# Mutant p53 stimulates chemoresistance of pancreatic adenocarcinoma cells to gemcitabine

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## ABSTRACT

Pancreatic adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths worldwide; PDAC is characterized by poor prognosis, resistance to conventional chemotherapy and high mortality rate. *TP53* tumor suppressor gene is frequently mutated in PDAC, resulting in the accumulation of mutated protein with potential gain-of-function (GOF) activities, such as genomic instability, hyperproliferation and chemoresistance. The purpose of this study was to assess the relevance of the p53 status on the PDAC cells response to the standard drug gemcitabine. We also examined the potential therapeutic effect of p53-reactivating molecules to restore the mutant p53 function in GEM treated PDAC cells. We showed that gemcitabine stabilized mutant p53 protein in the nuclei and induced chemoresistance, concurrent with the mutant p53-dependent expression of *Cdk1* and *CCNB1* genes, resulting in a hyperproliferation effect. Despite the adverse activation of mutant p53 by gemcitabine, simultaneous treatment of PDAC cells with gemcitabine and p53-reactivating molecules (CP-31398 and RITA) reduced growth rate and induced apoptosis. This synergistic effect was observed in both wild-type and mutant p53 cell lines and was absent in p53-null cells. The combination drug treatment induced p53 phosphorylation on Ser15, apoptosis and autophagosome formation. Furthermore, pharmacological inhibition of autophagy further increased apoptosis stimulated by gemcitabine/CP-31398 treatment. Together, our results show that gemcitabine aberrantly stimulates mutant p53 activity in PDAC cells identifying key processes with potential for therapeutic targeting. Our data also support an anti-tumoral strategy based on inhibition of autophagy combined with p53 activation and standard chemotherapy for both wild-type and mutant p53 expressing PDACs.

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## 1. Introduction

Pancreatic adenocarcinoma (PDAC) is the fourth leading cause of cancer-related deaths worldwide and one of the most aggressive and devastating human malignancies [1]. Because early symptoms of the disease are extremely rare, most patients present locally advanced disease and/or metastasis at the time of diagnosis, which prevents them from being considered as candidates for surgery. Standard treatments for advanced disease include monotherapy with gemcitabine (2′,2′-difluoro-2′-deoxycytidine; GEM) with a response rate of less than 20% [2]. Therefore, the identification of effective targets and novel therapeutic strategies to improve GEM effects in PDAC have been the topic of extensive investigation in the last few years [3]. The *p53* tumor suppressor gene is mutated in more than 50% of human cancers, including PDAC (50–75%), while the remaining 50% of tumors exhibit alterations in pathways regulating p53 functions [4–6]. Mutant p53 proteins (mp53) are incapable of recognizing wild-type p53 (wt p53) DNA binding sites in the promoter of p53 target genes and furthermore, some mutations acquire new and distinct oncogenic properties, which is generally referred to as "gain of function" (GOF) [7]. In particular, mp53 can reach the promoter of target genes through the interaction with sequence-specific transcription factors, such as NF-Y, E2F1, NF-kB and the Vitamin D receptor (VDR) [8–10]. Mutant p53 proteins have been shown to interfere with regulation of cell survival, DNA damage repair and drug resistance [11–14]. Therefore, activation of mp53-dependent pathways plays critical roles in tumor development, drug resistance and metastasis [15–18]. During the last few years, several attempts to identify p53-activating compounds have been made employing antibody assays and cell-based screening approaches [19]. These studies led to the identification of small molecules, such as CP-31398 and RITA, which have been shown to re-establish the wild-type transcriptionally competent conformation of mp53 proteins [20]. These

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molecules have been reported to activate wt p53 response in mp53-carrying tumors and to induce apoptotic cell death [21–25]. Recently, we showed that p53-reactivating molecules could inhibit the proliferation of pancreatic adenocarcinoma cells bearing mp53 and that their effect was attenuated by an AMPK/p53-mediated cyto-protective autophagy [26]. In this study, we have characterized, at the molecular level, the induction of mp53 transcriptional GOF activity with the associated chemioresistance in PDAC cell lines treated with the standard chemotherapeutic drug GEM. We also analyzed the effect of GEM in combination with CP-31398 and RITA in PDAC cell lines expressing wt or mp53. Our results revealed a novel role of mp53 protein in the aberrant regulation of the cell cycle in response to DNA-damaging agents in PDAC cells.

## 2. Materials and methods

### 2.1. Chemicals

Gemcitabine (2′,2′-difluoro-2′-deoxycytidine; GEM) was provided by Accord Healthcare (Milan, Italy) and CP-31398 dihydrochloride hydrate was obtained from Sigma (Milan, Italy). Both compounds were solubilized in sterile water. RITA [5,5′-(2,5-furandiyl)bis-2-thiophenemethanol; reactivation of p53 and induction of tumor cell apoptosis] was obtained from Sigma (Milan, Italy) and solubilized in DMSO. Chloroquine diphosphate [CQ; N4-(7-chloro-4-quinolinyl)-N1, N1-dimethyl-1,4-pentanediamine], and 3-methyladenine (3MA) were obtained from Sigma (Milan, Italy).

### 2.2. Cell culture

PaCa3 (wt p53), Panc1 (mp53-R273H), PaCa44 (mp53-C176S), Mia PaCa2 (mp53-R248W), SUIT-2 (mp53-R273H) and ASPC-1 (p53-null) human PDAC cell lines were grown in RPMI 1640 supplemented with 2 mM glutamine (Life Technologies, Milan, Italy), 10% FBS, and 50 μg/ml gentamicin sulfate (BioWhittaker, Lonza, Bergamo, Italy). Cell lines were incubated at 37 °C with 5% $CO_2$.

### 2.3. Cell proliferation assay

Cells were seeded in 96-well plates ($5 \times 10^3$ cells/well) and the day after were incubated with various compounds at the indicated times (see Figure legends). At the end of the treatments, cell growth was measured by Crystal Violet assay (Sigma, Milan, Italy) according to the manufacturer's protocol and absorbance measured by spectrophotometric analysis ($A_{595nm}$).

### 2.4. RNA extraction and qPCR

Total RNA was extracted using TRIzol Reagent (Invitrogen, Milan, Italy). One μg RNA was reverse transcribed using first-strand cDNA synthesis. RT-qPCR was performed in triplicate samples by SYBR Green detection chemistry with SYBR Green PCR Master Mix (Applied Biosystems) on a 7000 Sequence Detection System (Applied Biosystems). The following oligonucleotide sequences used were: hCdk1-forward, 5′-GGAAGGGGTTCCTAGTACTGC-3′ and Rev 5′- TGGA ATCCTGCATAAGCACA-3′; hCCNB1-forward 5′-GTTCCTACGGCCCCTG CT-3′ and Rev 5′-ATTTTGGCCTGCAGTTGTTC-3′; and GAPDH-forward 5′-TGTGTCCGTCG TGGATCTGA-3′ and Rev 5′-R-GATGCCTGCTTCACCA CCTT-3′. The following cycling conditions were used: 95 °C for 10 min, 40 cycles at 95 °C for 15 s, 60 °C for 1 min, and 72 °C for 30 s. The average of cycle threshold of each triplicate was analyzed according to the $2^{(-\Delta\Delta Ct)}$ method.

### 2.5. Transient transfection assays

Exponentially growing cells were seeded at $5 \times 10^3$ cells/well in 96-well plates and at $2.5 \times 10^5$ cells in 60 mm cell culture plates for proliferation assays and protein extraction respectively. To over-express mp53 in ASPC-1 p53-null cells, transfections were carried out using pcDNA3-mutp53R273H expression vector or its relative negative control (pcDNA3) [27]. Wt p53 and mp53 protein expression was transiently knocked-down by transfection with pRSUPER-p53 vector or its negative control (pRSUPER), kindly provided by Reuven Agami (The Netherlands Cancer Institute, Amsterdam) [28]. The knock-down of Beclin1 expression was obtained by transfecting cells with a specific Beclin1 siRNA: 5′-ACAGUGAAUUUAAACGACAGCAGCU-3′ and 5′-AGCUGCUGUCGUUUAAAUUCACUGU-3′ and with a siRNA-CTRL (negative control): 5′-CAGUCGCGUUUGCGACUGG-3′ purchased from Life Technologies (Monza MB, Italy). Cells were transfected by siRNAs at a final concentration of 50 nM using Lipofectamine 2000 (Life Technologies) for 24 h, according to the manufacturer's instructions. At the end of transfection time, the culture medium was changed and cells were treated with the compounds indicated in Figure Legends.

