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Withaferin A targets heat shock protein 90 in pancreatic cancer cells

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ABSTRACT

The purpose of this study is to investigate the efficacy and the mechanism of Hsp90 inhibition of Withaferin A (WA), a steroidal lactone occurring in *Withania somnifera*, in pancreatic cancer *in vitro* and *in vivo*. Withaferin A exhibited potent antiproliferative activity against pancreatic cancer cells *in vitro* (with IC_{50}s of 1.24, 2.93 and 2.78 μM) in pancreatic cancer cell lines Panc-1, MiaPaCa2 and BxPc3, respectively. Annexin V staining showed that WA induced significant apoptosis in Panc-1 cells in a dose-dependent manner. Western blotting demonstrated that WA inhibited Hsp90 chaperone activity to induce degradation of Hsp90 client proteins (Akt, Cdk4 and glucocorticoid receptor), which was reversed by the proteasomal inhibitor, MG132. WA-biotin pull down assay of Hsp90 using Panc-1 cancer cell lysates and purified Hsp90 showed that WA-biotin binds to C-terminus of Hsp90 which was competitively blocked by unlabeled WA. Co-immunoprecipitation exhibited that WA (10 μM) disrupted Hsp90–Cdc37 complexes from 1 to 24 h post-treatment, while it neither blocked ATP binding to Hsp90, nor changed Hsp90–P23 association. WA (3, 6 mg/kg) inhibited tumor growth in pancreatic Panc-1 xenografts by 30% and 58%, respectively. These data demonstrate that Withaferin A binds Hsp90, inhibits Hsp90 chaperone activity through an ATP-independent mechanism, results in Hsp90 client protein degradation, and exhibits *in vivo* anticancer activity against pancreatic cancer.

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1. Introduction

Pancreatic cancer is the fourth leading cause of cancer deaths in the United States [1,2] with 1- and 5-year survival of 23% and 4% [1]. The mortality rates associated with pancreatic cancer are almost equal to its incidence rates. The underlying mechanism of pancreatic tumor formation is rather complex. A number of biochemical and genetic abnormalities have been reported, which include mutations or over-expression of oncogenes (such as *KRAS*, *ERBB2*, and *AKT*) and tumor-suppressor genes (such as *P53*, *BRCA2*) [3–6]. In addition, over-expression of growth factors and their receptors, such as TGF-beta, VEGF, and EGFR [6], have also been linked to pancreatic cancer.

The treatment regimens for pancreatic cancer have no substantial improvement over the past few decades [7]. Currently, surgery is the main therapeutic option since chemotherapy and radiation only achieve minimal effects due to rapid progression, late diagnosis, and drug resistance of pancreatic cancer [1]. Unfortunately, only 15–20% of pancreatic cancer patients are amenable to curative resection while 80% of patients generally have nonresectable advanced or metastatic tumors [8]. Furthermore, even in patients with resectable disease, the overall 5-year survival is 15%. Currently, gemcitabine is the standard therapeutic drug for treatment of pancreatic cancer. However, it only improves the disease symptoms with no significant survival benefits. Thus, novel agents for prevention and treatment of pancreatic cancers are highly desired.

Natural products appear to be promising sources of drugs for cancer treatment [9]. Withaferin A (WA), a major active constituent purified from the Indian medicinal plant *Withania somnifera*, was shown to have antitumor, antiangiogenesis and radiosensitizing activity [10,11]. The anticancer activity of Withaferin A has been demonstrated in prostate cancer cells [12,13], breast cancer cells [14], leukemia cells [15], and melanoma cells [16]. It was shown that WA inhibits nuclear factor-κB (NF-κB) activation [11], induces apoptosis in prostate cancer cells through Par-4 induction [12], inhibits IκB kinase activation via a thioalkylation-sensitive redox mechanism [17], inhibits the chymotrypsin-like activity of proteasome [13], and targets the intermediate filament protein vimentin by covalently modifying the cysteine

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residue [18]. In addition, WA also targets Annexin II to induce Actin microfilament aggregation [19].

