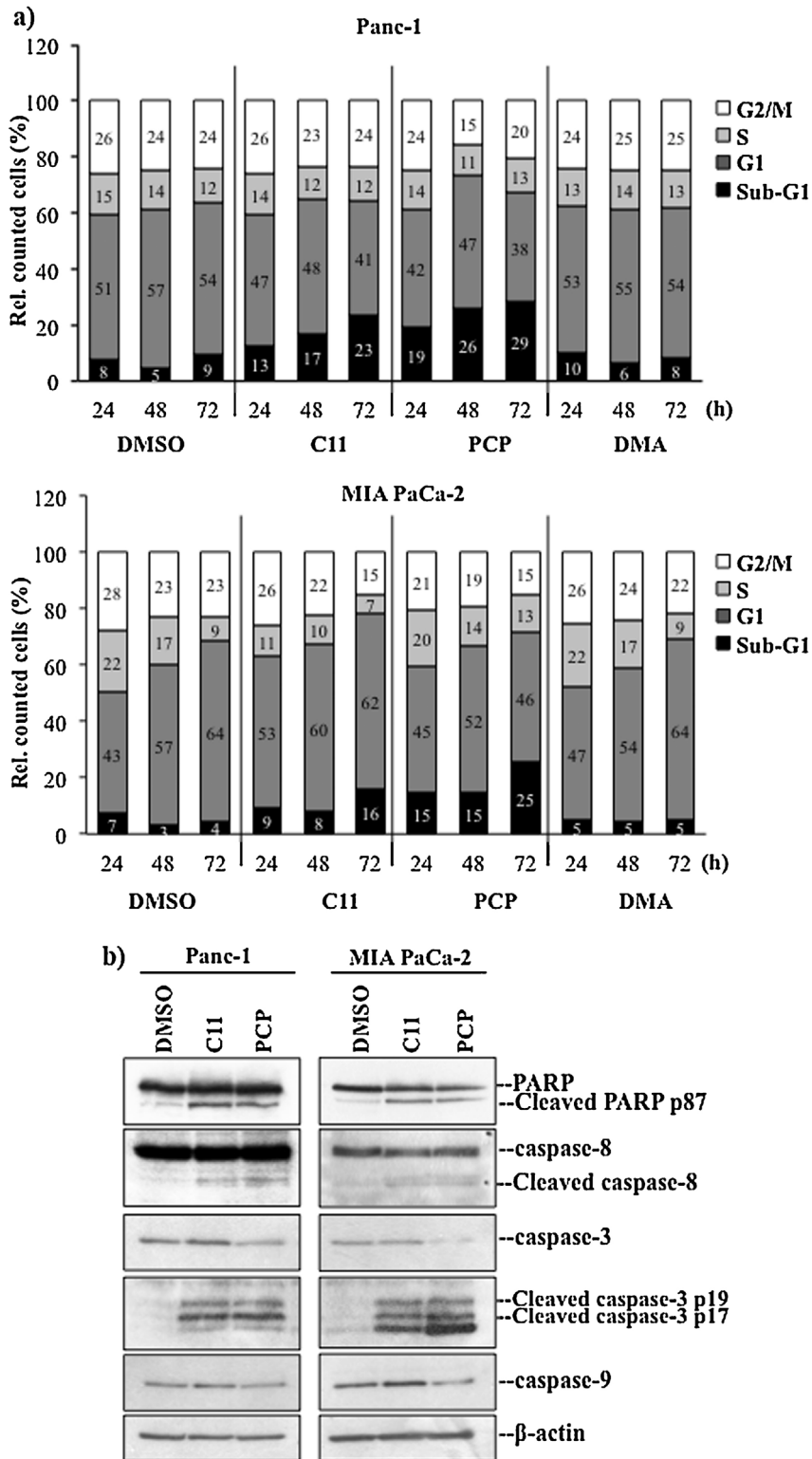
Fig. 3. Cell incubation with PCP induces apoptotic cell death in human pancreatic cancer cells. (a) Cells were incubated with DMSO or exposed to 100 μM C11, PCP and DMA, respectively, for variable incubation times. Cell cycle analysis was performed by flow cytometry. The distribution of cells in the various phases of the cell cycle is expressed in percentage. Cell death is indicated by the percentage of cells in the sub-G1 phase. (b) Western blot analysis of markers for apoptotic cell death from cells treated with 100 μM C11 and PCP, respectively, for 48 h. Control experiments are represented by the analysis of whole lysates from cells treated with DMSO. β-actin detection was performed for verifying equal loading. Experiments shown here, were performed three times obtaining similar results.
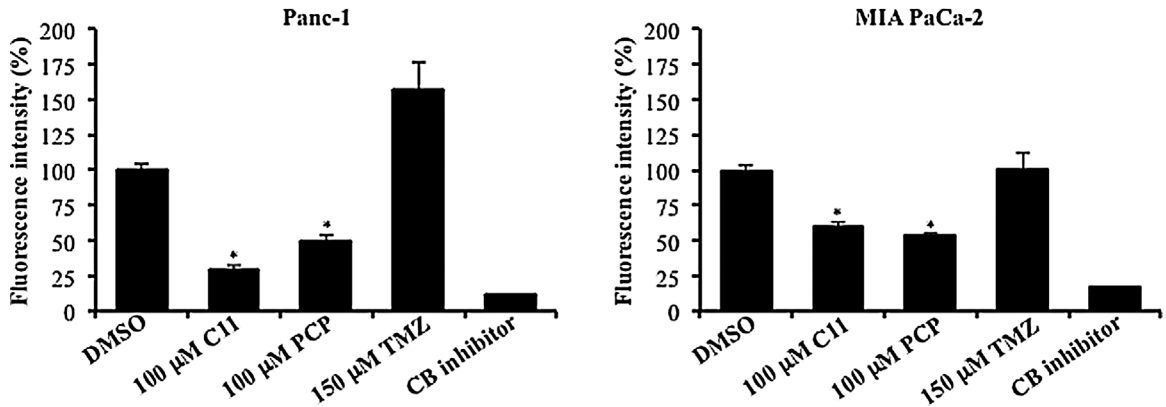