### 2.6. Drug combination studies

Drug combination studies were performed using the concentration ratios [GEM]:[CP-31398] = 1:8 and [GEM]:[RITA] = 1:16, which were chosen on the basis of GEM, CP-31398, or RITA $IC_{50}$ mean values. Taking into account the drug molar ratios, the ranges of concentration used were 50–2000 nM for GEM, 0.4–16 μM for CP-31398 and 0.8–32 μM for RITA. The Combination Index (CI) was calculated by the Chou–Talalay equation [29], which takes into account both the potency ($IC_{50}$) and the shape of the dose–effect curve, taking advantage of the CalcuSyn software (Biosoft, Cambridge, UK). The general equation for the classic isobologram is given by CI = (D)1/(Dx)1 + (D)2/(Dx)2 + [(D)1 · (D)2]/[(Dx)1·(Dx)2], where (Dx)1 and (Dx)2 in the denominator are doses (or concentrations) for D1 (drug 1) and D2 (drug 2) alone that gives x% growth inhibition, whereas (D)1 and (D)2 in the numerators are the doses of drug 1 and drug 2 in combination that also inhibit x% cell growth (i.e., isoeffective). CI/effect curves represent the CI versus the fraction (0–1) of cells killed by drug combinations: CI values below 1 indicate a synergistic effect of the drug combination. Dose Reduction Index ($DRI_{25}$, $DRI_{50}$, and $DRI_{75}$) represents the folds of dose reduction to obtain 25%, 50%, or 75% cell growth inhibition in combination setting as compared to each drug alone. In all the experiments, we obtained a linear correlation coefficient ($r$) > 0.90.

### 2.7. Immunoblot analysis

For total cell extracts, cells were harvested, washed in PBS, and re-suspended in lysis buffer in the presence of phosphatase and protease inhibitors (50 mM Tris–HCl pH 8, 150 mM NaCl, 1% Igepal CA-630, 0.5% Na-Doc, 0.1% SDS, 1 mM $Na_3VO_4$, 1 mM NaF, 2.5 mM EDTA, 1 mM PMSF, and $1\times$ protease inhibitor cocktail). After three freeze/thaw cycles and incubation on ice for 30 min, the lysates were centrifuged at $14,000 \times g$ for 10 min at 4 °C and the supernatant fractions were used for Western blot analysis. Nuclear extracts were prepared according to Osborn and colleagues [30]. Protein concentration was measured by Bradford reagent (Pierce, Milan, Italy) using bovine serum albumin as a standard. Protein extracts (50 μg/lane) were resolved on a 12% SDS-polyacrylamide gel and electro-blotted onto PVDF membranes (Millipore, Milan, Italy). Membranes were blocked in 5% low-fat milk in TBST (50 mM Tris pH 7.5, 0.9% NaCl, 0.1% Tween 20) for 1 h at room temperature and probed overnight at 4 °C with a mouse monoclonal anti-phospho (Ser15) p53 (1:1500 in blocking solution) (Cell Signaling, # 9286), mouse polyclonal anti-p53 (1:500) (Santa Cruz, sc-263), mouse monoclonal anti-TATA-box-binding protein (TBP) (1:500) (Merck-Millipore, SL-30-3-563), rabbit monoclonal anti-LC3

(1:1000) (Cell Signaling, # 2775), a rabbit polyclonal anti-Beclin1 (1:1000) (GeneTex, # GTX113039), or rabbit monoclonal glyceralde-hyde 3-phosphate dehydrogenase (GAPDH) (1:1000) (Cell Signaling, # 5174S). Horseradish peroxidase conjugated anti-mouse or anti-rabbit IgGs (1:8000 in blocking solution) (Upstate Biotechnology, Milan, Italy) were used as secondary antibodies. Immunodetection was carried out using chemiluminescent substrates (Amersham Pharmacia Biotech, Milan, Italy) and recorded using a HyperfilmECL (Amersham Pharmacia Biotech). ECL results were scanned and the amount of each protein band quantitated using NIH Image J software (http://rsb.info.nih.gov/nih-image/). Blots were probed with GAPDH as a loading control and the amount of each protein was normalized to the amount of GAPDH detected in the same extract.

### 2.8. Apoptosis assay

Cells were seeded in 96-well plates ($5 \times 10^3$ cells/well). Twenty-four hours later, cells were treated with various compounds at concentrations indicated in Figure legends for 48 h. At the end of the treatment, cells were fixed with 2% paraformaldehyde in PBS for 30 min at room temperature, washed twice with PBS and stained with annexinV/FITC (Bender MedSystem, Milan, Italy) in binding buffer (10 mM HEPES/NaOH pH 7.4, 140 mM NaCl, and 2.5 mM $CaCl_2$) for 10 min at room temperature in the dark. Cells were then washed with binding buffer and fluorescence was measured using a multimode plate reader ($Ex_{485nm}$ and $Em_{535nm}$) (GENios Pro, Tecan, Milan, Italy). The values were normalised on cell proliferation by Crystal Violet assay.

### 2.9. Autophagosome formation assay

To quantify the induction of autophagy, cells were incubated with the fluorescent probe monodansylcadaverine (MDC; Sigma, Milan, Italy). MDC is a selective marker for acidic vesicular organelles (AVOs), such as autophagic vacuoles and autolysosomes. Briefly, cells were seeded in 96-well plates ($5 \times 10^3$ cells/well) and treated with various compounds as indicated in Figure legends. At the end of the treatments, cells were incubated in culture medium containing 50 μM MDC at 37 °C for 15 min. Cells were then washed with Hanks buffer (20 mM Hepes pH 7.2, 10 mM glucose, 118 mM NaCl, 4.6 mM KCl, and 1 mM $CaCl_2$) and fluorescence was measured using a multimode plate reader ($Ex_{340nm}$ and $Em_{535nm}$) (GENios Pro, Tecan, Milan, Italy). The values were normalised on cell proliferation by Crystal Violet assay.
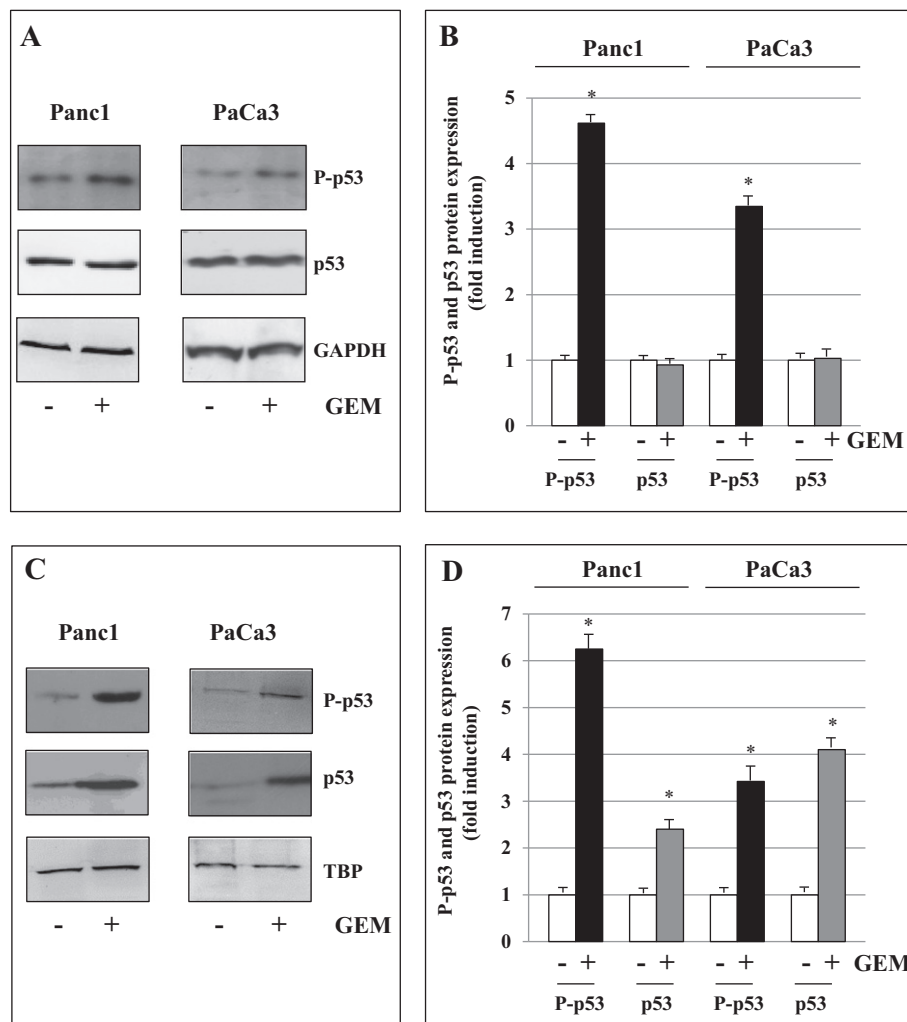


