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Histone deacetylase inhibitor trichostatin A and proteasome inhibitor PS-341 synergistically induce apoptosis in pancreatic cancer cells

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Abstract

Pancreatic cancer is a common and lethal malignancy. Pancreatic cancer cells overexpress multiple anti-apoptotic factors and death receptor decoys, and are strongly resistant to radiation and to 5-fluorouracil (5-FU)- or gemcitabine (Gem)-based chemotherapy regimens. We have found that low-dose proteasome inhibitor PS-341 and histone deacetylase inhibitor trichostatin A (TSA) synergistically induce cytotoxicity in a panel of eight diverse pancreatic cancer cell lines. Combining TSA with PS-341 effectively inactivated NFκB signaling, downregulated the predominant endogenous anti-apoptotic factor Bcl-XL overexpression, and disrupted MAP kinase pathway. The combined drug regimen effectively inflicted an average of 71.5% apoptotic cell death (55.2–80%) in diverse pancreatic cancer cell lines by activating the intrinsic apoptotic pathway. Conclusion: the TSA/PS-341 regimen may represent a potential novel therapeutic strategy for pancreatic cancer.

Keywords: Histone deacetylase inhibitor; Proteasome inhibitor; NFκB, Bcl-XL; Pancreatic cancer

Pancreatic cancer is a major unsolved health problem worldwide. Most people with pancreatic cancer die within 6 months of diagnosis due to lack of effective therapies [1,2]. Pancreatic cancer cells coexpress high levels of tumor necrosis factor (TNF) receptor 1 (TNF-R1), tumor necrosis factor-related apoptosis inducing ligand (TRAIL) receptor (TRAIL-R) and Fas, but are strongly resistant to apoptosis triggered by these death receptors [3]. The pancreatic cancer cells' insensitivity to TRAIL receptor- and Fas-mediated cytotoxicity is partly due to the overexpression of death receptor decoys DcR2 and DcR3. However, the predominant overexpression of Bcl-XL also plays a critical role in pancreatic cancer chemoresistance [3].

Clinical investigations indicate that 5-FU-based standard chemoradiation or Gem-based chemotherapies do not prolong overall survival for patients with pancreatic cancer [4–9]. In particular, proteasome inhibitor PS-341 does not improve the antitumor effect of Gem in patients with pancreatic cancer [10]. However, combining protein kinase B (PKB/Akt) inhibitor Wortmannin with Gem significantly suppresses the growth of tumor xenografts derived from a pancreatic cancer cell line [11,12]. With advanced technologies and understanding of cancer cell biology, a variety of new agents, including PS-341, that target specific molecular pathways in tumorigenesis have been developed and are being tested in clinical trials for other cancer systems [13–15].

The 26S proteasome, an ATP-dependent multi-enzyme complex, regulates the degradation of intracellular proteins, such as the NFκB transcription factor family, which are central to gene transcription, cell cycle progression, and apoptosis [13,14]. PS-341 is a potent and selective inhibitor of the 26S proteasome and can block NFκB activation and suppress tumor growth to some extent. Altered activities of histone acetyltransferases (HATs) and histone deacetylases (HDACs) have been identified in several types of cancer

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[15]. HATs and HDACs determine the acetylation status of histones and some oncogenic transcription and growth factors. The acetylation of these proteins affects the regulation of gene expression. Trichostatin A (TSA), an HDAC inhibitor, modifies chromatin structure and alters the biological functions of transcription factors involved in oncogenesis, inducing cancer cells to differentiation [13,14]. Heat shock protein-90 (Hsp90) regulates a wide range of cell processes, including cell cycle progression, proliferation, and apoptosis by regulating protein folding, stability, and degradation [16]. Hsp90 binds to Apaf-1, prevents apoptosome formation, and inhibits caspase-9 activation [17]. Hsp90 interacts with receptor interacting protein-1 (RIP-1), PKB/Akt [18], and extracellular signal regulated kinases type 1 and 2 (ERK1/2), and positively regulates NFκB activation and tumor survival pathways. Geldanamycin (GA), an Hsp90 inhibitor, disrupts Hsp90 chaperoning functions and enhances TNFα cytotoxic effects [18]. Another agent is doxorubicine (Dox), a transcription inhibitor, which can induce pro-apoptotic effects through regulation of NFκB RelA under certain conditions [19].

Most pancreatic cancer cell lines are strongly resistant to these drugs when administered individually. We hypothesized that an optimal combination of antitumor drugs would maximize killing of pancreatic cancer cells of diverse sources at low drug doses. In order to achieve this goal, we have investigated the cytotoxic effects of PS-341, GA, TSA, and Dox in eight invasive pancreatic cancer cell lines in the absence of pro-inflammatory cytokines, such as death receptor ligands. In particular, we have compared the efficacies of cytotoxicity of different combinatorial regimens among these drugs in pancreatic cancer cell lines. We have shown for the first time that TSA and PS-341 function synergistically and effectively kill an average of 71.5% of pancreatic cancer cells. This regimen causes the inactivation of NFκB signaling, downregulation of Bcl-XL overexpression, and disruption of the K-Ras-ERK MAP kinase pathways, and effectively induces apoptosis in diverse pancreatic cancer cell lines.

Materials and methods

Cell culture. Human pancreatic cancer cell lines (AsPC-1, BxPC-3, CFPAC-1, Capan-2, Mia PaCa-2, Panc-1, SU86, and SW1990) were maintained according to the instructions of American Type Culture Collection (Manassas, VA).