Fig. 4. Cathepsin B activity in whole cell lysates after treatment of cells with C11 and PCP reveals decreased enzyme activity. Cells were treated with DMSO (control experiment), C11 or PCP as indicated in the figure for 48 h. A positive control experiment was carried out by incubating cells with 150 μM temozolomide (TMZ) for 48 h. A negative control experiment was performed by adding 2 μl CB inhibitor to whole cell lysate (Cathepsin B assay kit, BioVision) for 2 h. The released fluorochrome AFC is expressed as percentage of fluorescence emitted/sample relative to release of reaction product in DMSO-treated cells. *$p < 0.005$ indicates values with statistically significant difference with respect to numbers obtained from DMSO-treated cells. Experiments were performed two times with four replicates each.
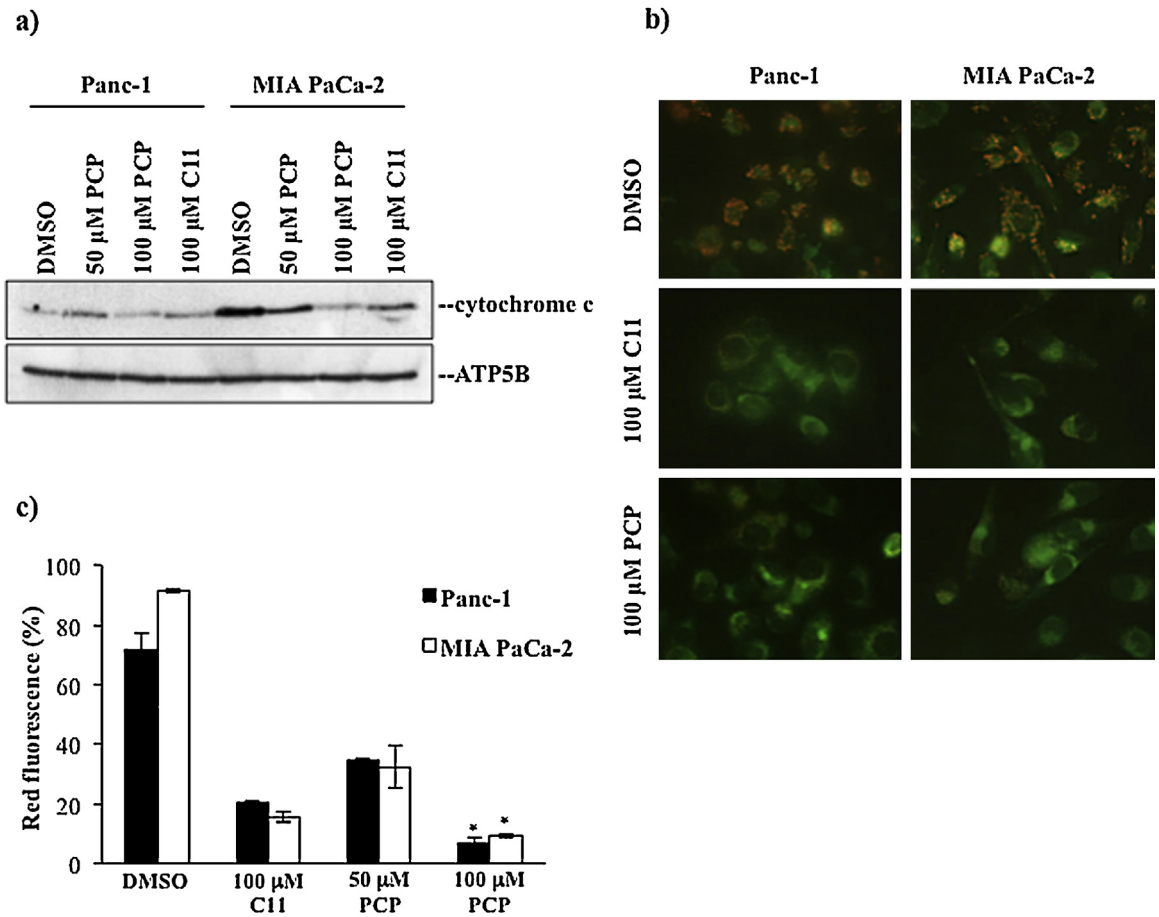