**Fig. 1.** Effect of GEM on mutant and wt p53 expression, phosphorylation, and nuclear localization. Panc1 and PaCa3 cells were treated with 5 μM GEM for 24 h. Whole-cell (A and B) or nuclear extracts (C and D) were used for western blot analyses with p53 antibodies (total and phosphor serine 15). GAPDH (B) or TBP (D) were used loading controls and to verify the purity of the extracts. The amount of protein in each extract was quantitated using NIH image J software and normalized to the amount of GAPDH or TBP protein in the same extract. *p < 0.05 GEM versus CTRL in both Panc1 and PaCa3 cells.

### 2.10. Statistical analysis

ANOVA analysis was performed by GraphPad Prism 5 software. $P$ value < 0.05 was indicated as statistically significant. Values are the means of three independent experiments ($\pm$SD).

## 3. Results

### 3.1. GEM induces phosphorylation and nuclear stabilization of both wt and mutant p53

To investigate whether GEM treatment modified the phosphorylation state of p53 protein, we incubated Panc1 and PaCa3 cell lines, expressing mp53R273H and wt p53 protein, respectively, with 5 μM GEM for 24 h. Fig. 1A and B show that GEM treatment induced Ser15 phosphorylation of both mutant and wt p53 proteins whereas their total protein levels remained unchanged. Western blot analysis of the nuclear extracts from both cell lines revealed that GEM stabilized the nuclear pool of both mutant and wt p53 proteins (and their corresponding Ser15-phosphorylated forms) (Fig. 1C and D). The purity of the nuclear extracts was confirmed by western blot using anti-nuclear TATA-box-binding protein (TBP) antibodies (Fig. 1C and D).

### 3.2. GEM induces mutant p53 target genes

It has been previously reported that the expression of some cell cycle-related NF-Y target genes has aberrantly increased by mp53 in response to adriamycin treatment [27]. This prompted us to investigate whether the incubation of pancreatic cancer cells with GEM could modulate the expression of cell cycle target genes. We observed significant increases in the mRNA expression levels of *CdK1* and *CCNB1* genes after GEM treatment in Panc1 (mp53) cells while they were significantly decreased in PaCa3 (wt p53) cells (Fig. 2A). To evaluate the contribution of endogenous mp53 to these cell cycle genes induction, Panc1 cancer cells were transiently transfected with pRSUPER (control) and pRSUPER-p53 to down-regulate mp53 expression as described in Material and Methods. Mutant p53 knock-down (KD) completely rescued *CdK1* and *CCNB1* gene induction by GEM in Panc1 cells, indicating that the activation of cell cycle genes by GEM was mediated by mp53 (Fig. 2A). On the other hand, the depletion of wt p53 expression in PaCa3 cells significantly increased *CdK1* and *CCNB1* gene expression, indicating the repressive role of the wt p53 on gene expression after GEM treatment (Fig. 2A). Overall, these findings are in agreement with previous data showing that DNA damage-induced p53 accumulation in wt p53 cells is critical for the cellular response to DNA damage [31,32]. Accordingly, mp53 depleted Panc1 cells showed decreases in *CdK1* and
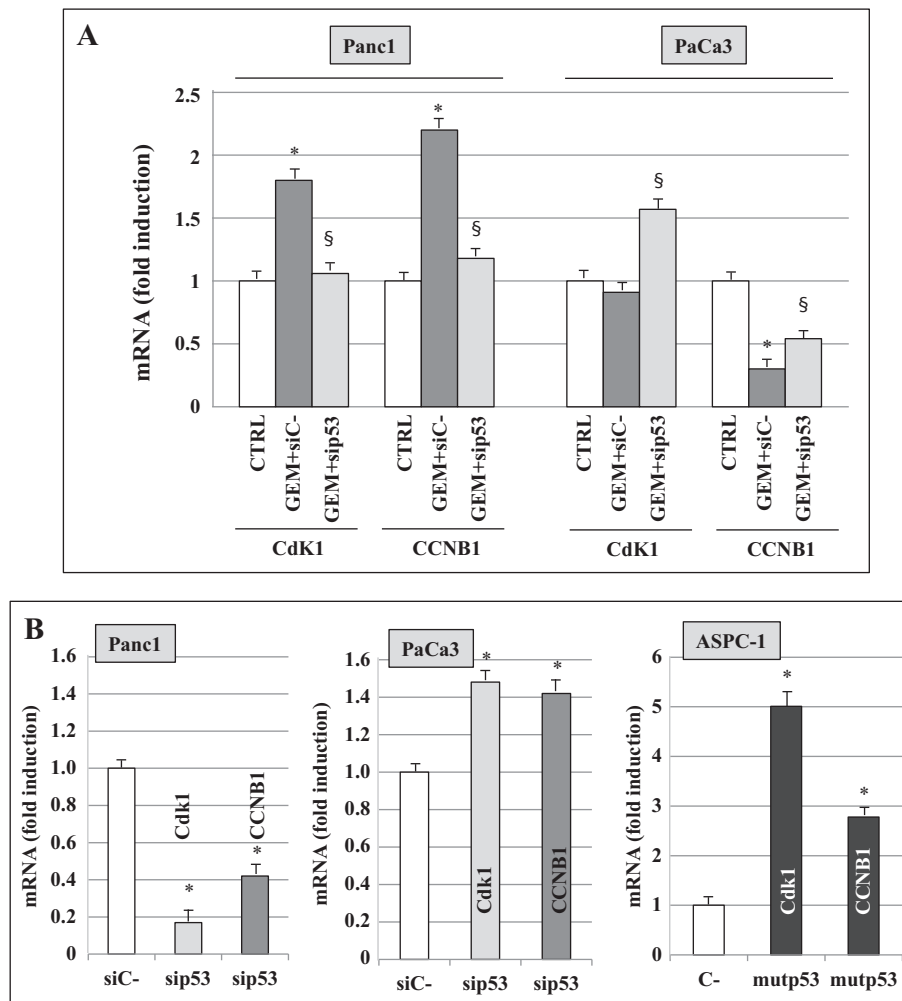


**Fig. 2.** Role of the p53 status on the expression level of CdK1 and CCNB1 mRNAs in control and GEM treated Panc1 and PaCa3 cells. Real-time PCR for CdK1 and CCNB1 was performed as described in "Materials and Methods" section. (A) Panc1 and PaCa3 cells were transfected with the vector alone or plasmid coding for siRNAp53 and a day after, cells they were treated with 5 μM GEM for 24 h. CdK1 and CCNB1 mRNA level were normalized to GAPDH mRNA. Statistical analysis: *p < 0.05 GEM + siC- versus CTRL; § p < 0.05 GEM + sip53 versus GEM + siC-. (B) Panc1 and PaCa3 cells were transfected as described in (A) whereas ASPC-1 cells were transfected with pCDNA-CTRL or pCDNA-mp53R273H plasmid. CdK1 and CCNB1 mRNA levels were normalized to GAPDH mRNA. *p < 0.05.