Antibodies, cytokines, drugs, and protein lysates. Polyclonal antibodies against human caspase-9 and extracellular signal regulated protein kinases (ERK1/2) were purchased from Cell Signaling Technology (Beverly, MA). Antibodies to human caspase-3, caspase-7, and caspase-8 were purchased from BD Pharmingen (San Diego, CA). mAb to human β-actin (AC-15) and human poly (ADP-ribose) polymerase (PARP) was obtained from Sigma (St. Louis, MO) and BIOMOL (Plymouth Meeting, PA), respectively. PS-341 was a gift from Millennium Pharmaceuticals (Cambridge, MA). Trichostatin A (TSA), Geldanamycin (GA), Doxorubicine (Dox), caspase inhibitors z-DEVD-FMK, and z-VAD-FMK were purchased from Sigma (St. Louis, MO). Protein samples for normal human pancreas tissues of three adult donors were provided by United States Biological (Swampscott, MA).

Cell viability assay. Cell sensitivity to drug treatments was measured by non-radioactive cell proliferation assays [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt (MTS) assays] from Promega (Madison, WI) following the manufacturer's instructions as described previously [3]. Statistical significances in cell viability between drug treated and control cells were evaluated by two-tailed Student's t test. A probability level of <0.05 is considered significant.

To determine the ongoing apoptotic process following drug treatment, we conducted Annexin-V (PE) immunostaining and FACS analysis according to manufacturer's instructions (BD Biosciences, San Diego, CA). Results from control cells (not treated with drugs) were utilized to normalize the background. Tests were done three times independently.

Caspase activity assay. The activation of executioner caspase-3 and -7 in cancer cells following drug treatments was determined using Apo-ONE Homogenous Caspase-3/7 Assay kit according to manufacturer's instructions from Promega (Madison, WI). Cancer cells in 96-well plates were incubated with specific drugs (six wells each) for 24 or 48 h. The assays were conducted simultaneously in the absence and presence of caspase inhibitors z-DEVD-FMK (10 μM) and z-VAD-FMK (40 μM) to normalize background caspase activities not induced by antitumor drugs. The results were statistically analyzed and presented as bar graphs.

Electrophoretic mobility shift assay (EMSA). Pancreatic cancer cells of exponential growth were incubated at 37 °C in a growth medium containing 500 nM TSA and 50 nM PS-341. At indicated time points following addition of drugs, nuclear extracts were prepared from control and drug treated cells by using a Nuclear Extract Kit from Active Motif (Carlsbad, CA). An EMSA kit from Panomics (Redwood, CA) was utilized to determine the binding activities of NFκB p65. Nuclear proteins (10 μg) from each sample were mixed with 10 ng biotinylated p65 double stranded oligonucleotide probes (5′-CATC GGAAATTTCCGGAAATT TCCGGAAATTTCCGGC-3′) in the presence of 1 μg Poly(I/C) DNA. The mixture was incubated at room temperature for 30 min. The binding complex was then resolved by using 6% polyacrylamide/glycerol gel as previously described [20]. For supershifts, 1 μl of diluted gel-shift grade goat anti-human p65 antibody from Santa Cruz (Santa Cruz, CA) was incubated with the nuclear extract for 20 min at room temperature prior to incubation with biotin-labeled probes. Additionally, pancreatic cancer cells were incubated in a growth medium containing TNFα (25 ng/ml) for 30 min. Nuclear extracts from TNFα-treated cells served as a positive control.

Reverse transcription polymerase chain reactions (RT-PCR). To determine Bcl-XL mRNA levels following drug treatment, genomic DNA-free total RNAs were prepared at indicated time points from control and drug treated pancreatic cancer cells by using RNeasy Mini Kit and the RNAse-free DNAse set from Qiagen (Valencia, CA). cDNA synthesis was conducted with 1.5 μg total RNA at 37 °C for 2 h by using Omniscript Reverse Transcription Kit of Qiagen (Valencia, CA) in the presence of random primers. cDNA was diluted in 1:100 in water. One microliters of diluted cDNA was submitted for PCR with Bcl-XL specific primers XLP1 (5′-CTGGTGGTTGACTTTCTCTCC-3′, located at 17 nucleotides downstream of the start codon in Bcl-XL mRNA) and XLP2 (5′-GGATGTCAGGTCACTGAATGC, located at nucleotide 309 downstream of the start codon in Bcl-XL mRNA), amplifying 314-bp fragment. PCR was conducted in a 50-μl reaction containing 1 × buffer in the presence of 240 μM dNTPs, 20 pmol each primer, 1.5 mM $MgCl_2$, and 5 U of Taq DNA polymerase (Invitrogen, Carlsbad, CA). The cycle was 95 °C for 1 min, 57 °C for 45 s, followed by 90-s extension at 68 °C for 30 rounds. The human β-actin was chosen as an internal control. Primers 5′β-actin (5′-ACCATGGATGATGATATCGC-3′) and 3′β-actin (5′-GCTCATTGTAGAAGGTGTGG-3′) were designed to amplify a 280-bp fragment.

Determination of synergy between PS-341 and TSA. The synergy between proteosome inhibitor PS-341 and HDAC inhibitor TSA in pancreatic cancer cells was determined by using isobolographic analysis [20,21]. To demonstrate whether PS-341 and TSA have additive and synergistic or antagonistic effects, pancreatic cancer cells were treated with serial combinations of various drug concentrations of PS-341

(10–1000 nM) and TSA (125–5000 nM). The cell viability assays were conducted at 37 °C for 48 h to determine drug concentrations for single and combined drug regimens that yielded a 50 ± 5% reduction of cell viability. The drug concentrations of single or combined regimens that produced this specific effect were used as coordinates to construct isobolograms. The sums of fractional inhibitory concentrations for these two agents (concentration of each agent in combination/concentrations of each agent alone) define additive (=1), synergistic (<1), and antagonist effects (>1). When the combination is additive, isobole (the line joining points that represent all combinations with the same effects, including the equally effective concentrations of PS-341 and TSA used alone) is a straight line. A synergistic combination yields a concave isobole, and an antagonistic combination gives a convex isobole.

