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Growth of the pancreatic cancer cell line PANC-1 is inhibited by protein phosphatase 2A inhibitors through overactivation of the c-Jun N-terminal kinase pathway

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ABSTRACT

Protein phosphatase 2A (PP2A) is a multimeric serine/threonine phosphatase that can dephosphorylate multiple kinases. It is generally considered to be a cancer suppressor as its inhibition can induce phosphorylation and activation of substrate kinases that mainly accelerate growth. We previously reported that cantharidin, an active constituent of a traditional Chinese medicine, potently and selectively inhibited PP2A, yet efficiently repressed the growth of pancreatic cancer cells through activation of the c-Jun N-terminal kinase (JNK) pathway. This suggested that activation of kinase pathways might also be a potential strategy for cancer therapy. In this study, we have confirmed that the basal activity of the phosphatidylinositol 3-kinase (PI3K)/JNK/activator protein 1 (AP-1) pathway promoted pancreatic cancer cell growth when stimulated by growth factors. Interestingly, although treatment with the PP2A inhibitors, cantharidin or okadaic acid (OA), amplified the PI3K-dependent activation of JNK, cell growth was repressed. We therefore hypothesised that a specific level of activity of the JNK pathway might be required to maintain the promitogenic function, as both repression and overactivation of JNK could inhibit cell proliferation. It was found that the JNK-dependent growth inhibition was independent of the activation of AP-1, but dependent on the repression of Akt. Although the PP2A inhibitors triggered overactivation of JNK and inhibited cell growth, excessively activated protein kinase C (PKC) improved cell survival. Combined treatment with a PP2A inhibitor and a PKC inhibitor produced a synergistic effect, which indicates a potentially promising therapeutic approach to pancreatic cancer treatment.

1. Introduction

Pancreatic cancer is one of the most lethal human cancers. The collective 1-year survival from the time of diagnosis at any stage is only 26%, while the 5-year survival falls to <5%.[1] The main reasons for such poor outcomes are delayed diagnosis (first diagnosis is often made in the advanced stages) and the overall resistance of this tumour to the available therapies. Even patients undergoing surgical therapy in the early stages often suffer from disease recurrence or

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metastatic disease.[2–4] Therefore, new treatments for this aggressive neoplasm are urgently needed.

Natural products and derivatives thereof have shown notable promise in cancer treatment. For instance, vinca alkaloids, taxanes and camptothecins have now become part of the standard repertoire in cancer chemotherapy.[5] In our previous study, we demonstrated that cantharidin, the active constituent of Chinese blister beetle,[6] inhibited the growth of pancreatic cancer cells through G_2/M cell-cycle arrest and apoptosis,[7] which suggested a potential role for cantharidin in the treatment of pancreatic cancer.

Cantharidin acts as a potent and selective inhibitor of a multimeric serine/threonine phosphatase, protein phosphatase 2A (PP2A).[8] The core enzyme of PP2A consists of a catalytic subunit (PP2Ac) and a regulatory subunit termed as A subunit (PP2Aa). A third regulatory B subunit can be associated with this core structure. At present, four different families of B subunits have been identified, modulating the substrate specificity of PP2A.[9,10] Interestingly, PP2A is generally considered to be a cancer repressor. Inhibition of PP2A has been thought to be cancer promoting by induction of phosphorylation and activation of several substrate kinases, including c-Jun N-terminal kinase (JNK), extracellular signal-related kinase (ERK), p38, Akt and protein kinase C (PKC) amongst others, most of which can accelerate growth.[9,10] However, some kinase-dependent growth inhibition pathways that are induced by treatment with PP2A inhibitors have recently been reported,[11,12] which has suggested that the activation of kinase pathways may not always be cancer promoting. In our previous study,[7] we found that ERK, JNK and p38 were activated following treatment with PP2A inhibitors. Inhibition of JNK, but not ERK or p38, reduced the cytotoxic effect of cantharidin, which suggested that cantharidin exerted its anticancer effect through the JNK-dependent pathway, although the detailed signalling transduction mechanism remained unclear.

JNK is evolutionarily conserved in animals. Although its basal activity appears to cause mitotic progression,[13–17] extensive work has been done to confirm the role of JNK in triggering growth inhibition.[18,19] This study investigated the mechanism of JNK-dependent toxicity in pancreatic cancer cells.

2. Materials and methods

2.1. Cell lines and cultures

The human pancreatic cancer cell lines PANC-1, CFPAC-1, Capan-1, HPAC, SW1990, Miapaca-2 and BxPC-3 were purchased from the American Type Culture Collection (Manassas, VA, United States of America (USA)). They were maintained in Dulbecco's minimum essential medium (DMEM; Gibco, Grand Island, NY, USA) supplemented with 10% foetal calf serum (FCS; Hyclone, Logan, UT, USA), 100 units/ml penicillin and 100 mg/ml streptomycin. The cultures were incubated at 37 °C in a humidified atmosphere with 5% CO_2. Cells were passaged every 2–3 days to obtain exponential growth.

2.2. Tissue specimens

Human pancreatic cancer tumours and adjacent normal tissues surgically removed and snap frozen in liquid nitrogen were obtained from Jiangsu Province Academy of Clinical Medicine, Institute of Tumour Biology. All human tissue samples were obtained and handled in accordance with an approved Institutional Review Board application (The Jiangsu Province Hospital Ethics Committee, The First Affiliated Hospital of Nanjing Medical University).

2.3. Reagents

Cantharidin, okadaic acid (OA), SP600125, LY294002 and GF109203X were purchased from Enzo Life Science International (Plymouth Meeting, PA, USA).

2.4. Plasmids

Molecular cloning revealed the existence of two mammalian PP2Ac isoforms, α and β, which share 97% identity in their primary sequence.[9,10] The wild-type PP2Ac α isoform (WT-PP2Acα) and β isoform (WT-PP2Acβ) were amplified by polymerase chain reaction (PCR) from PANC-1 cDNA using PrimerSTAR HS DNA polymerase, and the resulting PCR products were cloned into pcDNA3.1(+) (Invitrogen, Carlsbad, CA, USA). The constitutively active Akt1 (CA-Akt1), an Akt variant that lacks its pleckstrin homology domain but has the addition of a 14-amino acid *src* myristoylation signal, was constructed as described by Cross et al.[20] All plasmid constructs were confirmed by sequencing. The dominant-negative c-Jun mutant, TAM-67, was kindly provided by Nancy H. Colburn (Frederick, USA).[21] Plasmids were transiently transfected into cells using FuGENE HD (Roche, Indianapolis, USA) according to the protocol of the manufacturer using the transfection reagent: DNA ratio of 12:2.