CCNB1 gene expression (Fig. 2B). Furthermore, p53-null ASPC-1 cells transfected with the mp53 (R273H) expression plasmid showed a significant increase in CdK1 and CCNB1 gene expression (Fig. 2B). Notably, depletion of wt p53 expression in PaCa3 cells led to the induction of these transcripts (Fig. 2B). Collectively, these data indicated that in mp53 expressing PDAC cells GEM treatment resulted in the induction of mp53 cell cycle target genes, whereas in wt p53 expressing cells it downregulated the expression of these genes, consistent with the wt
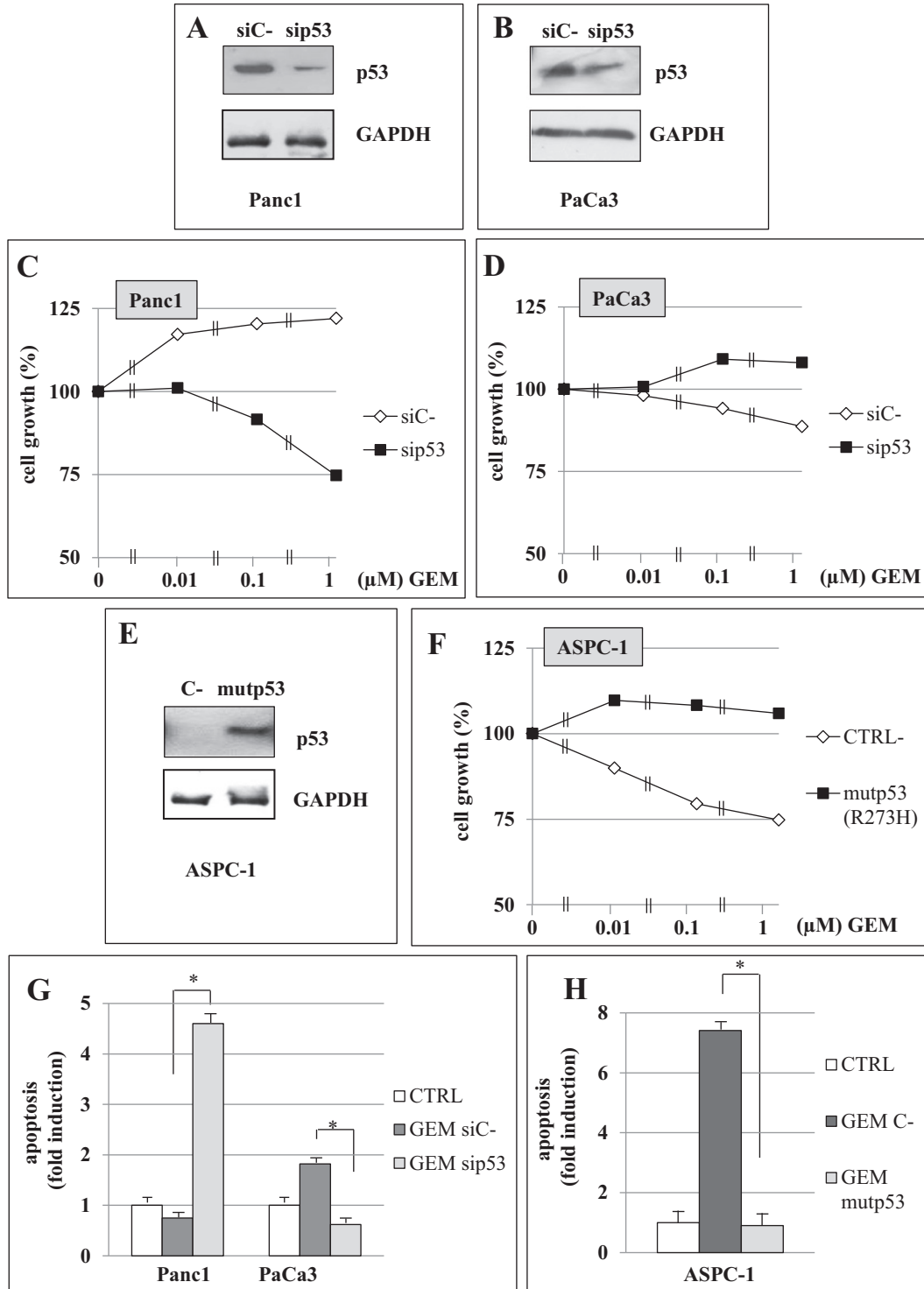


**Fig. 3.** Effect of mp53, wt p53 KD, and of mp53 overexpression on cell growth and apoptosis by GEM. Panc1 (A) and PaCa3 (B) cells were seeded in 100-mm diameter culture dishes and transfected with the plasmid coding for siRNAp53 or its negative control. Whole-cell extracts were used for western blot analyses of p53 expression. GAPDH expression was used as a control loading. Panc1 (C) and PaCa3 (D) cells were seeded in 96-well plates and 24 h later transfected with the plasmid coding for siRNAp53 or its negative control. The day after, culture medium was replaced with media containing increasing concentrations of GEM for 24 h. Cell growth was determined using the crystal violet colorimetric assay. (E) ASPC-1 cells were seeded in in 100-mm diameter culture dishes, incubated overnight, and transfected with the vector coding for mutp53 (pCDNA-mutp53R273H) or its negative control (pCDNA-CTRL). Whole-cell extracts were prepared and used for western blot analyses of p53 expression. GAPDH was used as a loading control. (F) ASPC-1 cells were seeded in 96-well plates, incubated overnight, and transfected as in (E). The day after culture medium was replaced with media containing increasing concentrations of GEM for 24 h. Cell growth was determined using the crystal violet colorimetric assay. (G and H) Cells were treated as described above and analyzed for apoptosis using the annexinV-FITC binding assay. *p < 0.05.

p53 role in cell-cycle checkpoint regulation after DNA damage [31,32]. To further evaluate whether the mp53 *Cdk1* and *CyclinB* target genes may be differentially expressed in pancreatic adenocarcinoma tissues, we queried public gene expression data repositories (GEO datasets-NCBI). The explorative analysis of the dataset from Zhang and colleagues [33] revealed that *CdK1* and *CCNB2* transcripts were substantially up-regulated in 45 pancreatic adenocarcinoma tissues, known to have a high frequency (ranging from 50% to 75%) of mutations in *TP53* gene, compared to their matched normal pancreas samples (Suppl. Fig. 1).

### 3.3. Dual opposite role of mutant and wt p53 on cell growth inhibition and apoptosis mediated by GEM

To investigate the functional role of mutant and wt p53 on PDAC cells response to GEM we evaluated the proliferation rate and the cell death in Panc1 and PaCa3 cells (Fig. 3A and B). Intriguingly, we observed