Fig. 5. Cytochrome c release following C11 or PCP treatment is cell type specific. (a) The content of isolated mitochondria from cells treated as indicated in the figure for 48 h was analyzed by Western blot using an antibody directed against cytochrome c. The detection of ATP synthase subunit β (ATP5B) was carried out as a control for equal loading. (b) Mitochondrial membrane potential was analyzed by cell staining with JC-1 reagent after the indicated treatments for 48 h. JC-1 monomeric form and aggregates in the mitochondria were detected using a BP 525/20; 635/40 filter allowing the simultaneous detection of green and red fluorescence emission. Cell images were taken at 400× magnification. (c) Quantification of the red fluorescence emission was carried out by flow cytometry and expressed in percentage relative to DMSO-treated cells. Results are presented as the average of three independent experiments. *$p < 0.005$ denotes statistical significant difference for measurements performed with cells treated with PCP with respect to control values.

3.4. PCP-mediated inhibition of CK2 is accompanied by activation of AKT1 and suppression of NFκB/p65 signaling pathway

Multiple lines of evidence have linked the PI3K/AKT, mitogen-activated protein kinases family (MAPK) and NFκB signaling pathways to chemoresistance of pancreatic cancer cell lines [28–33]. Given the importance of CK2 in the regulation of AKT, MAPKs and NFκB [5,34–37], we examined the effects of PCP on the phosphorylation levels of the major components of the aforementioned pathways. Treatment of cells with C11 and PCP, respectively, led to the inhibition of endogenous CK2 as shown by the decreased phosphorylation of the chaperone protein Cdc37 (Fig. 6a), a known CK2 substrate target [38], confirming the postulated inhibition of endogenous CK2 by PCP. The analysis by Western blot of major components of the PI3K/AKT signaling pathway revealed enhanced phosphorylation of both canonical regulatory AKT sites, i.e. T308 and S473, and the downstream protein target, i.e. GSK3β, as also indicated by the densitometric analysis of protein band signal intensity, suggesting that PCP activates rather than suppresses the PI3K/AKT signaling pathway (Fig. 6b). Analysis of the MAPK signaling pathway, revealed enhanced phosphorylation of the stress-activated Jun amino-terminal kinase (JNK) in both cell lines (Fig. 6c). Finally, treatment of cells with C11 and PCP, respectively, resulted in decreased phosphorylation of NFκB/p65 at the activating S536 and a concomitant reduction in total NFκB/p65 levels in MIA PaCa-2 cells (Fig. 6d) suggestsuggesting down-regulation of the NFκB-mediated signaling pathway. It has been shown that the pro-inflammatory cytokine tumor necrosis factor-α (TNFα) induces rapid phosphorylation of IκBα and its ubiquitin-induced degradation. This event is necessary for NFκB/p65 to be released from the complex with IκBα and for its relocation to the nucleus where it exerts transactivation functions by binding co-activator proteins [reviewed in [39]]. As incubation with PCP resulted in decreased phosphorylation of IKKβ-mediated phosphorylation of NFκB/p65 at the activating S536, we addressed the question whether PCP affected the TNFα-mediated translocation of NFκB/p65 into the nucleus. As shown in Fig. 7, treatment of both cell lines with TNFα led to the accumulation of NFκB/p65 in the nucleus with respect to DMSO- and PCP-treated cells, respectively, where NFκB/p65 appeared to localize in the cytoplasm. However, incubation of cells with PCP suppressed TNFα-induced migration of NFκB/p65 into the nucleus as indicated by the persistent signal in the cytoplasm.

4. Discussion

The study presented here, indicates that PCP is the active component of C11 exerting cytotoxic effects in human pancreatic cancer cell lines. Our previous studies showed that simultaneous silencing of the CK2 catalytic subunits by RNA interference enhances the sensitivity of these cell lines toward chemotherapeutic agents currently used in the clinics for the cure of advanced pancreatic cancer [[5], for a review see [40]]. We show here that PCP inhibits recombinant human CK2 in an ATP-competitive manner as well as the endogenously expressed enzyme as revealed by the decreased phosphorylation of the molecular chaperone Cdc37 at S13, a known cellular substrate target of CK2 [Fig. 6 [38]]. Evidence indicates that CK2 supports survival and confers resistance to chemotherapeutic treatment of cancer cells [reviewed in [2,6]]. Hence, PCP-mediated induction of cell death in human pancreatic cancer cells reported here, may be due, at least partially, to the inhibition of endogenous CK2. However, as protein kinase CK2 expression levels have been shown to be elevated in cancer and highly proliferating cells, we cannot exclude that other types of cancer cells would respond to PCP treatment in a similar fashion. The poor prognosis of pancreatic cancer is in part attributed to the presence of a subgroup of cancer stem cells which account for tumor recurrence due to their self-renewal, metastatic potential and resistance to cytotoxic drug treatment [19,20]. Interestingly, we show here that incubation of a sub-population of Panc-1 cells enriched in cancer stem cells with PCP induces a level of cytotoxicity comparable to the one observed in the cancer stem cells-depleted population suggesting that PCP treatment lowers the intrinsic resistance of cancer stem cells to cell death induction (Fig. 2).

In the present study, we show that PCP induces apoptotic cell death as demonstrated by cleavage of PARP and activation of caspase-3 (Fig. 3), both considered a hallmark of apoptosis [41,42]. Interestingly, the type of death signal generated in the two cell lines seems to differ and be cell type-dependent. In this respect, we show that PCP treatment of MIA PaCa-2 cells leads to activation of both the extrinsic and intrinsic caspase-mediated apoptotic pathways as indicated by the cleavage of caspase-8 and caspase-9, respectively, and the dose-dependent decreased level of cytochrome c in the mitochondria (Fig. 5). In the case of Panc-1 cells, induction of cell death is mediated solely by the death receptor-mediated caspase pathway as indicated by the cleavage of caspase-8 and lack of significant decrease in the levels of mitochondrial cytochrome c as compared to control cells.