In our preliminary study, we found that Withaferin A (WA) exhibited Hsp90 inhibition characteristics in pancreatic cancer cells by decreasing the levels of Hsp90 client proteins. Thus we intend to investigate the efficacy of WA and Hsp90 inhibition mechanisms in this study. Hsp90 is a molecular chaperone which mediates the folding, assembly, and maturation of many client proteins, including HER-2, EGFR, Akt, Raf-1, Cdk4, mutated P53, which are directly involved in the malignancy [20]. Hsp90 has three distinct domains. The N-terminal domain has the ATP binding site, the middle domain may interact with clients, and the C-terminal domain is responsible for dimerization of Hsp90. An additional ATP binding site is also found to be present in the C-terminus. The ATP binding sites act as a conformational switch to regulate Hsp90 chaperone activity [21]. In cancer cells, the newly synthesized oncogenic client proteins bind to Hsp90 to form an intermediate complex with other co-chaperones (such as Cdc37, Hop, Hsp70, Hsp40, and Hip). Upon ATP binding to Hsp90, client proteins and Hsp90 form a mature complex. This mature super-chaperone complex catalyzes the conformational maturation of client proteins [22]. These oncogenic client proteins stimulate cancer cell proliferation and survival. Several Hsp90 inhibitors, which block the ATP binding sites of Hsp90, have been developed and tested in preclinical and clinical models for their anticancer activity [23–26]. Geldanamycin (GA, and its derivatives 17-AAG, IPI-504, 17-DMAG), radicicol and derivatives, purines and derivatives, pyrazoles and isoxazoles, sulfanyl analogues, resorcinol-bearing compounds, and 2-aminopyrimidine-bearing derivatives block the N-terminal ATP binding pocket, whereas novobiocin (and its derivatives) and cisplatin block the C-terminal ATP binding pocket [23,27–31]. To date, many of these Hsp90 inhibitors have entered preclinical or phase I/II clinical studies [24,25,32,33].

In this study, we investigate the efficacy and mechanism of Withaferin A (WA) for Hsp90 inhibition and its use against pancreatic cancer. Our data suggest that Withaferin A exhibits potent cytotoxicity against pancreatic cancer cells both *in vitro* and *in vivo* xenograft models. The anticancer activity of WA is partially due to its direct binding to Hsp90 C-terminus, and inhibiting Hsp90 chaperone activity, inducing Hsp90 client protein degradation through an ATP-independent mechanism.

2. Materials and methods

2.1. Cell culture and reagents

Human pancreatic cancer cell lines Panc-1, BxPc3 and MiaPaCa2 were cultured in 10% FBS RPMI-1640 or 10% FBS DMEM at 37 °C and 5% CO_2. Withaferin A was purchased from Calbiochem, Inc. (San Diego, CA). The following antibodies were used for Western blot: Akt (Cell Signaling, Beverly, MA), Hsp70 and Hop (StressGen, Victoria, BC, Canada), Cdk4, Cdc37 and Hsp90 (Santa Cruz, Santa Cruz, CA), Actin and P23 (Abcam, Cambridge, MA). Monoclonal Hsp90 antibody H9010 for immunoprecipitation was purchased from Alexis Biochemicals (San Diego, CA), and purified Hsp90β protein for ATP binding assay was a kind gift of Dr. David Toft (Mayo Clinic, Rochester, MN).

2.2. MTS assay

Pancreatic cancer cells were seeded in 96-well plates at a density of 5000 cells per well. 24 h later the cells were treated with increasing concentrations of WA as indicated. MTS assay was performed to assess cell viability after 48 h incubation. The IC_{50} value for cytotoxicity was estimated by WinNonlin software (Pharsight, Mountain View, CA).

2.3. Apoptosis study

The Annexin V-EGFP Apoptosis Detection Kit was purchased from BioVision Research Products (Mountain View, CA) and used as recommended by manufacturer. Human pancreatic Panc-1 cells were treated with 1, 5, and 10 μM WA for 12 h, and stained with Annexin V-EGFP to analyze the phosphoserine inversion. Early apoptotic cells were observed with a fluorescence microscopy.