Results and discussion

PS-341 and TSA synergistically induce apoptosis in pancreatic cancer cells

Pancreatic cancer cells are strongly resistant to radiation, and to 5-FU- or Gem-based chemotherapy. These cancer cells are also strongly resistant to apoptosis triggered by antitumor agents PS-341, TSA, GA, and Dox when administered individually (data not shown), suggesting that combinatorial therapy may be a means to overcome chemoresistance in pancreatic cancer. In order to identify an optimal drug combination that would effectively control the growth of most pancreatic cancer cell lines, we determined the synergistic effects of PS-341, TSA, GA, Gem, and Dox in various settings of drug combinations and found that PS-341 and TSA synergistically reduced cell viability in pancreatic cancer cells (Fig. 1a). When PS-341 and TSA were administered alone, we had to use 2500 nM TSA or 100 nM PS-341 to achieve approximately 50% reduction of cell viability. When combined, however, 25 nM PS-341 and 350 nM TSA yielded a similar effect. According to mathematic calculation for quantitative analysis of combined effects of enzyme inhibitors established previously [21,22], the sums of fractional inhibitory drug concentrations for three arbitrarily chosen PS-341 and TSA combinations in induction of 50 ± 5% apoptosis were 0.425, 0.39, and 0.6. These sums were <1.

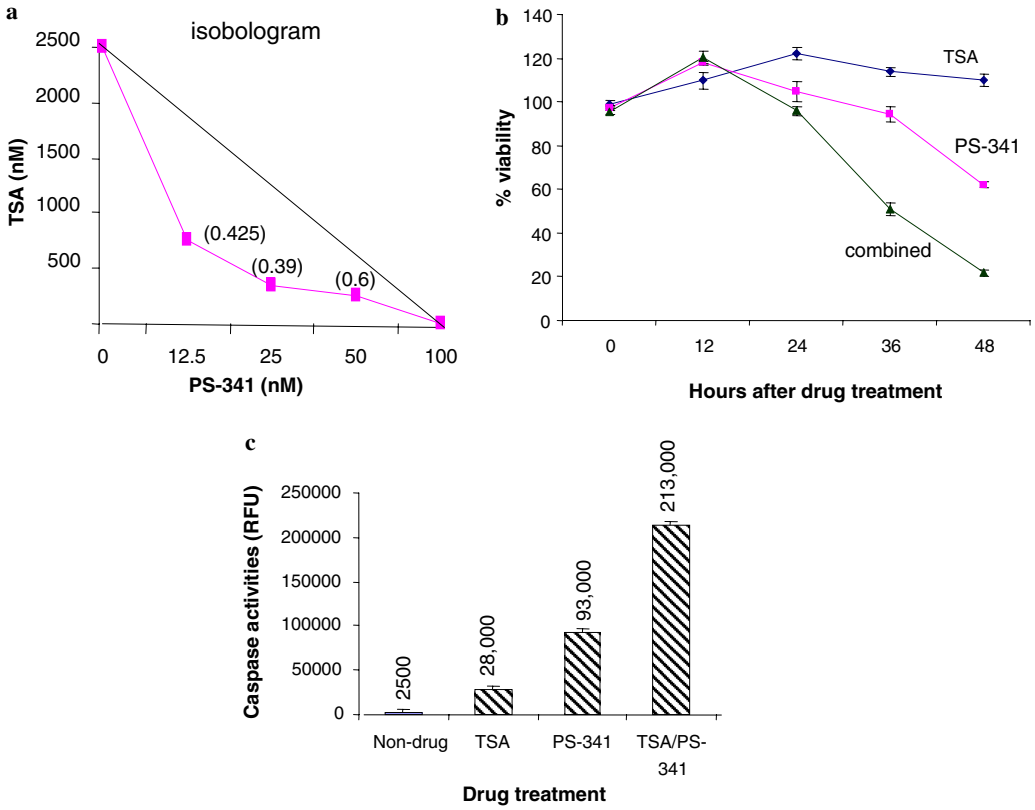

Fig. 1. Synergy of TSA and PS-341 in inducing apoptosis in pancreatic cancer cells. (a) TSA was diluted to 125–5000 nM, PS-341 diluted from 10 to 1000 nM. Panc-1 cells in 96-well plates were incubated with each drug dilution (four wells each) at 37 °C for 48 h followed by cell viability assays to determine drug concentration that could reduce cell viability by 50% ± 5 for TSA and PS-341, respectively. These analyses revealed that 100 nM PS-341 and 2500 nM TSA decreased Panc-1 cell viability by 50%, respectively. Based on these results, we diluted PS-341 to 12.5, 25, 50, and 100 nM. Medium with different concentrations of PS-341 were used to dilute TSA from 2500 to 125 nM to generate an array of TSA/PS-341 drug combinations. Cancer cells were then incubated with each drug dilution at 37 °C for 48 h for MTS assay to identify a specific combination that reduced cell viability by approximately 50%. Drug concentrations of single and combined regimens that produced this specific effect were used as coordinates to construct isobolograms. Numerical figures in brackets indicate the sums of fractional inhibitory drug concentrations for three arbitrarily chosen PS-341/TSA drug combinations that caused about 50% apoptosis. (b) TSA (500 nM) and PS-341 (50 nM) synergistically induced apoptosis in Panc-1 cells over a time course. (c) The activation of caspase-3 and -7 after drug treatments. Panc-1 cells were incubated with TSA (500 nM) and PS-341 (50 nM) alone or their combination. Forty eight hours later, caspase-3/7 enzyme activities were measured using fluorescein-based caspase activity assays. Error bars indicated standard deviations.