that silencing of mutant or wt p53 had a dual and opposite role on cells response to GEM: mp53 KD strongly sensitized cancer cells to GEM treatment overcoming drug resistance (Fig. 3C), while wt p53 KD res-cued the anti-proliferative effect of GEM (Fig. 3D). To ameliorate these results, we tested the effect of GEM with and without mp53 KD in other PDAC cell lines (PaCa44, MiaPaCa2, and SUIT-2) bearing different *TP53* gene mutations (see Material and Methods). In all of these cell lines we observed that mp53 KD strongly sensitized cancer cells to GEM treatment (Suppl. Fig. 2A, 2B, and 2C) and to GEM-stimulated apoptosis (Suppl. Fig. 3). Moreover, the ectopic expression of mp53 (R273H) protein in ASPC-1 cell line (p53 null) strongly inhibited the effect of GEM treatment (Fig. 3E and F). Similarly, we observed that mp53 KD stimulated GEM-mediated apoptosis, whereas wt p53 KD repressed GEM-mediated apoptosis (Fig. 3G). Finally, mp53 over-expression in p53-null ASPC1 cells completely suppressed apoptosis by GEM treatment (Fig. 3H).
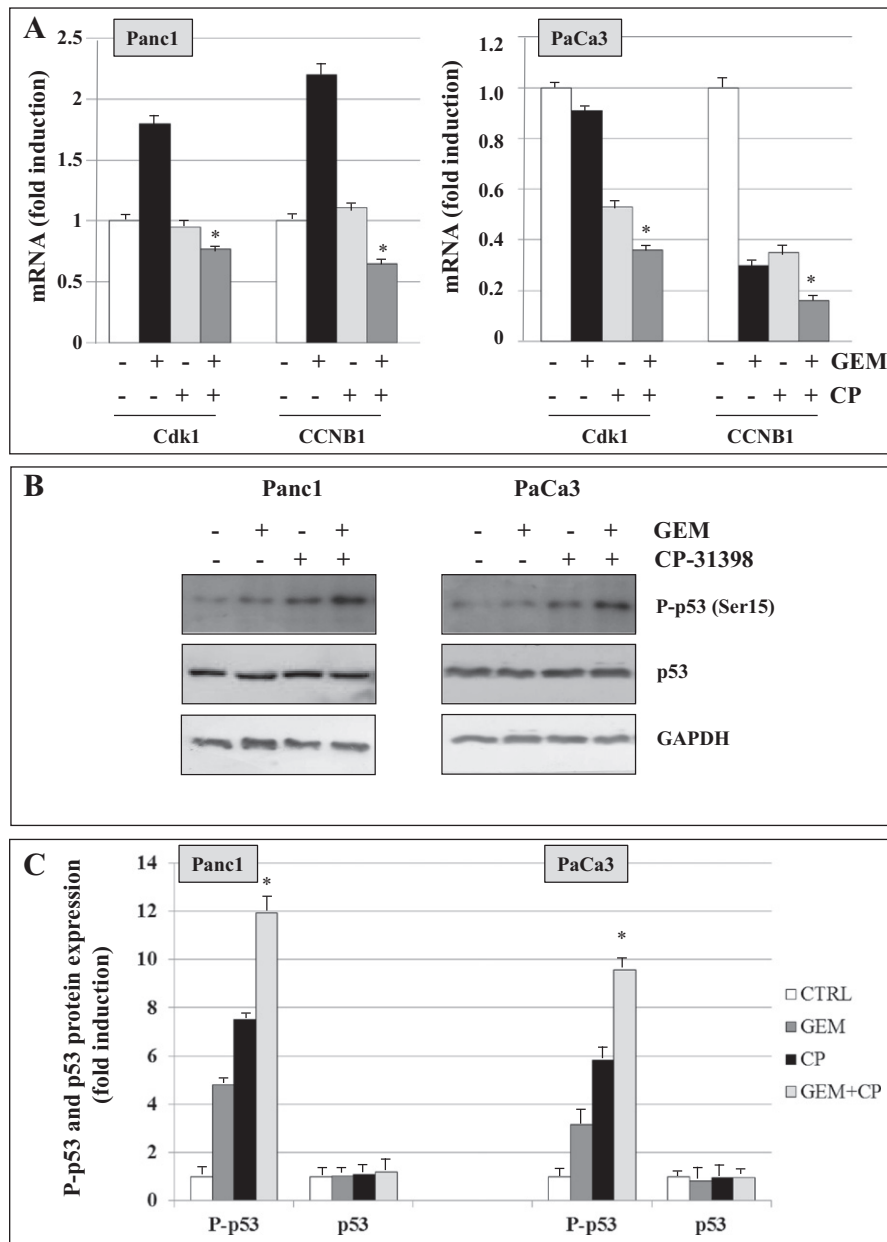


**Fig. 4.** Effect of GEM and CP-31398 on CdK1 and CCNB1 mRNA expression, p53 expression and p53 phosphorylation (Ser15). (A) Panc1 and PaCa3 cells were treated with 5 μM GEM and/or 40 μM CP-31398 for 24 h. CdK1 and CCNB1 expression was determined by qRT-PCR and normalized to GAPDH mRNA. *p < 0.05 GEM + CP versus GEM or CP alone. (B) Panc1 and PaCa3 cells were treated with 5 μM GEM and/or 40 μM CP-31398 for 48 h. Whole-cell extracts were processed for western blot analysis with p53 antibodies and GAPDH level was used as a loading control. (C) Quantitative evaluation of P-p53 and p53 in Panc1 and PaCa3 cells treated as described in (B). *p < 0.05 GEM + CP versus GEM or CP alone.

### 3.4. The p53-reactivating molecule CP-31398 blocks induction of mutant p53-related genes by GEM treatment

To overcome the chemoresistance to GEM treatment in PDAC cells expressing mp53 protein, we used the p53-reactivating molecule CP-31398 and analyzed the expression of *CdK1* and *CCNB1* genes. CP-31398 is able to stabilize a wt-like conformation of the mutant p53 proteins [22]. Incubation of Panc1 cells with CP-31398 in combination with GEM, strongly inhibited the mp53 target gene expression relative to the cells incubated with GEM alone. The drug combination significantly inhibited *Cdk1* and *CCNB1* gene expression in PaCa3 cells relative to GEM or CP-31398 treatment alone (Fig. 4A). To further address this issue, we analyzed p53 and P-p53 protein expression after GEM with or without CP-31398 treatment in both mutant and wt p53 cell lines. We observed that cells incubated with both GEM and CP-31398 showed an increase in the phosphorylation of Ser15 residue of p53 compared to untreated or single drug treated cells (Fig. 4B and C). The expression of total p53 protein did not change by any of these treatments (Fig. 4B and
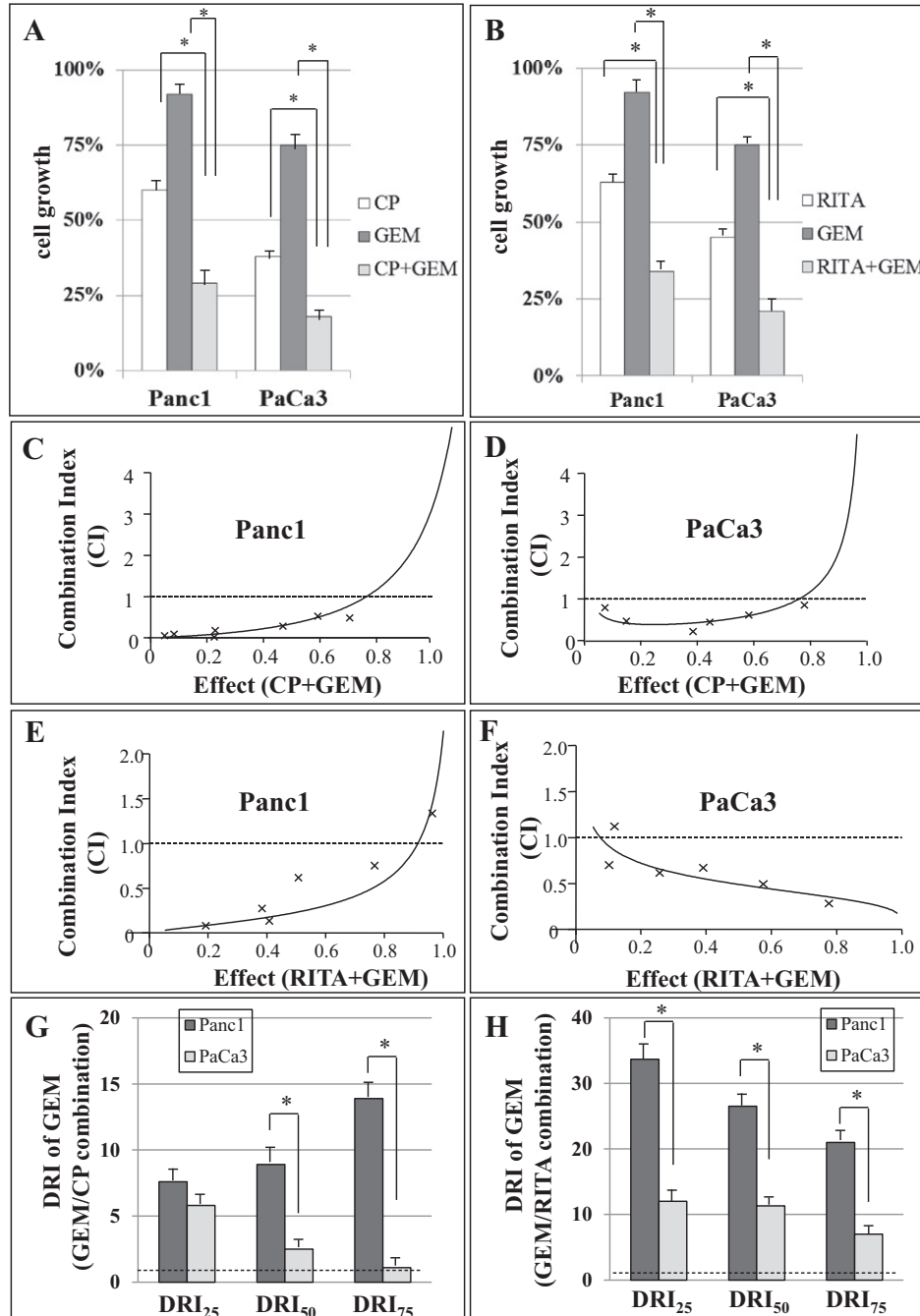