2.4. Withaferin A-biotin pull down assay

Withaferin A-biotin (WA-biotin) was prepared and used in the pull down assay as described previously [19]. Briefly, 500 μg of Panc-1 pancreatic cancer cell whole cell extracts or 5 μg of purified human Hsp90β, N-terminus Hsp90β, C-terminus Hsp90β and yeast Hsp90 were incubated with immobilized WA-biotin for 2 h at 4 °C in TNEK buffer (5 mM Tris, pH 7.4; NP-40 1%; EDTA 2 mM; KCl 200 mM) supplemented with protease inhibitors. To perform competition assay, the samples were preincubated with 100 μM WA for 1 h before add with WA-biotin. The beads were then washed with TNEK buffer for three times, and were boiled in loading buffer for 4 min to isolate the bound proteins. Western blot was carried out to analyze the levels of Hsp90 proteins.

2.5. ATP-sepharose binding assay

The assay was performed as previously described [34,35]. Total of 5 μg of human hsp90β protein with DMSO, WA or 17-AAG were incubated on ice in 200 μl incubation buffer consisting of 10 mM Tris–HCl, 50 mM KCl, 5 mM $MgCl_2$, 2 mM DTT, 20 mM Na_2MoO_4, 0.01% Nonidet P-40, pH 7.5. After 30 min, 25 μl of pre-equilibrated γ-phosphate-linked ATP-sepharose (Jena Bioscience GmbH, Jena, Germany) was added to tubes, which were then incubated at 37 °C for another 30 min with frequent mixing to resuspend the resin. Following incubation, the sepharose was washed, pelleted and analyzed by SDS-PAGE.

2.6. Co-immunoprecipitation and Western blotting assay

The general procedure for co-immunoprecipitation was described as follows. 500 μg of whole cell extracts was incubated with 5 μl H9010 anti-Hsp90 antibody or anti-P23 antibody for 1 h at 4 °C, rotating. 30 μl protein G agarose (Pierce, Rockford, IL) was added to each sample, and incubated for another 2 h at 4 °C. The beads were washed three times with PBS plus protease inhibitors. The beads were boiled in loading buffer for 4 min to isolate the bound proteins. Western blot was carried out to analyze the levels of coimmunoprecipitated proteins. Western blot was performed as previously described [35,36]. Non-reducing SDS-PAGE was used to analyze the disulfide-bonded protein as described previously [37]. Isolation of triton-soluble and triton-insoluble proteins was performed as described by Chen et al. [37].

2.7. Real-time PCR assay

RT-PCR is carried out as described previously [36]. Briefly, Panc-1 cancer cells are treated with 5 μM WA for 12 h. TRIzol reagents (Invitrogen, Carlsbad, CA) are used to extract total cellular RNAs as described in protocol provided by manufacturer. Superscript III first strand synthesis kit from Invitrogen is used to reverse transcribe the cDNA. Then the real-time PCR is carried out in ABI PRISM 7900T real-time PCR system (Perkin-Elmer, Branchburg, NJ) with SYBR Green PCR Master Mix (Applied Biosystems, Foster City, CA). The primers used in RT-PCR are as follows: Akt, forward, 5′-TCT ATG GCG CTG AGA TTG TG-3′, reverse, 5′-CTT AAT GTG CCC GTC CTT GT-3′; Cdk4, forward,

5′-GAA ACT CTG AAG CCG ACC AG-3′, reverse, 5′-GCC CTC TCA GTG TCC AGA AG-3′; glucocorticoid receptor (GR), forward, 5′-GAG AGG GGA GAT GTG ATG GA-3′, reverse, 5′-GTT TTC ACT TGG GGC AGT GT-3′. Internal standard β-actin, forward, 5′-GCT CGT CGT CGA CAA CGG CTC-3′; reverse, 5′-CAA ACA TGC TCT GGG TCA TCT TCT C-3′. mRNA levels are calculated as fold change of control. After completion of the RT-PCR, Ct values (cycle numbers in which signal intensity equal to the threshold value) will be obtained from the software. For each samples, ΔCt is calculated as $\Delta Ct = Ct_{Akt/Cdk4/GR} - Ct_{actin}$. Then ΔΔCt is calculated as $\Delta\Delta Ct = \Delta Ct_{treatment} - \Delta Ct_{control}$. The fold change of the Akt/Cdk4/GR mRNA levels relative to control group is calculated as $2^{-\Delta\Delta Ct}$.