When they were plotted onto the isobologram with relevant drug concentrations, the isobole displayed a concave shape (Fig. 1a), indicating that PS-341 and TSA acted synergistically in inducing cytotoxicity in pancreatic cancer cells. TSA alone at 500 nM did not significantly reduce cell viability (Fig. 1b). PS-341 at 50 nM reduced cell viability by 38% 48 h post treatment. Combining PS-341, however, with TSA significantly decreased cancer cell viability by 78.2% ($P < 0.001$) compared to TSA or PS-341 alone (Fig. 1b).

We also found that TSA and PS-341 acted synergistically in inducing apoptosis in other seven pancreatic cancer cell lines as well (data not shown). Combining TSA with PS-341 reduced cell viability by 55–80% (average of 71.5%) in all eight pancreatic cancer cell lines tested (Supplement, S1a–h). A previous study indicated that proteasome inhibitor PS-341 interacts synergistically with histone deacetylase inhibitors (suberoylanilide hydroxamic acid, SAHA) to induce apoptosis in Bcr/Abl$^+$ human leukemia cells [23]. Sodium butyrate and MG132 synergistically induce apoptotic effects in human retinoblastoma cells [24]. Our data are consistent with these findings and for the first time indicate that HDAC inhibitors and PS-341 synergistically induce apoptosis in pancreatic cancer cells.

The activation of executioner caspase-3 and -7 is another indicator of apoptosis. We utilized fluorescein-based caspase assays to determine the activities of caspase-3 and -7 in drug-treated pancreatic cancer cells in comparison with control counterparts (Fig. 1c). The reduction of cell viability after drug treatment according to the MTS assays was correlated with the extents of caspase activation. TSA alone did not significantly reduce cell viability (Fig. 1b) and only induced a low level of caspase activities (28,000 RFU) in comparison with control counterpart (2500 RFU). Apparently, this level of capsase activation was not enough to cause apoptosis in Panc-1 cells (Fig. 1b). PS-341 treatment induced caspase activities up to 93,000 RFU (Fig. 1c) and decreased cell viability by 38% (Fig. 1b). Combining TSA, however, with PS-341 induced caspase activities up to 213,000 RFU (Fig. 1c) and killed 78% of cancer cells (Fig. 1b).

In situ apoptosis TUNEL assay (S2) further demonstrated that TSA alone did not affect chromatin integrity 48 h after treatment. PS-341 treatment caused a fraction of cells to display positive TUNEL staining. The PS-341/TSA regimen, however, caused massive apoptotic cell death (S2). The above findings strongly supported the notion that TSA significantly enhanced PS-341 triggered apoptosis in pancreatic cancer cells. The data of cell viability from MTS apoptosis assays were well correlated with the extents of caspase activation and PARP cleavage according to immunoblot assays presented in Fig. 2. Our own studies and reports from other laboratories [25–27] indicated that the MTS assay was a reproducible method to measure cell proliferation as well as viability of pancre-