2.5. MTT assay

Cellular growth was evaluated by MTT (3-[4,5-dimethyltiazol-2-yl] 2,5-diphenyl-tetrazolium bromide) assay.[22] Cells were seeded into 24-well tissue culture plates at 5×10^4/well. After treatment, MTT (Sigma) was added to each well at a final concentration of 0.5 mg/ml, followed by incubation at 37 °C for 4 h. The medium was then removed and 800 μl of dimethyl sulphoxide (DMSO) was added to each well. The absorbance of the mixture was measured at 490 nm using a microplate ELISA reader (Bio-Rad Laboratories, Hercules, CA, USA). The relative cell viability was calculated as follows: relative cell viability = (mean experimental absorbance/mean control absorbance) × 100%.

2.6. Cell cycle analysis

Cell cycle analysis was performed with propidium iodide (PI) as described by Nicoletti et al.[23] Before treatment, the culture was deprived of serum for 24 h to synchronise the cell cycle.[24] After treatment, the cells were fixed with 80% cooled ethanol, and incubated with 0.5% Triton X-100 solution containing 1 mg/ml RNase A at 37 °C for 30 min. Next, PI (Sigma) was added into the wells at a final concentration of 50 μg/ml, followed by a 30-min incubation in the dark. The cellular DNA content was analysed by a fluorescence-activated cell sorter (FACS; Becton Dickinson, USA). Data were processed using

ModFit LT software (Verity Software House, Topsham, ME, USA).

2.7. Real-time PCR

Total RNA was extracted using Trizol reagent (Invitrogen) according to the protocol of the manufacturer. After spectrophotometric quantification, 1 μg total RNA in a 20-μl final volume was used for reverse transcription with AMV reverse transcriptase (Promega) according to the instructions of the manufacturer. cDNA aliquots corresponding to equal amounts of RNA were used for the quantification of mRNA by real-time PCR using the ABI Prism 7000 sequence detection system (Applied Biosystems, Foster City, CA, USA). The reaction system (20 μl) contained the corresponding cDNA, forward and reverse primers and SYBR Green PCR master mix (Applied Biosystems). All data were analysed using β-actin gene expression as an internal standard. The specific primers were as follows: (1) PP2Ac, forward, 5′-GTTCACCAAGGAGCTGGACCA-3′, reverse, 5′-CATGCACATCTCCACAGACAGTAAC-3′, product, 164 bp; and (2) β-actin, forward, 5′-TCATGAAGTGTGACGTGGACAT-3′, reverse, 5′-CTCAGGAGGAGCAATGATCTTG-3′, product, 158 bp.

2.8. Western blot

Rabbit anti-phospho-JNK (Thr183/Tyr185), rabbit anti-JNK, rabbit anti-phospho-Akt (Ser473), rabbit anti-Akt, rabbit anti-phospho-c-Jun (Ser63) and rabbit anti-c-Jun antibodies were purchased from Cell Signalling Technology (Beverly, MA, USA). Mouse anti-phospho-PKCα (Thr638), mouse anti-PKCα and mouse anti-β-actin antibodies were purchased from Santa Cruz Biotechnologies (Santa Cruz, CA, USA). Total protein was extracted using a lysis buffer containing 50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1 mM EDTA, supplemented with protease inhibitors (10 mg/mL leupeptin, 10 mg/mL aprotinin, 10 mg/mL pepstatin A, and 1 mM 4-[2-aminoethyl] benzenesulphonyl fluoride) and phosphatase inhibitors (1 mM NaF and 1 mM Na_3VO_4). The protein extract was loaded onto an SDS–polyacrylamide gel and size-fractionated by electrophoresis then transferred to polyvinylidene fluoride (PVDF) membranes (Bio-Rad Laboratories). After being blocked, the membranes were incubated overnight with primary antibodies at 4 °C. The protein expression was determined using horseradish peroxidase-conjugated antibodies followed by enhanced chemiluminescence (ECL) detection (Amersham Pharmacia Biotech, Buckinghamshire, United Kingdom (UK)). β-Actin was used as the internal control.

2.9. Luciferase reporter gene assay

The reporter plasmid, pAP-1-luc, which contained the activator protein 1 (AP-1)-enhancer consensus sequences [(TGACTAA) × 7] and AP-1-dependent firefly luciferase gene was purchased from Stratagene (La Jolla, CA, USA). The reporter plasmid, FKHR-luc, which contains the forkhead response element was kindly provided by M.J. Anderson (La Jolla, CA, USA).[25] The internal control plasmid, pRL-SV40, which contained the renilla luciferase gene was obtained from Promega (Madison, WI, USA). Cells were transiently cotransfected with the reporter plasmid (500 ng/well) and the pRL-SV40 plasmid (100 ng/well) for 8 h using FuGENE HD (3 μl/well) according to the protocol of the manufacturer. The medium was then renewed and treatments were started. After treatment, the cell lysates were subjected to the dual luciferase reporter assay (Promega) according to the recommendations of the manufacturer and the luciferase activities were measured with the TD-20/20 luminometer (Turner Designs, Sunnyvale, CA, USA). The results were expressed as relative luciferase activity, which is the ratio of firefly luciferase activity to renilla luciferase activity.

2.10. Statistical analysis

Each experiment was performed in triplicate at least. Results were expressed as the mean value ± standard deviation (SD). Statistical analysis was performed using unpaired Student's *t*-test. A *P* value less than 0.05 was considered significant.

3. Results

3.1. The basal activity of JNK promoted the growth of pancreatic cancer cells

Although we previously found that PP2A inhibitors repressed cell growth through excessive JNK activation,[7] several groups have reported that the basal activity of JNK promoted mitosis.[13–17] As shown in Fig. 1A–C, the JNK inhibitor SP600125, dose-dependently repressed the phosphorylation level of JNK, inhibited cell viability and triggered G_2/M cell cycle arrest, which indicated that basal activity of JNK promoted growth in pancreatic cancer cells.