2.8. Pancreatic tumor xenograft

The pancreatic tumor xenograft mouse model was used to test anticancer activity of WA similar to previous reports [35,36]. Briefly, 4–6-week-old nu/nu athymic female mice were obtained from Charles River Laboratories (Charles River, Wilmington, MA). Pancreatic cancer Panc-1 cells ($5–10 \times 10^6$) were mixed with reconstituted basement membrane (Collaborative Research, Bedford, MA) and inoculated s.c. to the right and left flanks of the mice. When the tumors became palpable ($\sim 100\ mm^3$), mice were randomly divided into different groups for treatment ($n = 6$/group). WA was dissolved in the vehicle (10% DMSO, 40% Cremophor/ethanol (3:1), and 50% PBS) [13], and administered at 6 or 3 mg/kg by i.p. injection for two continuous days. Then the dosing schedule was changed to two injections per week for 4 weeks. Tumor sizes and body weights were measured twice a week. After 30 days' drug treatment, and tumor sizes and body weights were monitored until 70 days.

3. Results

3.1. Withaferin A inhibits proliferation in pancreatic cancer cells

The antiproliferative effect of WA (Fig. 1A) against human pancreatic cancer cell lines was examined by MTS assay. A 48-h exposure to different concentrations of WA induced a dose-dependent inhibition in cell proliferation. WA exhibited high cytotoxicity against Panc-1 cells with an IC_{50} of 1.24 μM (Fig. 1B), whereas, WA showed relatively lower cytotoxicity against MiaPaCa2 and BxPc3, with IC_{50}s of 2.93 μM and 2.78 μM (Fig. 1C and D). Overall, WA showed potent antiproliferative effect against these three human pancreatic cancer cell lines.

3.2. Withaferin A induces apoptosis in pancreatic cancer cells

To illustrate that WA induces apoptosis in pancreatic cancer cells, Annexin V staining was conducted in Panc-1 cells with WA treatment. As shown in Fig. 2, the Annexin V positive staining cells accounted for 18.5 ± 1.68, 46.8 ± 5.22 and 68.1 ± 7.14 of the overall cell population in Panc-1 cells treated with 1, 5, and 10 μM WA for 12 h, respectively. In contrast, only marginal apoptotic cells were observed in control Panc-1 cells.

3.3. Withaferin A induces Hsp90 client protein degradation

WA exhibited potent cytotoxicity against pancreatic cancer cells and induced apoptosis in Panc-1 cells. To investigate the underlying mechanism, we screened a panel of protein level changes in Panc-1 cells in response to WA treatment. Hsp90 client proteins (Akt, Cdk4 and Glucocorticoid receptor (GR)) were observed to be decreased. These proteins exhibited time- and dose-dependent degradation in response to WA treatment (Fig. 3A and B). After exposure to 10 μM WA for 6 h, Akt and Cdk4 protein levels started to decrease by 1.88- and 1.95-fold. After 24 h treatment, the protein levels were undetectable. GR protein levels decreased even faster and became undetectable as early as 2 h after exposure to 10 μM WA (Fig. 3C). Previous studies have shown that these proteins are clients of Hsp90, and inhibiting Hsp90 chaperone activity would lead to the degradation of these proteins [35,38]. These data suggest that inhibition of Hsp90 chaperone activity might contribute to the anticancer activity of WA.

To further confirm inhibition of Hsp90 by WA, we examined two additional protein expression level changes, Hsp70 and Cdk2.