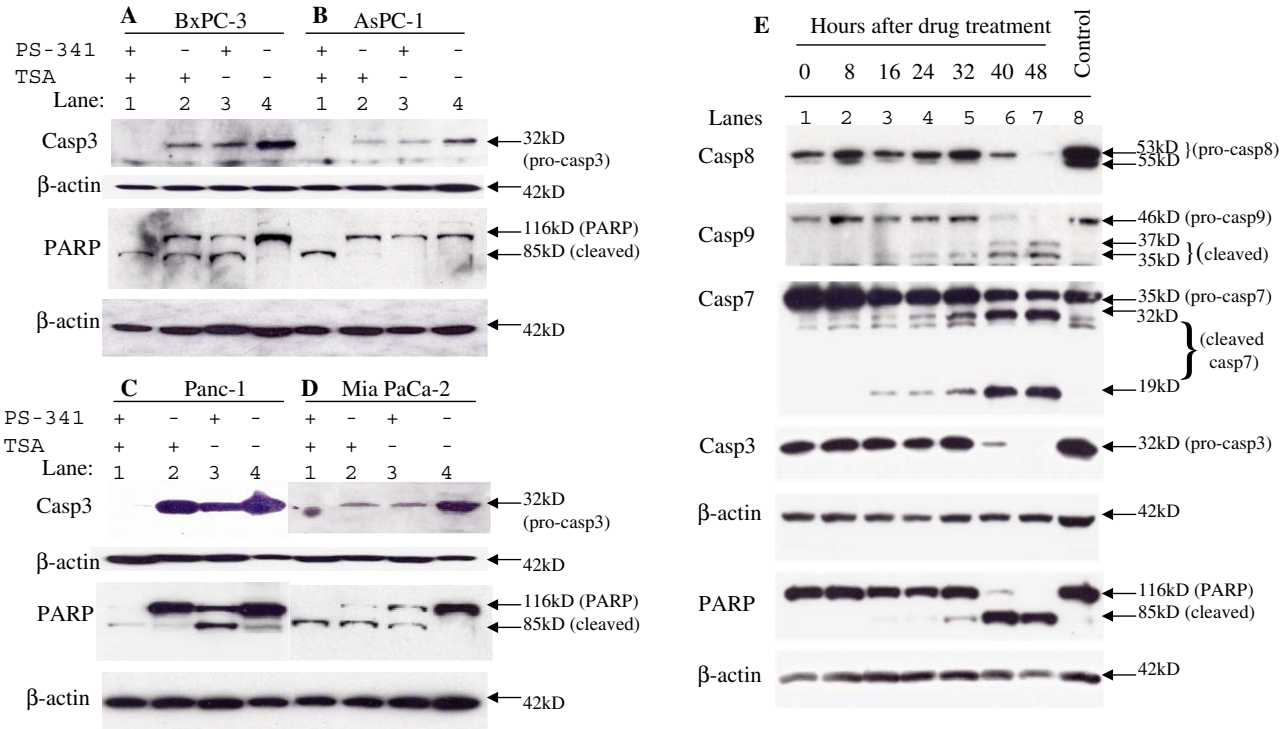

Fig. 2. Caspase activation and PARP cleavage assay. Pancreatic cancer cells were seeded into 12-well plates and were incubated with indicated drugs (50 nM PS-341 and 500 nM TSA) at 37 °C for 48 h. Protein lysates from control and drug-treated cells were analyzed by immunoblots with specific antibodies to determine the degradation of caspase-3 and PARP (A–D). Panel E: the extents of PS-341/TSA-triggered caspase activation and pro-PARP cleavage over a time course. were determined using immunoblot assays. Housekeeping gene β-actin was utilized to show equivalent protein loading.

atic cancer cells along with Annexin-V staining, caspase activation, and PARP cleavage assays.

Combining PS-341 with TSA effectively activated caspase cascades and enhanced PARP cleavage

To demonstrate further if the PS-341/TSA regimen could more effectively activate caspase cascades and cause the cleavage of PARP than single drugs, we determined the extent of caspase activation and PARP cleavage following treatment with TSA (500 nM) and PS-341 (50 nM) alone or combination (TSA/PS-341) in BxPC-3, Mia-PaCa-2, Panc-1, and AsPC-1 cells 48 h post drug treatment (Fig. 2A–D). PS-341 or TSAeffectively activated caspase-3 in BxPC-3 and Mia PaCa-2 cells, resulting in the substantial cleavage of PARP (Fig. 2A and D, and lanes 2 and 3). PS-341 and TSA alone also activated caspase-3 in AsPC-1 cells, but did not cause PARP cleavage (Fig. 2B, lanes 2 and 3). As for Panc-1 cells, PS-341 treatment partly activated caspase-3 and caused a fraction of PARP to degrade (Fig. 2C, lane 3). TSA alone had no effects on both caspase activation and PARP cleavage in Panc-1 cells (Fig. 2C, lanes 2 and 3). Combining TSA, however, with PS-341 effectively activated caspase cascades, and caused the complete degradation of PARP in the above four cell lines (Fig. 2A–D, lane 1).

Additional analysis of caspase activation and PARP cleavage in Capan-2, SU86, and SW1990 cell lines following treatments with TSA and PS-341 alone or combination showed that these cell lines yielded comparable results (data not shown) as described for Panc-1. Taken together, TSA significantly enhanced PS-341 triggered activation of caspase cascades, and the TSA and PS-341 combinatorial regimen more effectively caused PARP cleavage than single drugs in diverse pancreatic cancer cell lines.

We next conducted a time course study in Panc-1 cells following PS-341/TSA treatment in order to assess whether this combinatorial regimen activated the intrinsic or extrinsic apoptotic pathway. According to the onset of caspase cleavage, caspase-9 was activated at 16 h after drug treatment (Fig. 2E, lane 3). This process reached its peak at 40 h (Fig. 2E, lanes 1–7). The activation of executioner caspase-7 followed a similar pattern (Fig. 2E, lanes 1–7). However, the cleavage of PARP was significantly delayed until 32 h of drug treatment (Fig. 2E, lane 5) and reached its peak at 40 and 48 h (Fig. 2E, lanes 6 and 7). This phenomenon was consistent to progressive apoptotic cell death after drug treatment and the complete activation of caspase-9 and caspase-7. Interestingly, the protein levels of pro-caspase-8, an initiator of the extrinsic pathway, were not significantly reduced until 40 and 48 h of drug treatment (Fig. 2E, lanes 6 and 7), as was caspase-3 (Fig. 2E, lanes 1–7). These results suggested that PS-341/TSA might kill pancreatic cancer cells by activating the intrinsic apoptotic pathway. This point was further confirmed by RNA interference of caspase-8 that mediates the extrinsic apoptotic pathway (S3).

Combining PS-341 with TSA effectively blocked NFκB signaling

As discussed in the Introduction, PS-341 and TSA affect NFκB signaling by two distinct molecular mechanisms. In this study, we found that PS-341 and TSA synergistically induced apoptosis in pancreatic cancer cells. However, the molecular mechanism underlying this phenomenon was not known. RelA/p50 complex is the major NFκB factor that is involved in chemoresistance of pancreatic cancer [28]. In order to investigate the molecular mechanisms of apoptosis-triggered by the combined drugs, we examined the effects of the PS-341/TSA regimen on NFκB signaling in pancreatic cancer cells in comparison with PS-341 or TSA alone. NFκB promoter driven reporter gene assay showed that PS-341 (10 nM) suppressed both constitutive and TNFα-triggered NFκB activation by 11-fold (S4a,b). TSA (250 nM) enhanced constitutive NFκB signaling by 9-fold and TNFα-triggered NFκB signaling by 6-fold due to the suppression of nuclear HDAC activities [29]. These results are consistent with a previous report [29]. However, combining TSA (250 nM) with PS-341 (10 nM) inhibited constitutive NFκB signaling by 37-fold (S4a) and TNFα-triggered signaling by 82-fold (S4b). These data demonstrated for the first time that TSA significantly enhanced the inhibitory effects of PS-341 on NFκB signaling.