It has been shown that growth factors activate JNK through stimulation of the phospatidylinositol 3-kinase (PI3K) pathway,[26] a key signal transduction system that participates in carcinogenesis.[27] We previously reported that growth of pancreatic cancer cells could be repressed by treatment with wortmannin, a PI3K inhibitor,[28] and a similar result was found in this study (Fig. 1D). Treatment with the PI3K inhibitor LY294002 inhibited cell viability in a time-dependent fashion. As shown in Fig. 1E and F, LY294002 not only repressed the JNK phosphorylation that was stimulated by growth factors, but also weakened the growth inhibition effect of SP600125, which suggested that the basal activity of JNK promoted cell growth due to stimulation by growth factors/PI3K pathway.c-Jun is the most studied substrate of JNK. Phosphorylated c-Jun can form either a homodimer or a heterodimer with Fos or activating transcription factor 2 (ATF-2) to form AP-1 complexes that serve as transcription factors to alter expression of the genes that affect cell proliferation.[13] As shown in Fig. 1G and H, treatment with SP600125 inhibited phosphorylation of c-Jun and reduced the transcriptional activity of AP-1. Overexpression of the dominant negative form of c-Jun, TAM-67, not only suppressed cell viability, but also attenuated the growth inhibition effect of SP600125 (Fig. 1I), which indicated that the JNK pathway accelerated proliferation in a manner dependent on c-Jun/AP-1.

PP2A appears to be a negative regulator of JNK. Applying real-time PCR, pancreatic cancer tumours and adjacent normal tissues ($n = 11$), as well as the pancreatic cancer cell lines were analysed for the expression of the PP2A catalytic subunit

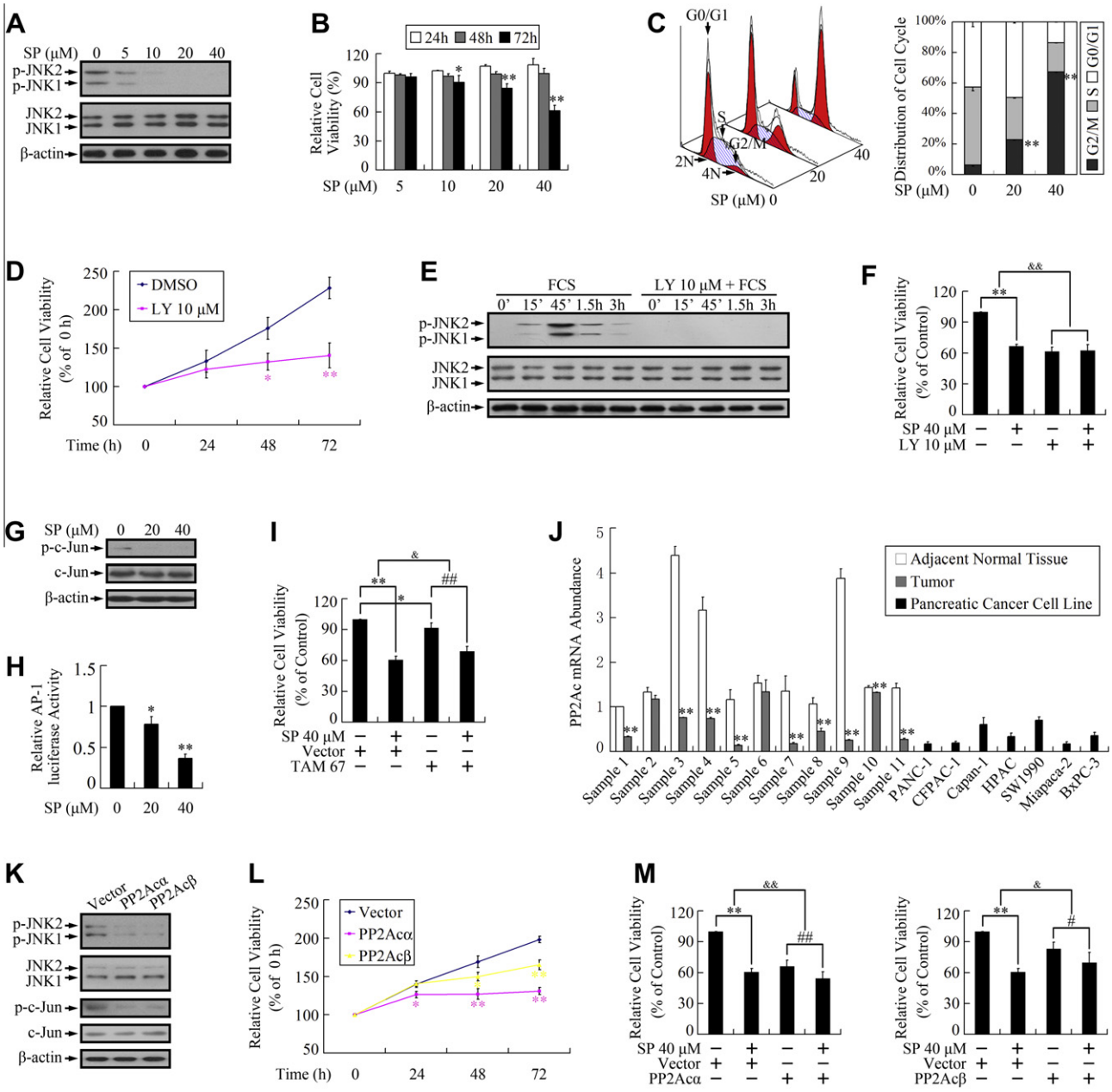

Fig. 1 – Growth factors promote pancreatic cancer cell growth through the phospatidylinositol 3-kinase (PI3K)/c-Jun N-terminal kinase (JNK)/activator protein 1 (AP-1) pathway. (A) Treatment with SP600125 dose-dependently represses the phosphorylation level of JNK. (B) The inhibition of cell viability by SP600125 is dose and time dependent. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus respective control groups. (C) SP600125 triggers G_2/M cell cycle arrest. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group. (D) Treatment with LY294002 time-dependently inhibits cell growth. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus respective control groups. (E) LY294002 represses the phosphorylation of JNK triggered by growth factors. (F) LY294002 attenuates the growth inhibition effect of SP600125. $^{**}P < 0.01$ versus control group; $^{\&\&}P < 0.01$ between folds induction. (G) Treatment with SP600125 dose-dependently inhibits phosphorylation of c-Jun. (H) Treatment with SP600125 dose-dependently represses transcriptional activity of AP-1. (I) Overexpression of the dominant negative form of c-Jun, TAM-67, attenuates the growth inhibition effect of SP600125. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus control group; $^{\#\#}P < 0.01$ versus TAM-67 group; $^{\&}P < 0.05$ between folds induction. (J) Expression of PP2Ac is repressed in pancreatic cancers and the seven pancreatic cancer cell lines. $^{**}P < 0.01$ versus the respective adjacent normal tissues. (K) Overexpression of PP2Ac represses the phosphorylation of JNK and c-Jun. (L) Overexpression of PP2Ac time-dependently inhibits cell growth. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus respective control groups. (M) Overexpression of PP2Ac attenuates the growth inhibition effect of SP600125. $^{**}P < 0.01$ versus respective control groups; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus PP2Ac groups; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ between folds induction.