**Fig. 5.** Growth inhibitory effect of GEM and p53-reactivating molecules (CP-31398 or RITA). Panc1 and PaCa3 cells were seeded in 96-well plates, incubated overnight, and treated with 2.5 μM GEM and/or 20 μM CP-31398 (molar ratio 1:8) for 48 h (A) or with 2.5 μM GEM and/or 40 μM RITA (molar ratio 1:16) for 48 h (B). Cell proliferation was measured by Crystal Violet colorimetric assay. *p < 0.05. (C-F) CI/fractional effect curves obtained by CalcuSyn software for Panc1 and PaCa3 cells treated with GEM/CP-31398 or GEM/RITA combinations. The fractional effect (x-axis) is the % of cell growth inhibition given by each drug combination maintaining the same molar ratio and increasing drug concentrations. Combination index (CI) values obtained by the same combinations are reported on the y-axis. CIs < 1 indicate synergistic effects. 1:8 molar ratio of GEM/CP-31398 and the 1:16 molar ratio of GEM/RITA were chosen based on the previously determined $IC_{50}$ mean values of GEM, CP-31398, or RITA. (G and H) Dose Reduction Index (DRI) values of GEM when used in combination with CP-31398 or RITA. $DRI_{25}$ $DRI_{50}$, and $DRI_{75}$ indicate the fold reduction of GEM concentration in combination settings as compared to GEM alone in order to obtain 25%, 50%, or 75% cell growth inhibition, respectively. *p < 0.05.

C). Together, these data strongly suggest that chemotherapeutics can stimulate mp53 GOF activity and that the p53-reactivating molecules can reverse this stimulation and increase wt p53-like activity in cancer cells bearing mp53. In contrast, in cancer cells bearing wt p53, treatment with p53-reactivating molecules can further stimulate p53 activity.

### 3.5. GEM and p53-reactivating molecules synergistically inhibit pancreatic cancer cell proliferation

The antiproliferative effect of GEM in combination with each one of the two p53-reactivating molecules CP-31398 and RITA, was examined on Panc1 and PaCa3 cell lines. In both cell lines, treatment with CP-31398 or RITA in combination with GEM significantly decreased cell growth as compared to single treatments (Fig. 5A and B). To evaluate the potential synergistic effect of GEM treatment in combination with CP-31398 or RITA, we analyzed cell growth inhibition curves using CalcuSyn software (see Materials and Methods). Based on the software requirements, in these experiments, we increased the treatment period to 48 h in order to avoid proliferation exceeding 100% as we noted earlier with 24 h GEM treated Panc1 cells (see Fig. 3C). Fig. 5C- F show a dose-dependent growth analyses in cells treated with different concentrations of GEM and/or p53-reactivating molecules for 48 h. The results of these analyses showed that the combination treatment induced a significant synergistic antiproliferative effect in cancer cells as revealed by the curves of the combination index (CI) values versus the fraction (0–1) of cells killed by the combination drug treatment (Fig. 5C- F). The <1 CI values represented a synergistic effect at both low and high concentrations of the drugs. The $CI_{50}$ values were 0.41 and 0.51 for GEM/CP-31398 combination and 0.28 and 0.49 for GEM/RITA combination in Panc1 and PaCa3, respectively (Table. 1). To better describe the antiproliferative synergism between GEM and p53-reactivating molecules, Table 1 also includes the reduction folds related to the drug concentration to obtain 50% cell growth inhibition in combination setting as compared to each drug alone ($DRI_{50}$). Intriguingly, these analyses showed that DRIs of GEM/CP-31398 and GEM/RITA combination treatments were much higher in mp53 Panc1 cells than in wt p53 PaCa3 cells (Fig. 5G and H). This suggest that, despite the synergistic effect of combination drug treatment in both cell types, patients bearing m*TP53* gene may benefit more from this treatment regimen than those bearing wt *TP53* gene. Of note, no synergistic effects of combination drug treatment was observed in the p53-null ASPC-1 cell line (Suppl. Fig. 4A and 4B), suggesting that this combinatory drug approach could be ineffective in patients with *TP53* gene deleted.

### 3.6. GEM and p53-reactivating molecules strongly induce apoptosis and autophagy

CP-31398 or RITA treatments have been reported to have a significant effect on apoptotic cell death and induction of autophagy in tumour cells [26,34]. To investigate the possible effects of GEM/CP-31398 combined treatment in apoptosis and autophagy, we analyzed the apoptotic response (annexinV-FITC binding) and the amount of autophagosome formation (monodansylcadaverine staining) in both Panc1 and PaCa3 cell lines treated with GEM and/or CP-31398 for 48 h. The combined GEM/CP-31398 treatment led to induction of both apoptosis and autophagy at much higher levels than that observed with the single treatments (Fig. 6A and B). The induction of autophagy was further confirmed by examining the level of LC3 protein in the drug-treated cultures of Panc1 and PaCa3 cells using an anti-LC3 antibody. The level of LC3-II protein, the phosphoethanolaminated active form of the autophagosome protein LC3-I, was significantly increased after GEM/CP-31398 combination treatment (Fig. 6C). This result supports the formation of autophagosomes, which, after fusion with lysosomes, constituted acidic vesicular organelles (AVOs) and were detected by monodansylcadaverine staining (Fig. 6B).

### 3.7. Autophagy induced by GEM/CP-31398 has a pro-survival effect

To analyze the role of autophagy stimulation by GEM/CP-31398 drug combination on cell proliferation and apoptosis, we treated both Panc1 and PaCa3 cell lines with the combined treatment in the absence or presence of chloroquine (CQ) or 3-methyladenine (3-MA), the pharmacological inhibitors of autophagy. The results showed that apoptosis induced by GEM/CP-31398 treatment was significantly increased by the addition of CQ or 3-MA in both cell lines (Fig. 7A). Consistently, despite the non-cytotoxic concentrations of CQ or 3-MA (Suppl. Fig. 5A), they both were able to reduce the formation of autophagosomes in the GEM/CP-31398 treated cells (Suppl. Fig. 5B) and furthermore, to significantly sensitize both cell lines to the antiproliferative effect of GEM/CP-31398 (Fig. 7B). Similar results have been obtained using a genetic approach to inhibit autophagy. Indeed, the knock-down of Beclin1, a protein with a pivotal role in the autophagic machinery, by siRNA transfection (Fig. 7C) significantly increased cell growth inhibition stimulated by GEM/CP combined treatment (Fig. 7D). Together, these results indicated that, at least in this cellular context, autophagy had a protective and pro-survival role and that a full effect of GEM/CP-31398 drug combination was achieved when autophagy was inhibited.

### 4. Discussion

To date, many clinical trials have failed to demonstrate an improvement in the overall survival of pancreatic cancer patients treated with GEM in combination with different drugs. Extensive research is currently focused on the identification of novel potential therapeutic targets to overcome GEM resistance of PDAC [35]. Accordingly, high-throughput and proteomic tools are extensively used to unravel the early mechanisms of pancreatic cancer onset and chemoresistance, as well as, to discover biomarkers to aid in prevention, prediction, and development of personalized medicine for treatment of this neoplasm [36]. The p53 tumor suppressor protein is a master transcriptional regulator that controls several key physiological pathways, such as cell cycle arrest, apoptosis, senescence, DNA damage response and metabolism [37–39]. *TP53* gene is mutated in more than 50% of infiltrating PDACs.