We next treated CFPAC-1 cells with PS-341 (50 nM) or TSA (500 nM) alone or combination for 48 h followed by immunoblot assays to determine the total cellular RelA expression level. PS-341 or TSA alone did not significantly affect RelA protein levels (S4c). However, RelA protein levels started to decrease 24 h following TSA/PS-341 treatment and gradually diminished at 40–48 h S4d, lanes (1–7).

To determine the effects of TSA/PS-341 regimen on nuclear RelA binding activities, we conducted a sensitive EMSA assay by using biotinylated oligonucleotide probes containing conserved RelA binding sequences. An irrelevant biotin-labeled control probe was used to confirm the specificity of RelA binding (Fig. 4a and b). Nuclear extracts from Panc-1 cells in the absence of TNFα stimulation showed binding activities to the biotin-labeled p65-specific probe (Fig. 3a, lane 4), suggesting that NFκB signaling was constitutively activated. Compared to control cells (not treated with drugs, Fig. 3a, lane 4), TSA/PS-341 treatment significantly reduced nuclear RelA binding activities between 24 and 40 h following drug treatment (Fig. 3a, lanes 5–9). Nuclear RelA binding activities were depleted 48 h post drug treatment. The binding activities to biotin-labeled RelA probes could be abolished by 3-fold excessive cold p65 probe (Fig. 3a and b, lanes 2).

In order to determine whether nuclear proteins binding to biotin-p65 probes were human RelA transcription factors, we used 20 μg nuclear extract for each binding and supershift assay. The proteins binding to the biotin-labeled RelA probe could be supershifted by RelA specific antibodies (Fig. 3b, lanes 1, and 5–8), but did not supershift the protein complex binding to the irrelevant biotin-labeled

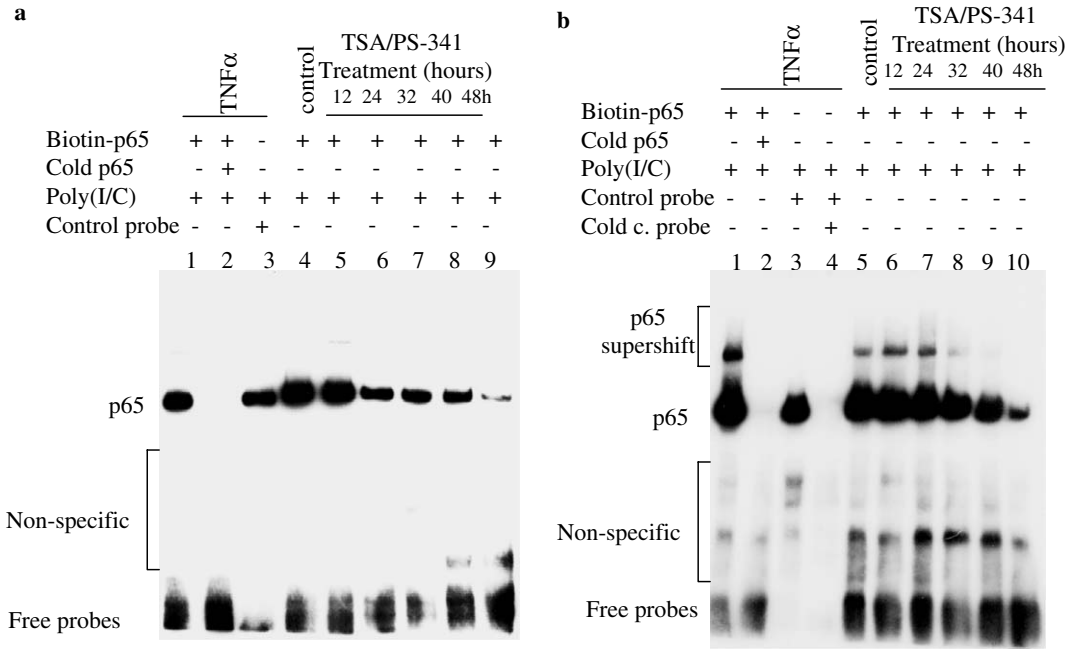

Fig. 3. Effects of TSA/PS-341 on nuclear NFκB binding activities. (a) Panc-1 cells were incubated with TSA (500 nM) and PS-341 (50 nM) combination. Nuclear extracts were prepared from treated cells at indicated time points. Protein sample from TNFα-treated cells served as a positive control. Protein sample from non-treated cells were utilized for comparison to determine whether the combined drug regimen affected nuclear NFκB binding activities. For each binding reaction, 10 μg nuclear proteins was incubated with biotinylated RelA oligonucleotide probe in the presence of Poly(I/C). To determine the binding specificity, 3-fold excessive cold RelA probes were used for competitive binding. An irrelevant control biotin-labeled oligonucleotide probe was used as a negative control. The binding complex was resolved by using PAGE/glycerol gel. Binding bands were developed using an EMSA kit from Panomics. (b) Anti-human RelA polyclonal antibodies were used to determine the specificity of nuclear proteins binding to biotin-labeled RelA probes (Materials and methods). The binding activities were analyzed similarly.

control probe (Fig. 3b, lane 3), indicating that nuclear proteins binding to the p65 probe were RelA specific. However, protein complex binding to biotin-p65 probe in lanes 9 and 10 (Fig. 3b) displayed no supershift. This phenomenon was likely caused by decreased RelA binding activities in these samples, and was consistent with the depletion of total RelA protein levels (S4d, lanes 6 and 7) at these time points. The time frame for the reduction of nuclear RelA binding activities coincided with the depletion of total cellular RelA and massive apoptosis in pancreatic cancer cells, suggesting that the suppression of NFκB signaling by TSA/PS-341 regimen might contribute to TSA/PS-341 triggered apoptosis.