(PP2Ac; Fig. 1J). PP2Ac transcripts were found in all cancer tumours, adjacent normal tissues and cell lines. In the tumours, suppression of PP2Ac expression was detected in nine out of 11 pairs. PP2Ac expression in the pancreatic cancer cell lines

was also at a low level. Furthermore, overexpression of PP2Ac repressed the phosphorylation of JNK and c-Jun (Fig. 1K), inhibited cell proliferation (Fig. 1L), and attenuated the growth inhibition effect of SP600125 (Fig. 1M), which suggested that the downregulation of PP2A might play a part in the growth of pancreatic cancer through activation of the JNK pathway.

In summary, we have demonstrated that growth factors promoted pancreatic cancer growth through the PI3K/JNK/c-Jun/AP-1 pathway, and downregulation of PP2A increased the activity of JNK and accelerated proliferation.

3.2. *Treatment with PP2A inhibitors induced growth inhibition of pancreatic cancer cells through overactivation of JNK in a manner independent of AP-1*

Although the downregulation of PP2A promoted the phosphorylation of JNK and accelerated proliferation, a small quantity of PP2A was probably necessary for the survival of the pancreatic cancer cells. We previously reported that treatment with cantharidin resulted in almost complete inhibition of PP2A activity and induced persistent overactivation of JNK.[7] As presented in Fig. 2A and B, PP2A inhibitors caused growth inhibition that was dose and time dependent, but that was attenuated by SP600125, which suggested that the overactivated JNK became cytotoxic.

Overactivation of JNK induced higher phosphorylation levels of c-Jun, which could be repressed by SP600125 (Fig. 2C). Treatment with PP2A inhibitors upregulated the transcriptional activity of AP-1, which could be attenuated by SP600125 (Fig. 2D) or by overexpression of TAM67 (Fig. 2E). However, overexpression of TAM67 had no effect on the cytotoxicity of cantharidin or OA (Fig. 2F), which suggested that although PP2A inhibitors activated AP-1, the JNK-dependent growth inhibition was not executed downstream through AP-1.

3.3. *PP2A inhibitors inhibited cell growth through growth factors/PI3K/JNK pathway*

One hypothesis was that cytotoxic JNK triggered by cantharidin was stimulated by stress signals, as treatment with cantharidin can induce oxidative stress and activation of JNK, which is the main responder to stress signals, also termed stress activated protein kinase (SAPK). However, we have shown that cantharidin inhibited cell growth independent of oxidative stress induction.[7] As the growth factors could activate JNK in a PI3K-dependent way, we further investigated whether JNK overactivation amplified by the PP2A inhibitors could also be stimulated by growth factors and mediated by PI3K.

As shown in Fig. 3A and B, when the cells were deprived of serum, the inhibition of cell viability induced by the PP2A inhibitors, which was dose and time dependent, could no longer be attenuated by SP600125, which indicated that JNK was not cytotoxic without the stimulation of growth factors.

Phosphorylation of JNK triggered by the PP2A inhibitors could be repressed by LY294002, the PI3K inhibitor (Fig. 3C). Fig. 3D presented the effects of LY294002 and/or SP600125 on the cytotoxicity of PP2A inhibitors. The groups in Fig. 3D could be divided into four pairs, which were respectively pretreated with solvent (DMSO), LY294002, SP600125, and the combination of LY294002 and SP600125. The relative cell viabilities of cantharidin treated groups versus respective controls in each pairs were 28.20(±4.82)%, 47.88(±3.09)%, 60.64(±2.92)% and 50.23(±4.24)%. The data of OA groups were 25.55(±1.55)%, 10.95(±4.01)%, 63.75(±9.74)% and 10.93(±4.80)%. The effect of pretreatment with LY294002 presented significant difference from pretreatment with SP600125, but showed no difference from cotreatment with LY294002 and SP600125, which indicated that the effect of SP600125 could be blocked by LY294002 and PI3K acted upstream of JNK (Fig. 3D). LY294002 showed a protective effect against cantharidin, although this effect was weaker than that with SP600125 (Fig. 3D). Interestingly, LY294002 increased the toxicity of OA (Fig. 3D), which suggested that there could be other kinases involved, in addition to JNK, both downstream of PI3K and acting as the substrate for PP2A (Fig. 3E). The promitogenic effect of these kinases, unlike JNK, was strengthened when the cells were also treated with PP2A inhibitors, but their promitogenic effect was weaker than the antimitogenic effect of JNK when the cells were treated with cantharidin. However, on treatment with OA, the promitogenic effect of these kinases dominated. The toxicity of PP2A inhibitors was therefore the result of the combined forces of several kinases. Activation of some promitogenic kinases may weaken the anticancer effect of PP2A inhibitors.

3.4. *PP2A inhibitors suppressed cell growth through JNK-dependent inhibition of Akt*

Further attempts were made to identify the promitogenic kinase downstream of PI3K. Akt/protein kinase B (PKB) is a major effector of PI3K in cancers,[29] and functions as a critical promoter of cell survival and proliferation.[29,30] Akt is also the substrate of PP2A,[9] so it was initially investigated as the possible promitogenic kinase.

Surprisingly, Akt was found to be inhibited when cells were treated with PP2A inhibitors. Interestingly, the inhibition of Akt depended on the activation of JNK (Fig. 4A). Overexpression of CA-Akt1 attenuated the cytotoxic effect of PP2A inhibitors (Fig. 4B), which indicated that the JNK-dependent growth inhibition was executed through repression of Akt.