**Table 1**
Combination Index (CI) and Dose Reduction Index (DRI) values for GEM/CP-31398 or GEM/RITA combinations.

| CELL LINES | Molar Ratio | $CI_{50}$ | $DRI_{50}$ | r |
|---|---|---|---|---|
| Panc1 | GEM:CP 1:8 | 0.41 | GEM: 8.9<br>CP: 1.8 | 0.95 |
| PaCa3 | GEM:CP 1:8 | 0.51 | GEM: 2.5<br>CP: 9.0 | 0.97 |
| Panc1 | GEM:RITA 1:16 | 0.28 | GEM: 26.5<br>RITA: 4.1 | 0.91 |
| PaCa3 | GEM:RITA 1:16 | 0.49 | GEM: 11.3<br>RITA: 2.5 | 0.97 |

$CI_{50}$ was calculated for 50% cell growth inhibition in the combined treatment by isobologram analyses performed with the CalcuSyn software. $DRI_{50}$ represents the folds of dose reduction to obtain 50% cell growth inhibition in combination setting as compared to each drug alone.

Importantly, *in vivo* studies performed in mice have shown that the re-activation of p53 could lead to tumor stasis/regression even in the presence of multiple tumor-associated genetic alterations [40,41]. These observations have paved the way for the identification of small molecules able to transform mp53 proteins in tumors into forms that can perform wild-type p53 tumor suppressor functions in cancer cells [42]. We have recently demonstrated that p53-reactivating small molecules can significantly reduce cell proliferation of both wt and mp53 PDAC cell lines, without having any effect on p53-null cancer cells [26]. Here, we showed that GEM treatment stabilized mp53 (and its serin 15 phosphorylated form) in the nuclei, induced the expression of mp53 cell cycle target genes (*CdK1* and *CCNB1*; Figs. 1 and 2), and stimulated pancreatic cancer cell resistance to GEM treatment (Fig. 3). Our data is consistent with the observations that non-functional p53 affects the sensitivity of cancer cells to GEM [43] and that restoration of p53 function by various approaches in tumors allows activation of molecular pathways that improve the response to GEM [44,45]. Accordingly, some studies demonstrated that exposure to GEM enhances the transcriptional activity of p53 and that GEM-induced apoptosis in cancer cells is mediated by a p53-dependent transcriptional activation of cell death-related genes [46,47]. Here, we showed that the combined treatment of PDAC cells with GEM and p53-reactivating molecules (CP-31398 and RITA) synergistically inhibited cell proliferin in both wt and mp53 tumor cells but not in p53-null cancer cells (Fig. 5 and Suppl. Fig. 4). These results may be explained by the observations that: i) in wt-p53 cells, p53-reactivating molecules can further stimulate p53 activity induced by GEM; ii) in mp53 cells, these compounds can stabilize a wt-like conformational status of p53 by modifying the mutant conformation, enabling them to respond to GEM-mediated stimulation; iii) in p53-null cells, the absence of the protein hampers the effect of p53-reactivating molecules and their synergistic effect with GEM. We also showed that combination GEM/CP-31398 or RITA drug treatment increased the sensitivity of the cancer cells to minimum GEM concentration, which in vivo can translate into decreased chemotherapy-related side effects. The reduction of GEM concentration in combined drug treatment approaches is much more pronounced in mp53 PDAC cells than in wt p53 cells, suggesting that GEM resistance in mp53 cells can efficiently be overcome by p53-reactivating molecules. Our data showed that GEM/CP-31398 treatment was also able to strongly induce p53 phosphorylation on Ser15 residue (Fig. 4), which has been described to play a role in the regulation of p53-mediated apoptosis [48]. Ser15 phosphorylation has been shown to result in the accumulation of p53 via the inhibition of its interaction with MDM2 and degradation [49]. However, we did not observe any significant increase of the total level of p53 protein, suggesting that, at least under these experimental condition, GEM/CP-31398 effect was not mediated by inhibition of the MDM2-p53 interaction.

Autophagy is a mechanism that cells adopt following nutrient deprivation or stressful stimuli to remove proteins and organelles via lysosomal pathway, where they are degraded and recycled [50]. It has been reported that autophagy can be stimulated by up-regulation of a number of autophagy-related genes via a p53-mediated mechanism [51]. However, the role of autophagy in cancer is still controversial, mainly due to its dual function, which can protect cancer cells during a stressful episode or promote cell death known as "autophagic cell death" [52–54]. In this study, we also investigated the involvement of autophagy in PDAC cells treated with GEM/CP-31398 combination. Our results clearly showed that the antiproliferative effect of GEM/CP-31398 treatment was paired with a strong autophagosome formation (Fig. 6). This was further confirmed by pharmacological and genetic inhibition of autophagy, which strongly enhanced apoptotic cell death by GEM/CP-31398 treatment, indicating autophagy as a protective and survival mechanism for cancer cells. Our results are consistent with recent studies describing that the autophagy inhibitor verteporfin, identified in a screen for chemicals that prevent autophagosome formation and approved by FDA, enhanced the antitumor activity of GEM in a PDAC

model [55]. Remarkably, Yang S. et al. demonstrated that pancreatic cancers have constitutively activated autophagy and a profound requirement for this process, making them uniquely sensitive to autophagy inhibition [56]. Additionally, Fijii et al. reported the clinicopathological significance of autophagy in PDAC by demonstrating that LC3 (an autophagy marker) positivity was correlated with shorter disease-free and poor overall survival [57]. In this context, the role of the p53 mutational status is still discussed. In an autochthonous model of pancreatic cancer driven by oncogenic *Kras* and the stochastic LOH of *Tp53*, Yang A. et al. elegantly demonstrated that autophagy has a
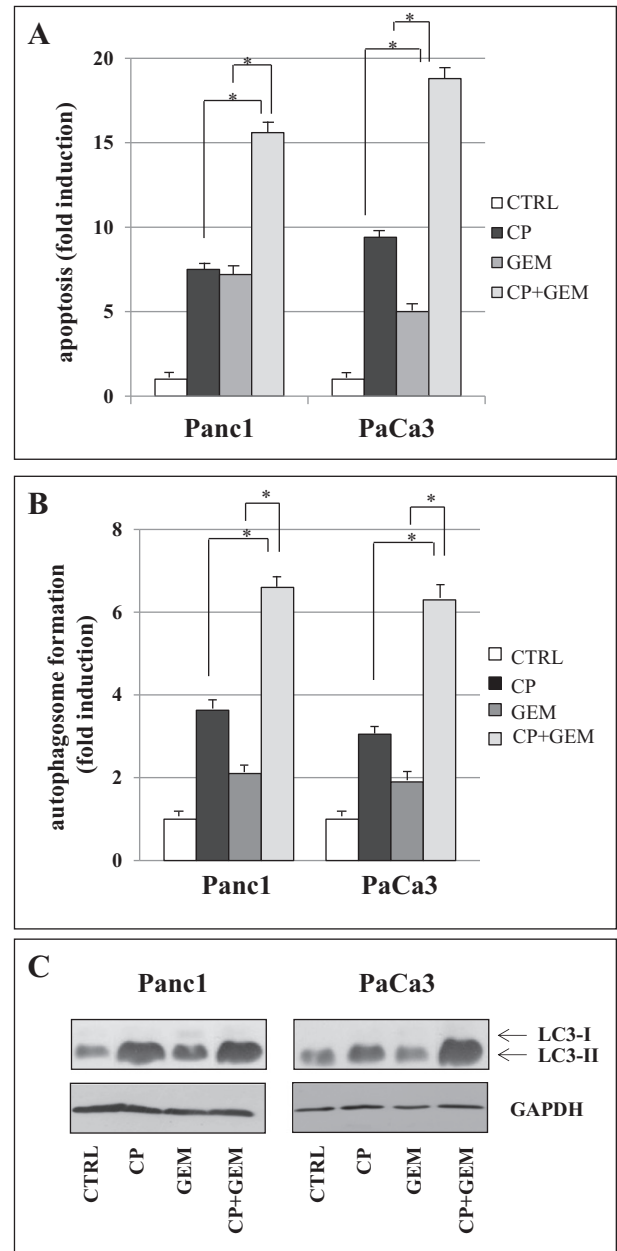