Loss of mitochondrial membrane potential is believed to occur during activation of death pathways and accompanied by cytochrome c release. As shown in Fig. 5, both cell lines lose their membrane potential during C11 or PCP-induced apoptosis as indicated by the remarkable decrease in the JC-1 red fluorescence signal. Surprisingly, it appeared that decreased $\Delta\Psi_m$ did not correlate with cytochrome c release in Panc-1 cells suggesting that these two events occur independently from each other. In support of these data, Johnson et al. [43] proposed that the mitochondria contribute to the activation of death pathways at various levels and that release of cytochrome c and mitochondria depolarization are separate and independent events depending on where the contribution of the mitochondria in the death pathway resides.

The analysis of intracellular signaling pathways that have been shown to be de-regulated in pancreatic cancer supporting growth and conferring chemoresistance, suggest that the cytotoxic properties of PCP are not solely confined to the inhibition of CK2 but also to alteration of other intracellular signaling molecules. In this respect, phosphorylation of JNK was found up-regulated. JNK is part
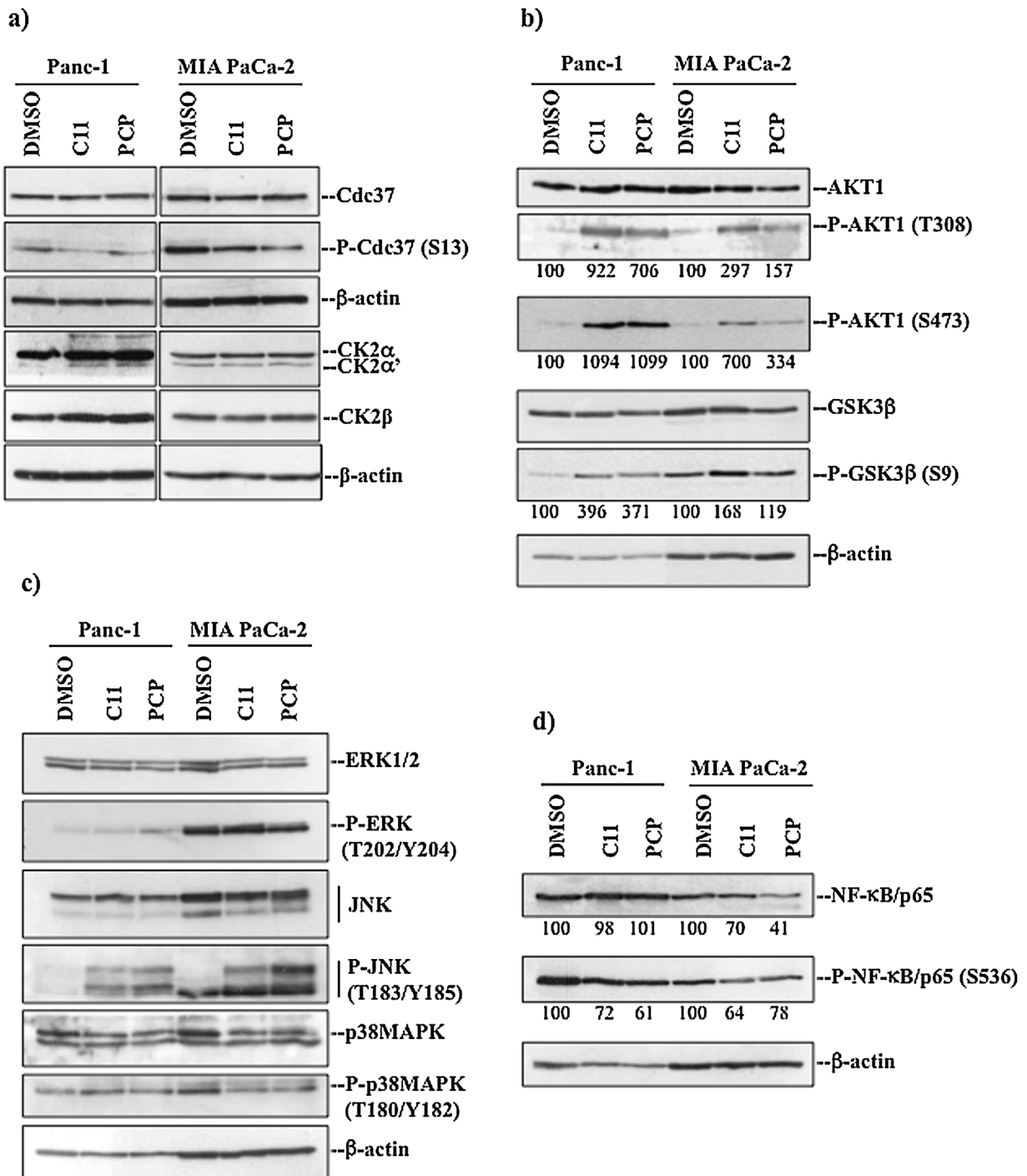
Fig. 6. Analysis of cell signaling pathways de-regulated in human pancreatic cancer cells. (a–d) Whole lysate from cells treated as indicated in the figure for 48 h were analyzed by Western blot employing antibodies directed against the indicated proteins or their phosphorylated form, belonging to the PI3K/AKT, MAPK and NFκB signal transduction pathways, respectively. Analysis of the expression and phosphorylation status of Cdc37, an endogenous substrate of CK2, and expression levels of the CK2 subunits is also shown. In all experiments, β-actin was detected as control for equal loading. Numbers below the blots refer to quantitation of band signal intensity with ImageJ software. Experiments were performed at least three times obtaining similar results.