Akt has multiple downstream targets.[29,30] To evaluate whether the repression of Akt could affect the downstream pathways, the changes in the transcriptional activity of forkhead family transcription factor (FKHR), a major target of Akt were measured.[30] Akt represses the activity of FKHR, which can inhibit cell survival.[30] As shown in Fig. 4C and D, treatment with the PP2A inhibitor induced the activation of FKHR, which could be attenuated by SP600125 or by overexpression of CA-Akt1.

3.5. *Activation of PKC weakened the cytotoxic effect of PP2A inhibitors*

PKC, an accelerant for pancreatic cancer cell growth, also acts downstream of PI3K[31] and is a substrate of PP2A.[9] The role of PKC in the treatment of cells with PP2A inhibitors was therefore further investigated.

Treatment with PP2A inhibitors induced the phosphorylation of PKC, which could be attenuated by LY294002 (Fig. 5A).

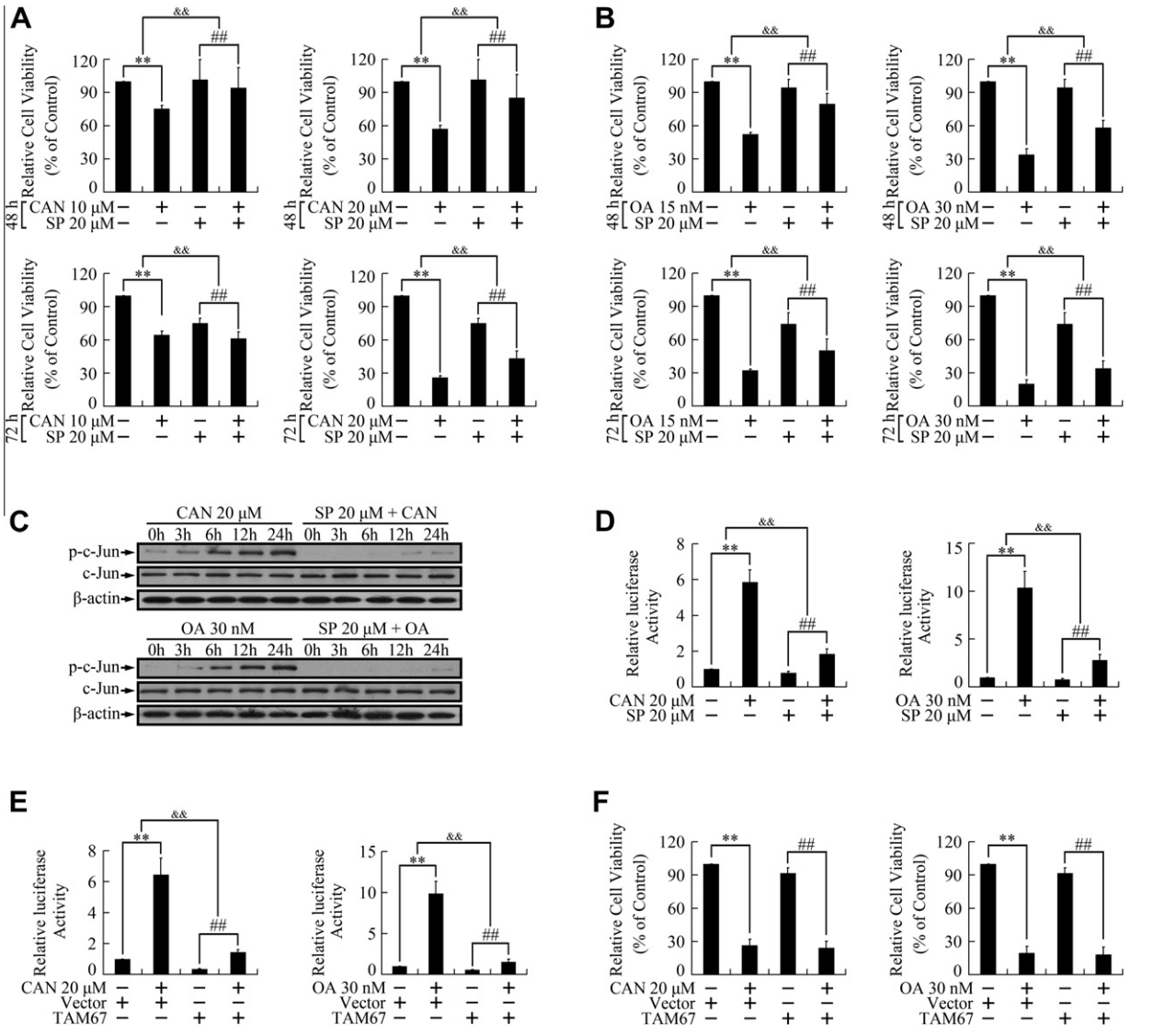

Fig. 2 – Protein phosphatase 2A (PP2A) inhibitors induce growth inhibition of pancreatic cancer cell line PANC-1 in a JNK-dependent manner. (A) and (B) The inhibitory effect, which is dose and time dependent, of cantharidin (A) or okadaic acid (OA; B) can be attenuated by SP600125. $^{**}P < 0.01$ versus respective control groups; $^{\#\#}P < 0.01$ versus SP600125 groups; $^{\&\&}P < 0.01$ between folds induction. (C) PP2A inhibitors induce higher phosphorylation levels of c-Jun, which can be repressed by SP600125. (D) and (E) PP2A inhibitors upregulate the transcriptional activity of activator protein 1 (AP-1), which can be attenuated by SP600125 (D) or overexpression of TAM67 (E). $^{**}P < 0.01$ versus respective control groups; $^{\#\#}P < 0.01$ versus the SP600125 or TAM-67 groups; $^{\&\&}P < 0.01$ between folds induction. (F) The growth inhibition effect of cantharidin or OA cannot be attenuated by overexpression of TAM67. $^{**}P < 0.01$ versus respective control groups; $^{\#\#}P < 0.01$ versus TAM-67 group.

The cytotoxic effect of PP2A inhibitors could be strengthened by GF109203X, a selective inhibitor of PKC, which indicated that the promitogenic effect of PKC was strengthened by treatment with PP2A inhibitors (Fig. 5B). The activation of PKC obliterated the anticancer effect of PP2A inhibitors, which suggested that the combination of PP2A inhibitors and PKC inhibitors might be synergistic, and therefore this might be a promising stratagem for cancer treatment.