The presence of combined caspase inhibitors (40 μM z-VAD-FMK and 10 μM z-DEVD-FMK) in the culture medium effectively blocked the enzyme activities of activated caspase-3 and -7 in TSA/PS-341 treated pancreatic cancer cells (S5c), but did not block RelA depletion (S5a and b), suggesting that PS-341/TSA-triggered downregulation of endogenous RelA was not a consequence of caspase activation.

The TSA/PS-341 treatment downregulated Bcl-XL

Bcl-XL is an NFκB dependent anti-apoptotic factor. The endogenous Bcl-XL is predominantly overexpressed in pancreatic cancer cells and plays a critical role in chemoresistance in pancreatic cancer according to our previous study [3]. To determine whether TSA and PS-341 alone or combination affected cellular Bcl-XL expression, we performed immunoblot assay following drug treatment of Panc-1 cells. TSA at 500 nM had no significant effect on Bcl-XL expression compared to control counterpart 48 h post drug treatment (Fig. 4a, lanes 1 and 3). PS-341 (50 nM) decreased Bcl-XL protein level (Fig. 4a, lane 2), but did not cause its depletion. Combining TSA with PS-341, however, caused Bcl-XL depletion (Fig. 4a, lane 4). When the Capan-2 and SU86 pancreatic cancer cell lines were treated under identical conditions, we obtained similar results (data not shown), suggesting that TSA/PS-341-triggered Bcl-XL depletion is not a random event. Fig. 4b (lanes 1–8) showed that Bcl-XL protein levels were decreased 24 h (Fig. 4b, lane 4) after treatment with the combined drug regimen and gradually disappeared at 40–48 h (Fig. 4b, lanes 6 and 7). The depletion of Bcl-XL proteins in cancer cells treated with the PS-341/TSA regimen was correlated with increased apoptosis and caspase activation as shown in relevant panels of Figs. 1, 2, S1–2. Blocking executioner caspase-3 and -7 activities using combined caspase inhibitors (S5c) upon the PS-341/TSA treatment merely slowed down, but did not block Bcl-XL depletion (S5a and b), suggesting that PS-341/TSA-triggered Bcl-XL downregulation was not a consequence of caspase-mediated protein degradation.