of a family of protein kinases activated in response to a wide range of cellular stresses [44]. Hence, increased phosphorylation observed following C11 or PCP treatment might represent a stress response accompanying activation of the

apoptotic cell death signaling as previously postulated [45]. Unexpectedly, the anti-proliferative response of PCP correlated with increased phosphorylation of AKT S473 and T308 and a mild effect on AKT protein expression levels
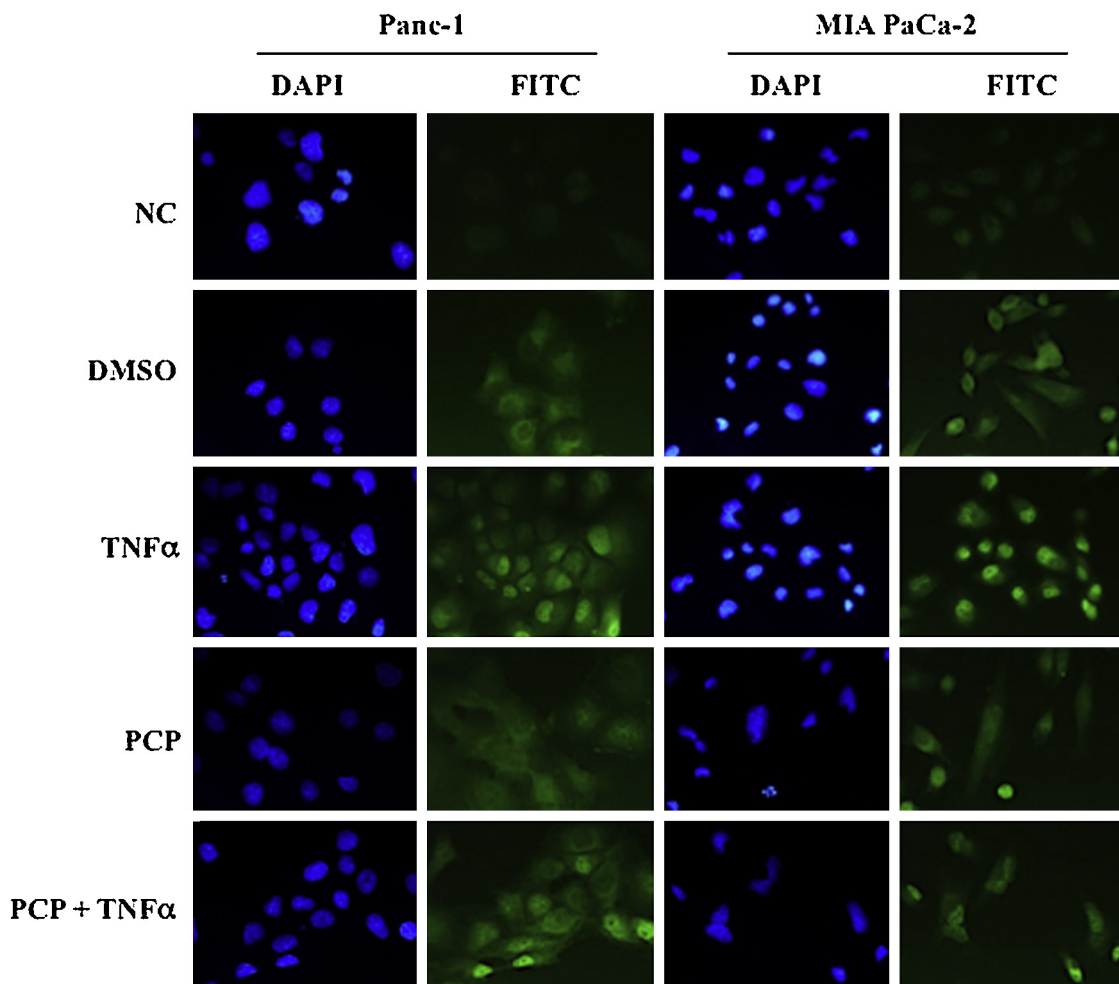
Fig. 7. Cell incubation with PCP influences negatively the TNFα-induced NFκB/p65 translocation into the nucleus. Cells were left untreated or incubated with 100 μM PCP for 48 h. Where indicated, cells were added 20 ng/ml TNFα in the last 15 min of incubation time. After fixation, cells were subjected to immunofluorescence analysis. Nuclei were visualized by DAPI staining. Negative control (NC) refers to cells immunostained with only the biotinylated secondary antibody and FITC-conjugated streptavidin. Original magnification: 400×. At least 50 cells were analyzed per treatment condition.

in Mia PaCa-2 cells (Fig. 6b). At a first glance these results may appear contradictory as the PI3K/AKT signaling pathway has been linked to cell growth and survival and, thus, one would expect that this signaling cascade would remain unaltered or be suppressed during induction of cell death. However, recent data suggested that cellular outcome in tumor cell treatment cannot be predicted by changes in the phosphorylation status of AKT as induction of its phosphorylation in the presence of the broad anti-tumor agent everolimus (RAD0019) has been shown to occur as a results of mTORC1 (rapamycin-sensitive kinase complex containing raptor) inhibition in a rictor-dependent manner [46]. Further supporting this, a negative feedback loop has been described, where mTOR/S6K1 activation results in PI3K signaling inhibition by suppressing the insulin receptor-dependent cascade [47–49]. Hence, it remains to be determined whether the anti-proliferative response in cells incubated with PCP is accompanied by mTORC1 inhibition and whether suppression of AKT phosphorylation at S473 can be induced by rictor down-regulation.