4. Discussion

JNK is a kinase, a protein phosphorylase enzyme, that is evolutionarily conserved in animals.[18] The majority of published evidence supports a role for JNK in conveying apoptotic responses when cells are exposed to cytokines or stress.[13] However, increasing numbers of reports have shed new light on the role of basal JNK in neoplastic progression.[13–17] In this study, the basal activity of JNK was found to be promitotic in pancreatic cancer cells, while the PP2A inhibitors induced overactivation of JNK and suppressed cell growth.

It has been shown that JNK in different cases can both promote and suppress tumours depending on the upstream stimulation.[32] When stimulated by growth factors, JNK promotes cell growth, whereas when activated by a stress trigger, it inhibits proliferation.[32] However, we previously reported that the cytotoxicity of cantharidin was executed in an

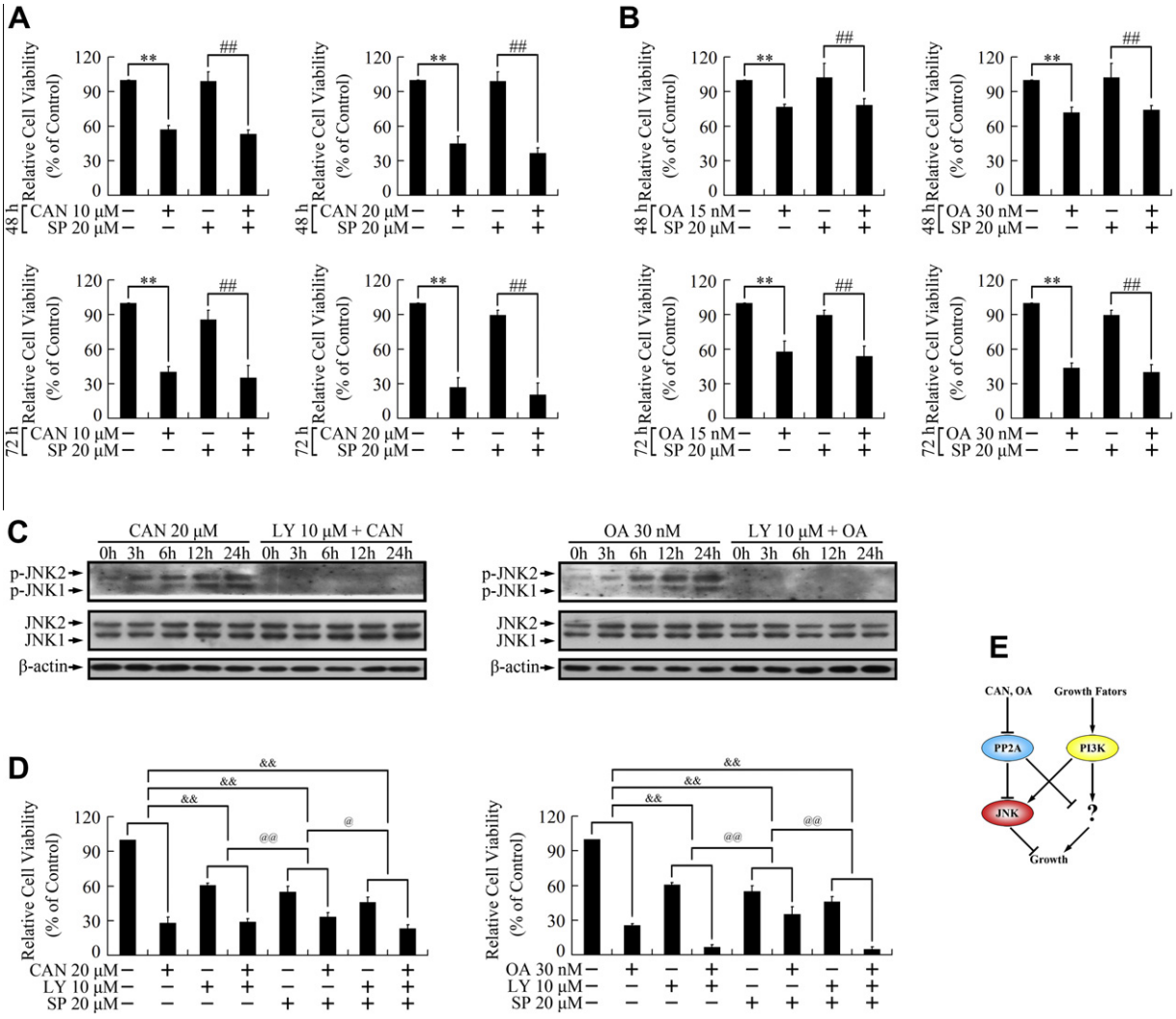

Fig. 3 – Protein phosphatase 2A (PP2A) inhibitors inhibit PANC-1 cell growth through growth factors/phospatidylinositol 3-kinase (PI3K)/c-Jun N-terminal kinase (JNK) pathway. (A) and (B) The inhibitory effect, which is dose and time dependent, of cantharidin (A) or okadaic acid (OA) (B) cannot be attenuated by SP600125 when the serum is deprived. $^{**}P < 0.01$ **versus respective control groups;** $^{\#\#}P < 0.01$ **versus SP600125 group. (C) LY294002 represses the phosphorylation of JNK triggered by PP2A inhibitors. (D) The effect of pretreatment with LY294002 shows no difference from cotreatment with LY294002 and SP600125. LY294002 shows a protective effect against cantharidin, although this effect is weaker than SP600125. LY294002 increases the toxicity of OA.** $^{@}P < 0.05$; $^{@@}P < 0.01$ **and** $^{\&\&}P < 0.01$ **between folds induction. (E) There may be some promitogenic kinases both downstream of PI3K and acting as the substrates for PP2A.**

oxidative-stress independent manner,[7] which suggested that the PP2A inhibitors-strengthened JNK activation was not stimulated by stress triggers. In the present study, we found that with the same upstream signal (growth factors/PI3K pathway), the basal activity of JNK accelerated proliferation but the overactivated JNK arrested cell cycle progression, which indicated that the function of JNK was determined not only by the nature of the upstream stimulation but also by the level of activity.

Akt is the most well known kinase downstream of PI3K and promotes cell growth in pancreatic cancer cells. Theoretically, Akt should be activated when cells are treated with PP2A inhibitors. However, in this study we found that overactivated JNK exhibited a depressive effect on Akt, which suggested that the regulation of the kinase pathways may be different in various cell types.