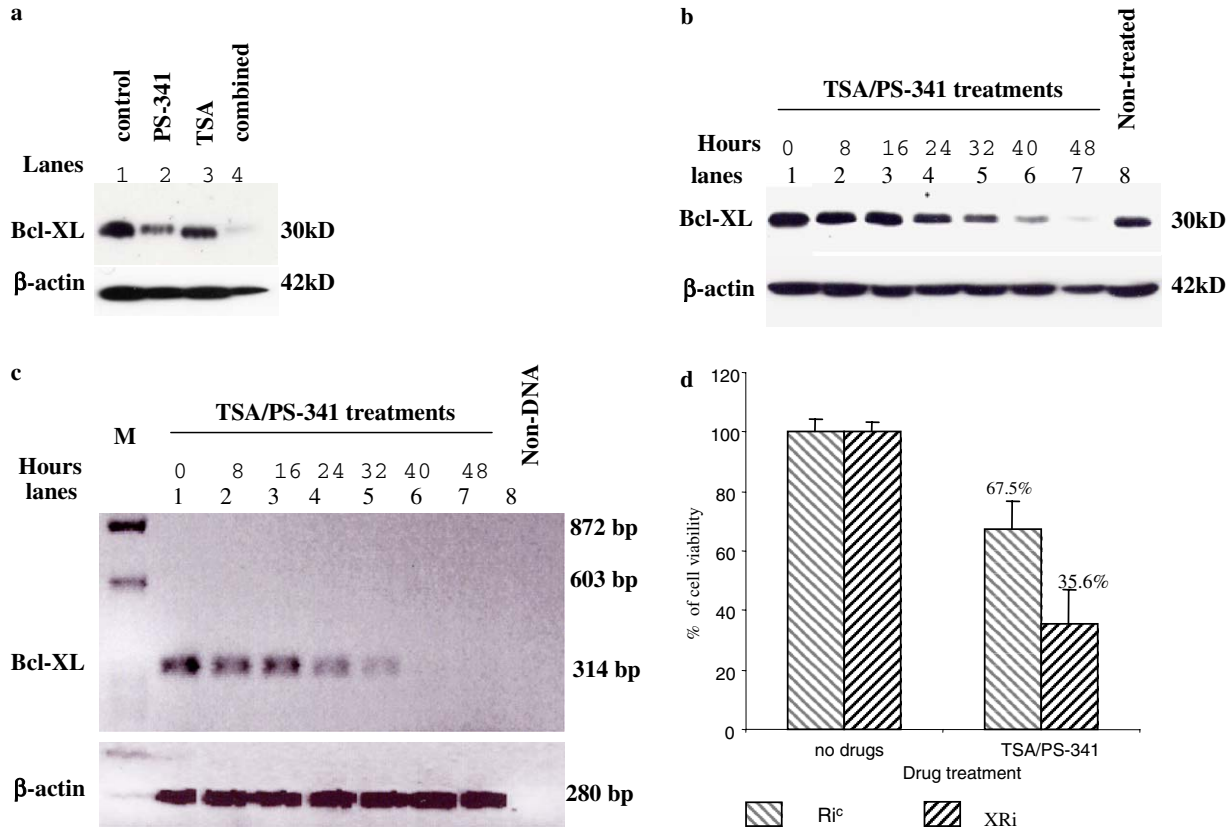

Fig. 4. Regulation of Bcl-XL expression by the TSA and PS-341 combinatorial therapy. (a) Panc-1 cells were incubated with PS-341 (50 nM), TSA (500 nM), and combination at 37 °C for 48 h. Bcl-XL protein levels were analyzed by immunoblot assays. (b) Protein samples were prepared at indicated time points for immunoblot assays with Bcl-XL antibodies. (c) Panc-1 cells in 6-cm dishes were incubated with TSA/PS-341. RNAs from drug-treated cells at indicated time points were assayed by RT-PCR with Bcl-XL specific DNA primers. Ten percentage of PCR products were analyzed using agarose gel electrophoresis. DNA bands followed by ethidium bromide staining and photography. β-Actin genes were amplified for internal cDNA synthesis control. (d) Panc-1 cells were transduced with Bcl-XL knockdown (XRi) and a control retroviral construct (Ri^C) [3]. Mixed cell populations of Bcl-XL knockdown or control cells sorted for eGFP marker were incubated with the PS-341 (10 nM)/TSA (250 nM) regimen (four wells each) 37 °C for 48 h. Cell viabilities were determined by MTS assays.

To further investigate whether the decrease of Bcl-XL protein levels was associated with downregulation of Bcl-XL gene transcription, we conducted a RT-PCR assay over a time course and analyzed the Bcl-XL mRNA levels following drug treatment (Fig. 4c, lanes 1–8). The transcription of Bcl-XL mRNA decreased at 24 h after TSA/PS-341 treatment and became undetectable at 40 h, whereas the mRNA level of housekeeping gene β-actin was unaffected. The time frame for the downregulation of Bcl-XL transcription was consistent with the reduction of Bcl-XL protein expression, and also coincided with significant decrease of nuclear NFκB RelA binding activities, suggesting that TSA/PS-341 triggered apoptosis in pancreatic cancer cells might be associated with the suppression of NFκB signaling and the depletion of the anti-apoptotic factor Bcl-XL (refer next section). In comparison, TSA alone showed no effects on Bcl-XL mRNA levels at similar time points according to RT-PCR assay (data not shown). PS-341 alone slightly decreased, but did not cause the depletion of Bcl-XL mRNA (data not shown).

Downregulation of the endogenous Bcl-XL increased the sensitivity of pancreatic cancer cells to TSA-PS-341 regimen

Pancreatic cancer cells overexpress Bcl-XL that blocks both intrinsic and extrinsic apoptosis pathways [3]. Treating pancreatic cancer cells with a combined drug regimen containing 50 nM PS-341 and 500 nM TSA caused Bcl-XL depletion (Fig. 4), resulting in high levels of apoptosis in pancreatic cancer cells (Fig. 1). We attempted to show if exogenous expression of Bcl-XL using an irrelevant foreign promoter, such as CMV or HSV TK promoters, could suppress TSA/PS-341 triggered apoptosis in pancreatic cancer cells. It was desirable that eukaryotic foreign promoters to be used would not be affected by these drugs. However, we found that TSA/PS-341 treatment suppressed CMV and TK promoter-driven reporter gene activities (data not shown), indicating that these promoters are not suitable for such tests. It is unknown which promoter is not affected by the TSA/PS-341 regime. We know that normal pancreatic tissues do not express Bcl-XL [3], and a

higher-dose TSA (500 nM) and PS-341 (50 nM) combinatorial regimen caused Bcl-XL depletion. Treating pancreatic cancer cells with a lower dose of the combined regimen (10 nM PS-341 and 250 nM TSA) only slightly reduced Bcl-XL protein levels and induced a low level of apoptosis (data now shown). To investigate whether the endogenous Bcl-XL overexpression confers on resistance to PS-341/TSA-triggered apoptosis, we created Bcl-XL depleted pancreatic cancer cells using retrovirus-based RNA interference as described in our previous report [3]. We incubated Bcl-XL knockdown pancreatic cancer cells (XRi) and control counterparts (Ri^c) with PS-341 (10 nM) and TSA (250 nM) combination for 48 h to determine the cell viability (Fig. 4d). This regimen killed 64.4% of Bcl-XL depleted cancer cells compared to control counterparts (32.5%, $P < 0.02$), indicating that the Bcl-XL overexpression indeed played a role in resistance to TSA/PS-341 induced killing, which suggested that Bcl-XL depletion following a higher dose of PS-341 (50 nM)/TSA (500 nM) treatment might partly contribute to the onset of apoptosis in pancreatic cancer cells. Additionally, we found that pancreatic cancer cell lines overexpressed ERK1/2 (S5a). The TSA/PS-341 treatment also inactivated Ras-MAP kinase pathway by depleting several key components of MAP kinase cascades, including K-Ras, MEK1/2, phosphorylated MEK, and ERK1/2 (S6b and c).

Conclusions

PS-341 and TSA combinatorial chemotherapy inactivates NF-κB signaling, downregulates anti-apoptotic factor Bcl-XL, disrupts the ERK MAP kinase pathway, and effectively induces apoptosis in diverse pancreatic cancer cell lines. It may represent a potential novel therapeutic strategy for pancreatic cancer.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bbrc.2006.07.185.

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