The NFκB signaling pathway is implicated in the regulation of numerous cellular functions including inflammation, proliferation, stress-response and programmed cell death control. Moreover, its de-regulation has been linked to chemoresistance of pancreatic cancer cells. We have examined the effect of PCP on the activation of NFκB/p65. Our data demonstrate that PCP leads to decreased phosphorylation of NFκB/p65 at S536 and reduction of its protein expression levels in MIA PaCa-2 cells. NFκB/p65 phosphorylation at S536 results in nuclear localization and stimulation of NFκB transactivation functions. We show here, that the TNFα-mediated stimulation of NFκB/p65 is suppressed in the presence of PCP providing mechanistic evidence that the anti-proliferative and pro-apoptotic effects of PCP are associated with inhibition of the NFκB signaling pathway.

Apart from the carcinogenic properties of PCP reported in previous work, this study shows that PCP exerts toxic effects in human pancreatic cancer cells involving

mitochondria damage, activation of apoptosis-related proteins and lysosomal cysteine proteases.

5. Conclusion

Data reported here, are consistent with the involvement of three major pro-survival signaling cascades, i.e. the PI3K/AKT/mTOR, MAPK and NF-κB pathways but also with the inhibition of a nodal pro-survival kinase, i.e. protein kinase CK2. These data aim to provide initial insight into the anti-proliferative effects of PCP in pancreatic cancer cells and form the basis for more advanced studies on the mechanism of action of chlorinated aromatic compounds in vivo.

Conflict of interest

The authors declare that there are no conflicts of interest.

Acknowledgments

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.toxrep.2014.10.027.

References

[1] J. Zhang, P.L. Yang, N.S. Gray, Targeting cancer with small molecule kinase inhibitors, Nat. Rev. Cancer 9 (2009) 28–39, http://dx.doi.org/10.1038/nrc2559.

[2] B. Guerra, O.-G. Issinger, Protein kinase CK2 in human diseases, Curr. Med. Chem. 15 (2008) 1870–1886.

[3] N.A. St-Denis, D.W. Litchfield, Protein kinase CK2 in health and disease: from birth to death: the role of protein kinase CK2 in the regulation of cell proliferation and survival, Cell. Mol. Life Sci. 66 (2009) 1817–1829, http://dx.doi.org/10.1007/s00018-009-9150-2.

[4] G. Wang, G. Unger, K.A. Ahmad, J.W. Slaton, K. Ahmed, Downregulation of CK2 induces apoptosis in cancer cells – a potential approach to cancer therapy, Mol. Cell. Biochem. 274 (2005) 77–84.

[5] J.N. Kreutzer, M. Ruzzene, B. Guerra, Enhancing chemosensitivity to gemcitabine via RNA interference targeting the catalytic subunits of protein kinase CK2 in human pancreatic cancer cells, BMC Cancer 10 (2010) 440, http://dx.doi.org/10.1186/1471-2407-10-440.

[6] S. Tawfic, S. Yu, H. Wang, R. Faust, A. Davis, K. Ahmed, Protein kinase CK2 signal in neoplasia, Histol. Histopathol. 16 (2001) 573–582.

[7] A. Bevenue, H. Beckman, Pentachlorophenol: a discussion of its properties and its occurrence as a residue in human and animal tissues, Residue Rev. 19 (1967) 83–134.

[8] Y.J. Wang, J.K. Lin, Estimation of selected phenols in drinking water with in situ acetylation and study on the DNA damaging properties of polychlorinated phenols, Arch. Environ. Contam. Toxicol. 28 (1995) 537–542.

[9] U. Juhl, I. Witte, W. Butte, Metabolism of pentachlorophenol to tetrachlorohydroquinone by human liver homogenate, Bull. Environ. Contam. Toxicol. 35 (1985) 596–601.

[10] Y.J. Wang, Y.S. Ho, J.H. Jeng, H.J. Su, C.C. Lee, Different cell death mechanisms and gene expression in human cells induced by pentachlorophenol and its major metabolite, tetrachlorohydroquinone, Chem. Biol. Interact. 128 (2000) 173–188.

[11] O.I. Aruoma, B. Halliwell, E. Gajewski, M. Dizdaroglu, Copper-ion-dependent damage to the bases in DNA in the presence of hydrogen peroxide, Biochem. J. 273 (Pt. 3) (1991) 601–604.

[12] C.P. Carstens, J.K. Blum, I. Witte, The role of hydroxyl radicals in tetrachlorohydroquinone induced DNA strand break formation in PM2 DNA and human fibroblasts, Chem. Biol. Interact. 74 (1990) 305–314.

[13] Y.J. Wang, C.C. Lee, W.C. Chang, H.B. Liou, Y.S. Ho, Oxidative stress and liver toxicity in rats and human hepatoma cell line induced by pentachlorophenol and its major metabolite tetrachlorohydroquinone, Toxicol. Lett. 122 (2001) 157–169.