PKC, another kinase downstream of PI3K, was found to be overactivated in cells treated with PP2A inhibitors. The promitotic effect of PKC was strengthened along with the upregulation of its activity. Activation of PKC obliterated the anticancer effect of the PP2A inhibitors. The combination of PKC inhibitor and PP2A inhibitors produced a stronger cytotoxic effect on pancreatic cancer cells, which indicated the possibility of some attractive combination chemotherapy regimens with the combination of PP2A inhibitors and the inhibitors of the growth-promoting kinases. Further studies are required to investigate whether there are other PP2A inhibitors that can stimulate promitotic kinases, in addition to PKC.

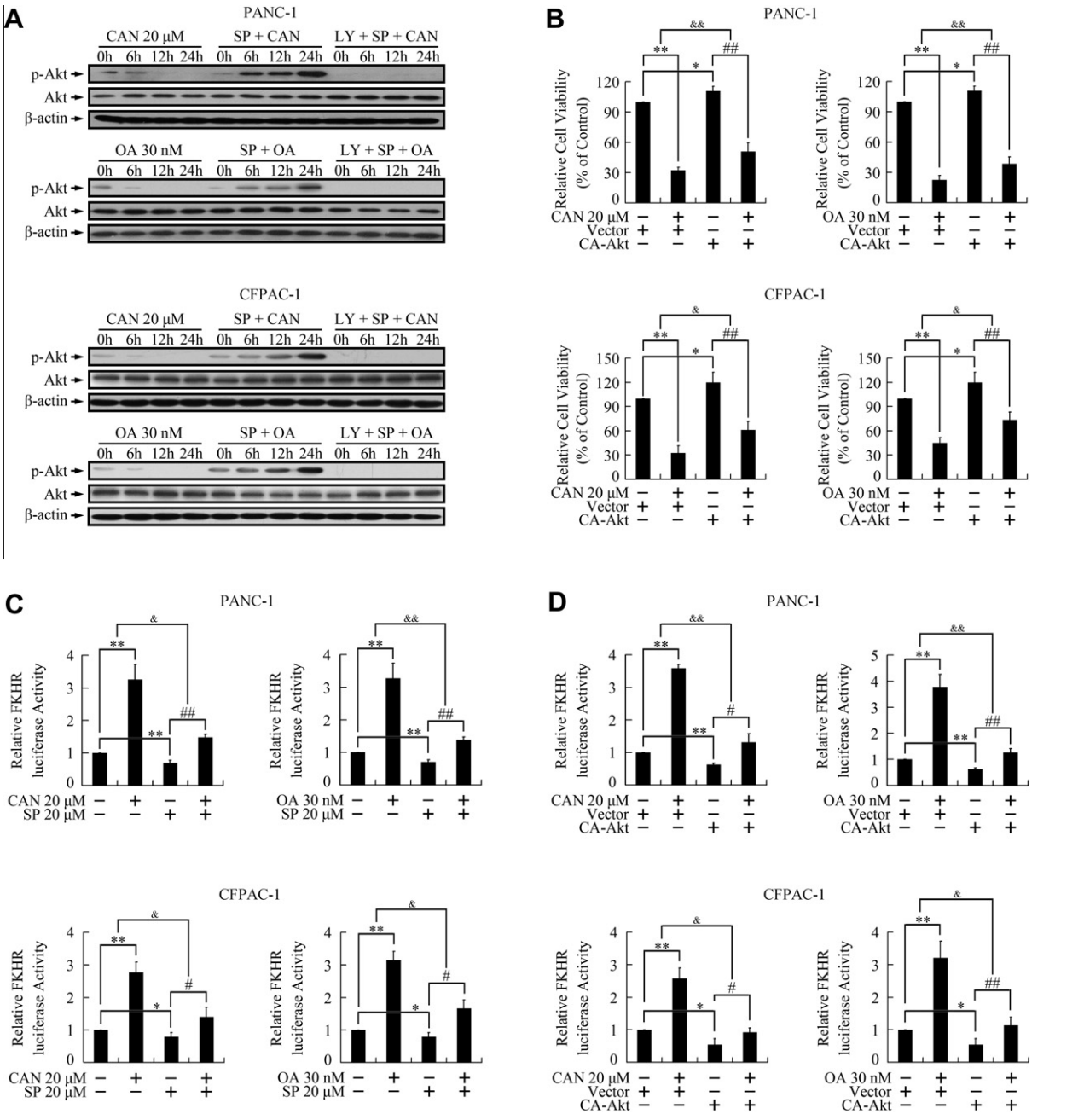

Fig. 4 – Protein phosphatase 2A (PP2A) inhibitors repress cell growth in PANC-1 and CFPAC-1 cell lines through c-Jun N-terminal kinase (JNK)-dependent inhibition of Akt. (A) PP2A inhibitors repress the phosphorylation of Akt. The phosphorylation level of Akt is increased by PP2A inhibitors when pretreated with SP600125. This upregulation can be further inhibited by LY294002. (B) Overexpression of the constitutively active Akt1 (CA-Akt1) attenuates the cytotoxic effect of PP2A inhibitors. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus respective control groups; $^{\#\#}P < 0.01$ versus CA-Akt1 group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ between folds induction. (C) and (D) Treatment with PP2A inhibitors induces the activation of forkhead family transcription factor (FKHR), which can be attenuated by SP600125 (C) or overexpression of CA-Akt1 (D). $^{*}P < 0.05$ and $^{**}P < 0.01$ versus respective control groups; $^{\#}P < 0.05$ and $^{\#\#}P < 0.01$ versus SP600125 group or CA-Akt1 group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ between folds induction.

To date, we have investigated several mitosis-related kinases in cells treated with PP2A inhibitors, including ERK, p38,[7] JNK, Akt and PKC, all of which are substrates of PP2A. Activation of ERK and p38 did not affect cell growth[7]; however, activation of JNK was found to be antimitotic and it repressed the activity of Akt. The growth-promoting effect of PKC was enhanced along with the upregulation of its activity. Hence, PP2A, the phosphatase which regulates the activity of JNK and other kinases at appropriate levels, is of paramount importance in maintaining cellular function (Fig. 5C).