**Fig. 6.** Effect of GEM and CP-31398 on apoptosis and autophagy. (A) Panc1 and PaCa3 cells were seeded in 96-well plates, incubated overnight, and treated with 2.5 μM GEM and/or 20 μM CP-31398 for 48 h. Apoptosis was analyzed using annexinV binding assays. *p < 0.05. (B) Cells were seeded in 96-well plates, incubated overnight, and treated with 1 μM GEM and/or 8 μM CP-31398 for 48 h. Autophagosome formation was assayed by the incorporation of monodansylcadaverine (MDC) probe. *p < 0.05. (C) Cells were seeded in 100-mm diameter culture dishes, incubated overnight, and treated with 5 μM GEM and/or 40 μM CP-31398 for 48 h. Whole-cell extracts were processed for western blot analysis of the autophagic marker LC3. GAPDH protein level in the same extract was used as a control loading.

critical role in pancreatic cancers and that inhibition of autophagy may have clinical utility in the treatment of these tumors, independent of p53 status [58]. On the other hand, Rosenfeldt et al. reported that the p53 status can determine the role of autophagy in pancreatic cancer development, demonstrating that the autophagy inhibitor chloroquine can enhance tumor formation in mouse models lacking p53, while in the presence of functioning wt p53, genetic ablation of autophagy can block progression of pre-malignant lesions to high-grade pancreatic intraepithelial neoplasia and PDAC [59].

In summary, we have provided a novel functional link between GEM treatment, already used as conventional therapy in pancreatic adenocarcinoma, and autophagic inhibitors associated with p53-reactivating molecules. Our data might contribute: a) to better define the molecular events underlying GEM chemoresistance in mp53 pancreatic tumour
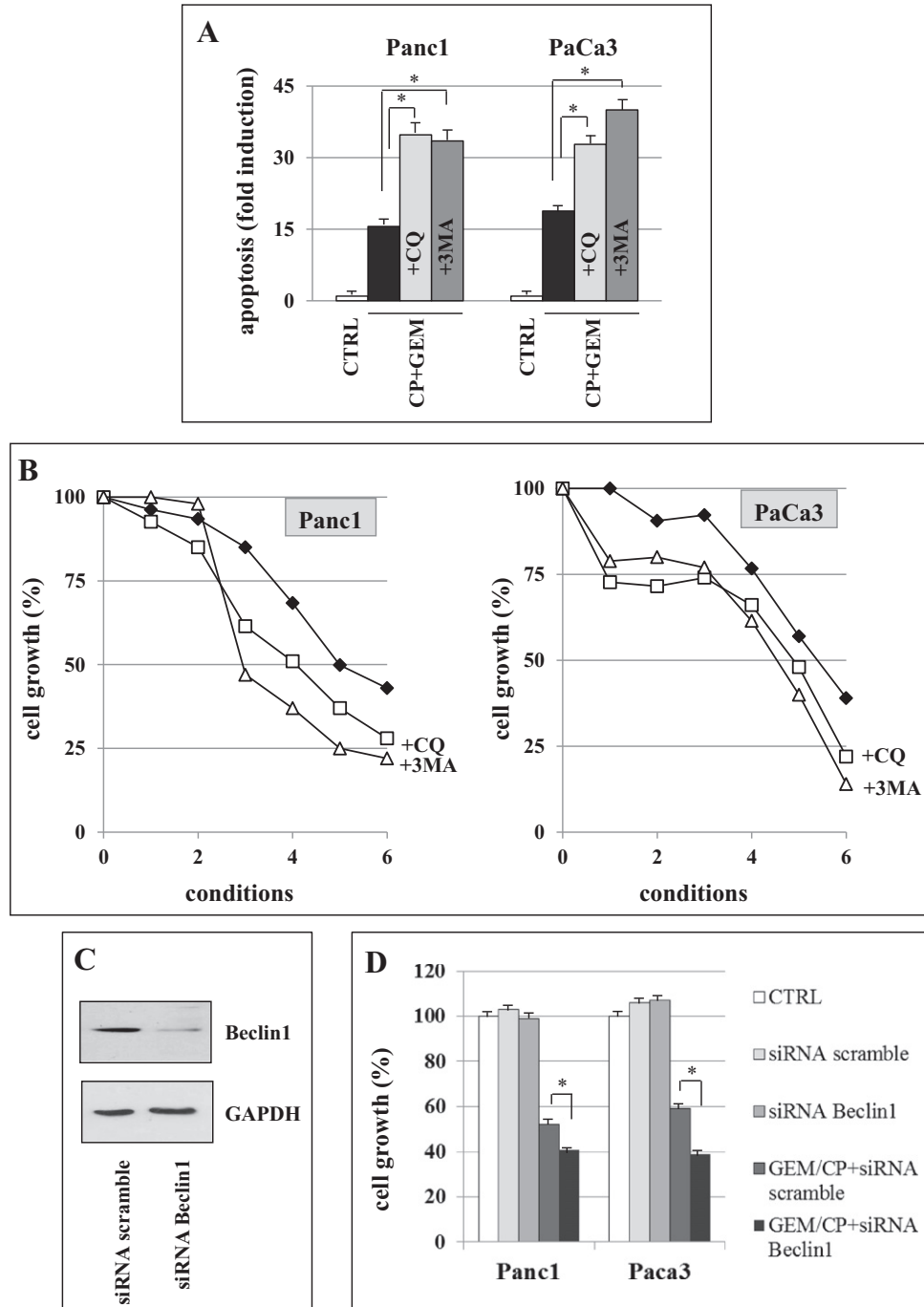


Fig. 7. Role of autophagy in the antiproliferative effect of GEM/CP-31398 combination treatment. (A) Cells were seeded in 96-well plates, incubated overnight, and treated with 2.5 μM GEM plus 20 μM CP-31398 for 48 h in the absence or presence of 10 μM chloroquine (CQ) or 2.5 mM 3-methyladenine (3MA). Apoptosis was assessed using the annexin V binding assay. *p < 0.05. (B) Cells were seeded in 96-well plates, incubated overnight, and treated with six increasing concentrations of GEM/CP-31398 combination (1:8 fixed molar ratio) for 48 h in the absence or presence of 10 μM CQ or 2.5 mM 3MA. The following concentrations used were: 50 nM GEM + 0.4 μM CP (condition 1); 100 nM GEM + 0.8 μM CP (condition 2); 200 nM GEM + 1.6 μM CP (condition 3); 500 nM GEM + 4 μM CP (condition 4); 1 μM GEM + 8 μM CP (condition 5); 2 μM GEM + 16 μM CP (condition 6). Cell proliferation was determined using the Crystal Violet colorimetric assay. (C) Panc1 cells were seeded in 60-mm diameter culture dishes, incubated overnight, and transfected with Beclin1 siRNA (siBECN1) or negative control siRNA for 24 h. Beclin1 expression was analyzed by western blot using whole-cell extracts and Beclin1 antibody. GAPDH protein level in the same extract was used as a loading control. (D) Cells were seeded in 96-well plates, incubated overnight, and transfected with Beclin1 siRNA or negative control siRNA. The day after, cells were treated with 1 μM GEM + 8 μM CP for a further 48 h. Cell proliferation was determined using the Crystal Violet colorimetric assay. *p < 0.05.

cells and consequently to tailor more accurately target specificity and b) to design therapeutic protocols for the use of autophagic inhibitors to sensitize pancreatic adenocarcinoma cells to treatments with GEM and p53-reactivating molecules.

## Acknowledgments

## Appendix A. Supplementary data

Supplementary data to this article can be found online at http://dx. doi.org/10.1016/j.bbamcr.2014.10.003.

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