[14] J. Folch, M. Yeste-Velasco, D. Alvira, la Torre, A.V. de, M. Bordas, M. López, F.X. Sureda, V. Rimbau, A. Camins, M. Pallàs, Evaluation of pathways involved in pentachlorophenol-induced apoptosis in rat neurons, Neurotoxicology 30 (2009) 451–458, http://dx.doi.org/10.1016/j.neuro.2009.02.001.

[15] B. Wispriyono, M. Matsuoka, H. Igisu, Effects of pentachlorophenol and tetrachlorohydroquinone on mitogen-activated protein kinase pathways in Jurkat T cells, Environ. Health Perspect. 110 (2002) 139–143.

[16] C.W. Yde, B.B. Olsen, D. Meek, N. Watanabe, B. Guerra, The regulatory β-subunit of protein kinase CK2 regulates cell-cycle progression at the onset of mitosis, Oncogene 27 (2008) 4986–4997, http://dx.doi.org/10.1038/onc.2008.146.

[17] B. Guerra, K. Iwabuchi, O.-G. Issinger, Protein kinase CK2 is required for the recruitment of 53BP1 to sites of DNA double-strand break induced by radiomimetic drugs, Cancer Lett. (2013), http://dx.doi.org/10.1016/j.canlet.2013.11.008.

[18] B.B. Olsen, O.-G. Issinger, B. Guerra, Regulation of DNA-dependent protein kinase by protein kinase CK2 in human glioblastoma cells, Oncogene (2010) 1–11, http://dx.doi.org/10.1038/onc.2010.337.

[19] S. Gou, T. Liu, C. Wang, T. Yin, K. Li, M. Yang, J. Zhou, Establishment of clonal colony-forming assay for propagation of pancreatic cancer cells with stem cell properties, Pancreas 34 (2007) 429–435, http://dx.doi.org/10.1097/MPA.0b013e318033f9f4.

[20] C. Li, D.G. Heidt, P. Dalerba, C.F. Burant, L. Zhang, V. Adsay, M. Wicha, M.F. Clarke, D.M. Simeone, Identification of pancreatic cancer stem cells, Cancer Res. 67 (2007) 1030–1037, http://dx.doi.org/10.1158/0008-5472.CAN-06-2030.

[21] S. Gobeil, C.C. Boucher, D. Nadeau, G.G. Poirier, Characterization of the necrotic cleavage of poly(ADP-ribose) polymerase (PARP-1): implication of lysosomal proteases, Cell Death Differ. 8 (2001) 588–594, http://dx.doi.org/10.1038/sj.cdd.4400851.

[22] Y. Yang, S. Zhao, J. Song, Caspase-dependent apoptosis and -independent poly(ADP-ribose) polymerase cleavage induced by transforming growth factor beta1, Int. J. Biochem. Cell Biol. 36 (2004) 223–234.

[23] N. Aggarwal, B.F. Sloane, Cathepsin B: multiple roles in cancer, Proteomics Clin. Appl. 8 (2014) 427–437, http://dx.doi.org/10.1002/prca.201300105.

[24] M.R. Buck, D.G. Karustis, N.A. Day, K.V. Honn, B.F. Sloane, Degradation of extracellular-matrix proteins by human cathepsin B from normal and tumour tissues, Biochem. J. 282 (Pt. 1) (1992) 273–278.

[25] M. Niedergethmann, B. Wostbrock, J.W. Sturm, F. Willeke, S. Post, R. Hildenbrand, Prognostic impact of cysteine proteases cathepsin B and cathepsin L in pancreatic adenocarcinoma, Pancreas 29 (2004) 204–211.

[26] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309–1312.

[27] N. Zamzami, P. Marchetti, M. Castedo, D. Decaudin, A. Macho, T. Hirsch, S.A. Susin, P.X. Petit, B. Mignotte, G. Kroemer, Sequential reduction of mitochondrial transmembrane potential and generation of reactive oxygen species in early programmed cell death, J. Exp. Med. 182 (1995) 367–377.

[28] A. Arlt, A. Gehrz, S. Müerköster, J. Vorndamm, M.-L. Kruse, U.R. Fölsch, H. Schäfer, Role of NF-kappaB and Akt/PI3K in the resistance of pancreatic carcinoma cell lines against gemcitabine-induced cell death, Oncogene 22 (2003) 3243–3251, http://dx.doi.org/10.1038/sj.onc.1206390.

[29] A. Arlt, J. Vorndamm, M. Breitenbroich, U.R. Fölsch, H. Kalthoff, W.E. Schmidt, H. Schäfer, Inhibition of NF-kappaB sensitizes human pancreatic carcinoma cells to apoptosis induced by

etoposide (VP16) or doxorubicin, Oncogene 20 (2001) 859–868, http://dx.doi.org/10.1038/sj.onc.1204168.

[30] R.A. Fryer, B. Barlett, C. Galustian, A.G. Dalgleish, Mechanisms underlying gemcitabine resistance in pancreatic cancer and sensitisation by the iMiD™ lenalidomide, Anticancer Res. 31 (2011) 3747–3756.

[31] S.S.W. Ng, M.S. Tsao, S. Chow, D.W. Hedley, Inhibition of phosphatidylinositide 3-kinase enhances gemcitabine-induced apoptosis in human pancreatic cancer cells, Cancer Res. 60 (2000) 5451–5455.

[32] S.S. Ng, M.S. Tsao, T. Nicklee, D.W. Hedley, Wortmannin inhibits pkb/akt phosphorylation and promotes gemcitabine antitumor activity in orthotopic human pancreatic cancer xenografts in immunodeficient mice, Clin. Cancer Res. 7 (2001) 3269–3275.