It is noteworthy that, PP2A is generally considered to be a cancer repressor, as most of the substrate kinases are

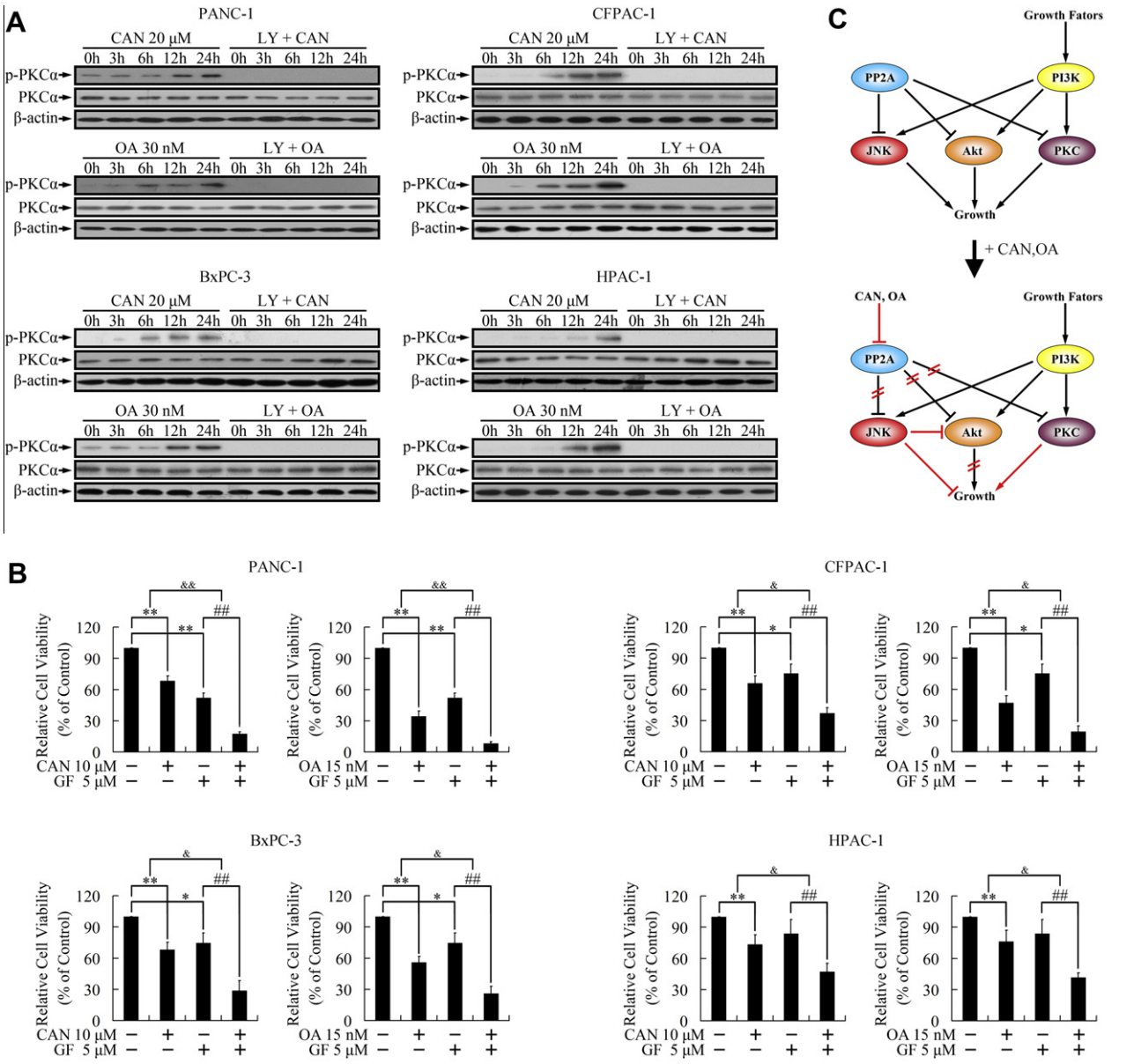

Fig. 5 – Activation of protein kinase C (PKC) promotes cell survival from the cytotoxicity of protein phosphatase 2A (PP2A) inhibitors. (A) Treatment with PP2A inhibitors induces the phosphorylation of PKC, which can be attenuated by LY294002. (B) The cytotoxic effect of PP2A inhibitors can be strengthened by GF109203X. $^{*}P < 0.05$ and $^{**}P < 0.01$ versus respective control groups; $^{\#\#}P < 0.01$ versus GF109203X group; $^{\&}P < 0.05$ and $^{\&\&}P < 0.01$ between folds induction. (C) Cell signalling pathways regulated by PP2A and phospatidylinositol 3-kinase (PI3K).

promitotic. In this study, decreased PP2A expression was found in pancreatic cancer samples. The growth of pancreatic cancer cells was inhibited by the overexpression of PP2A, which suggested that repression of PP2A in normal cells could induce kinase activation and promote carcinogenesis. However, the function of even the diminished level of PP2A was still very important in cancer cells. Excess inhibition of PP2A induced a higher level of activation of the kinases, and their respective functions changed; the effect of JNK was reversed. Thus, the function of the overactivated kinases could be the opposite of their function with basal activity. So PP2A, the expected tumour suppressor[10] is also an important regulator, not only in normal cells, but also in tumours.

This phenomenon is consistent with the 'Yin–Yang' theory, which is an ancient Chinese philosophy nowadays accepted around the world.[33,34] The principle of Yin and Yang, the balance between opposing natural forces, has been emphasised as a fundamental property of cellular growth regulation.[35] Such a balance creates a dynamic state, derangements of which can seriously upset the metabolic balance and induce tumourigenesis or death.[34,35] Protein kinases and phosphatases play opposing roles in the control of protein phosphorylation and signalling, and consequently in the regulation of cell growth and proliferation.[36] Repression of phosphatases (Yin) results in activation of kinases (Yang) and can be oncogenic in normal cells. In terms of cancer cells,

if the phosphatases (Yin) are completely inhibited, the balance of Yin and Yang cannot be maintained neither and the cells will die.

Many anticancer drugs function by blocking growth signalling pathways, so that cancer cells are made to 'starve' to death, while PP2A inhibitors overactivated the kinase pathways and terminated cancer cells through excessive 'Yang-invigoration',[33] which may bring new light to the treatment of pancreatic cancer.

Statements of novelty and interest

(1) JNK may accelerate or inhibit the growth of pancreatic cancer cells depending on its stimulation of the upstream pathways and its level of activity.
(2) Combination treatment with a PP2A inhibitor and a PKC inhibitor can produce a synergistic effect, which indicates a potential promising therapeutic approach.

Conflict of interest statement

None declared.

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