[33] Y. Wang, Y. Zhou, H. Zhou, G. Jia, J. Liu, B. Han, Z. Cheng, H. Jiang, S. Pan, B. Sun, Pristimerin causes G1 arrest, induces apoptosis, and enhances the chemosensitivity to gemcitabine in pancreatic cancer cells, PLoS ONE 7 (2012) e43826, http://dx.doi.org/10.1371/journal.pone.0043826.

[34] G. Di Maira, M. Salvi, G. Arrigoni, O. Marin, S. Sarno, F. Brustolon, L.A. Pinna, M. Ruzzene, Protein kinase CK2 phosphorylates and upregulates Akt/PKB, Cell Death Differ. 12 (2005) 668–677, http://dx.doi.org/10.1038/sj.cdd.4401604.

[35] R. Romieu-Mourez, E. Landesman-Bollag, D.C. Seldin, G.E. Sonenshein, Protein kinase CK2 promotes aberrant activation of nuclear factor-kappaB, transformed phenotype, and survival of breast cancer cells, Cancer Res. 62 (2002) 6770–6778.

[36] R. Romieu-Mourez, E. Landesman-Bollag, D.C. Seldin, A.M. Traish, F. Mercurio, G.E. Sonenshein, Roles of IKK kinases and protein kinase CK2 in activation of nuclear factor-kappaB in breast cancer, Cancer Res. 61 (2001) 3810–3818.

[37] F. Vazquez, S. Ramaswamy, N. Nakamura, W.R. Sellers, Phosphorylation of the PTEN tail regulates protein stability and function, Mol. Cell. Biol. 20 (2000) 5010–5018.

[38] Y. Miyata, E. Nishida, CK2 controls multiple protein kinases by phosphorylating a kinase-targeting molecular chaperone, Cdc37, Mol. Cell. Biol. 24 (2004) 4065–4074.

[39] N.D. Perkins, Integrating cell-signalling pathways with NF-kappaB and IKK function, Nat. Rev. Mol. Cell Biol. 8 (2007) 49–62, http://dx.doi.org/10.1038/nrm2083.

[40] J. Iovanna, M.C. Mallmann, A. Gonçalves, O. Turrini, J.-C. Dagorn, Current knowledge on pancreatic cancer, Front. Oncol. 2 (2012) 6, http://dx.doi.org/10.3389/fonc.2012.00006.

[41] S.H. Kaufmann, S. Desnoyers, Y. Ottaviano, N.E. Davidson, G.G. Poirier, Specific proteolytic cleavage of poly(ADP-ribose) polymerase: an early marker of chemotherapy-induced apoptosis, Cancer Res. 53 (1993) 3976–3985.

[42] M. Tewari, L.T. Quan, K. O'Rourke, S. Desnoyers, Z. Zeng, D.R. Beidler, G.G. Poirier, G.S. Salvesen, V.M. Dixit, Yama/CPP32 beta, a mammalian homolog of CED-3, is a CrmA-inhibitable protease that cleaves the death substrate poly(ADP-ribose) polymerase, Cell 81 (1995) 801–809.

[43] B.W. Johnson, E. Cepero, L.H. Boise, Bcl-xL inhibits cytochrome c release but not mitochondrial depolarization during the activation of multiple death pathways by tumor necrosis factor-alpha, J. Biol. Chem. 275 (2000) 31546–31553, http://dx.doi.org/10.1074/jbc.M001363200.

[44] J. Cui, M. Zhang, Y.-Q. Zhang, Z.-H. Xu, JNK pathway: diseases and therapeutic potential, Acta Pharmacol. Sin. 28 (2007) 601–608, http://dx.doi.org/10.1111/j.1745-7254.2007.00579.x.

[45] P. Dent, A. Yacoub, P.B. Fisher, M.P. Hagan, S. Grant, MAPK pathways in radiation responses, Oncogene 22 (2003) 5885–5896, http://dx.doi.org/10.1038/sj.onc.1206701.

[46] M. Breuleux, M. Klopfenstein, C. Stephan, C.A. Doughty, L. Barys, S.-M. Maira, D. Kwiatkowski, H.A. Lane, Increased AKT S473 phosphorylation after mTORC1 inhibition is rictor dependent and does not predict tumor cell response to PI3K/mTOR inhibition, Mol. Cancer Ther. 8 (2009) 742–753, http://dx.doi.org/10.1158/1535-7163.MCT-08-0668.

[47] A. Carracedo, P.P. Pandolfi, The PTEN-PI3K pathway: of feedbacks and cross-talks, Oncogene 27 (2008) 5527–5541, http://dx.doi.org/10.1038/onc.2008.247.

[48] K.E. O'Reilly, F. Rojo, Q.-B. She, D. Solit, G.B. Mills, D. Smith, H. Lane, F. Hofmann, D.J. Hicklin, D.L. Ludwig, J. Baselga, N. Rosen, mTOR inhibition induces upstream receptor tyrosine kinase signaling and activates Akt, Cancer Res. 66 (2006) 1500–1508, http://dx.doi.org/10.1158/0008-5472.CAN-05-2925.

[49] Q. Yang, K.-L. Guan, Expanding mTOR signaling, Cell Res. 17 (2007) 666–681, http://dx.doi.org/10.1038/cr.2007.64.