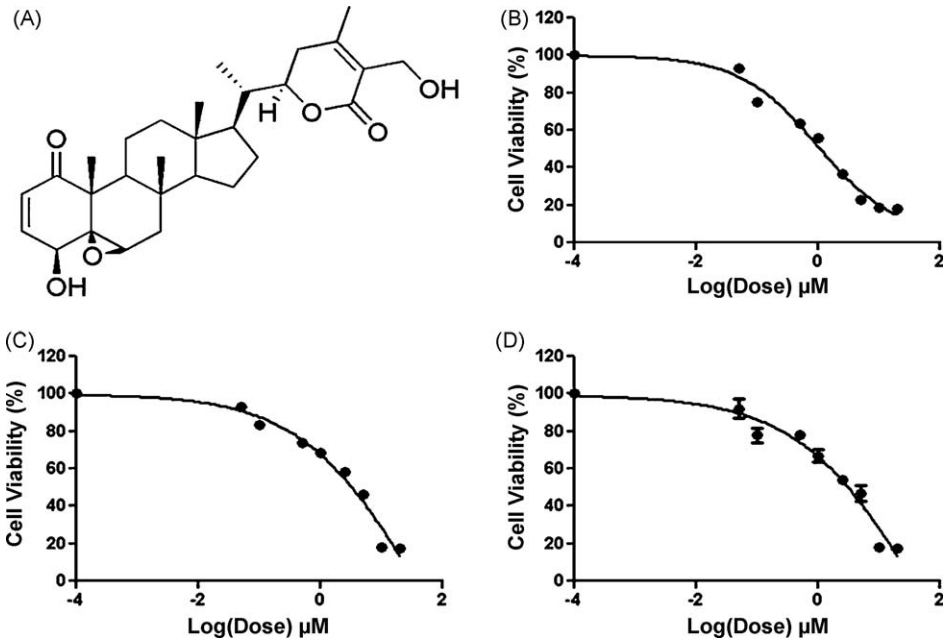

Fig. 1. (A) chemical structure of WA. (B) Effect of WA treatment on viability of Panc-1 cells. Panc-1 cells were seeded in 96-well plates at a density of 5000 cells per well. 24 h later the cells were subjected to WA treatment with concentrations of 0.05, 0.1, 0.5, 1.0, 2.5, 5, 10, and 20 μM. MTS assay was performed to assess cell viability after 48 h incubation. (C) Effect of WA treatment on viability of MiaPaCa2 cells. Viability of MiaPaCa2 cells after WA treatment was assessed similar to (B). (D) Effect of WA treatment on viability of BxPc3 cells. Viability of BxPc3 cells after WA treatment was assessed similar to (B).

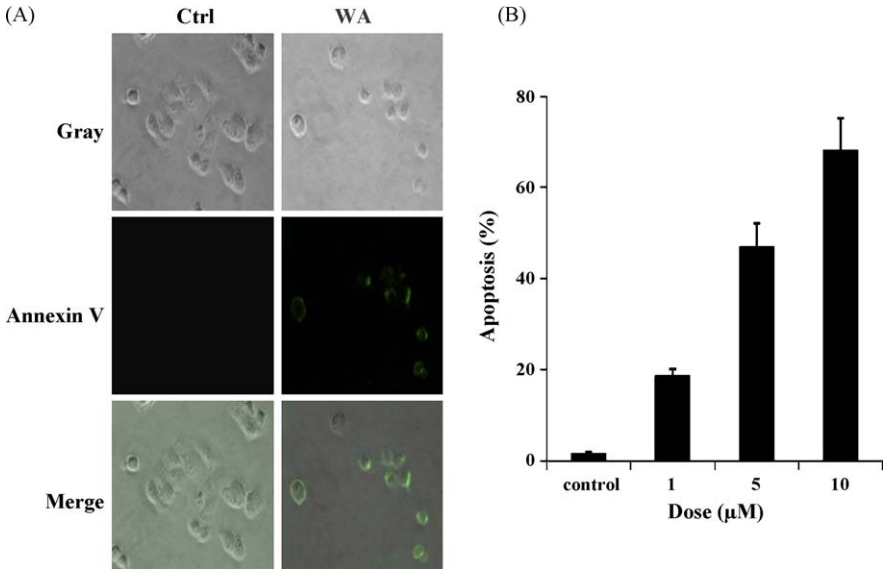

Fig. 2. WA induces apoptosis in Panc-1 cells as determined by Annexin V staining. (A) Representative images from one of four independent experiments. Panc-1 cells were treated with 1, 5, and 10 μM WA for 12 h. Cells were stained with Annexin V-EGFP (green). Apoptotic cells were observed under fluorescent microscope. (B) Quantification of WA induced apoptotic cells. The percentage of apoptotic cells was calculated as Annexin-EGFP positive cells divided by the total cancer cells.

The induction of Hsp70 is another molecular signature in response to Hsp90 inhibition [39]. As shown in Fig. 3D, 10 μM WA increased the protein level of Hsp70 by 13.47-fold after 6 h while without affecting the Hsp90 protein level. To demonstrate WA specifically inhibits Hsp90, a non-Hsp90 client protein Cdk2 was examined. Indeed, Cdk2 levels were not significantly altered after WA treatment (Fig. 3E). These data demonstrate that WA inhibited Hsp90 chaperone activity.

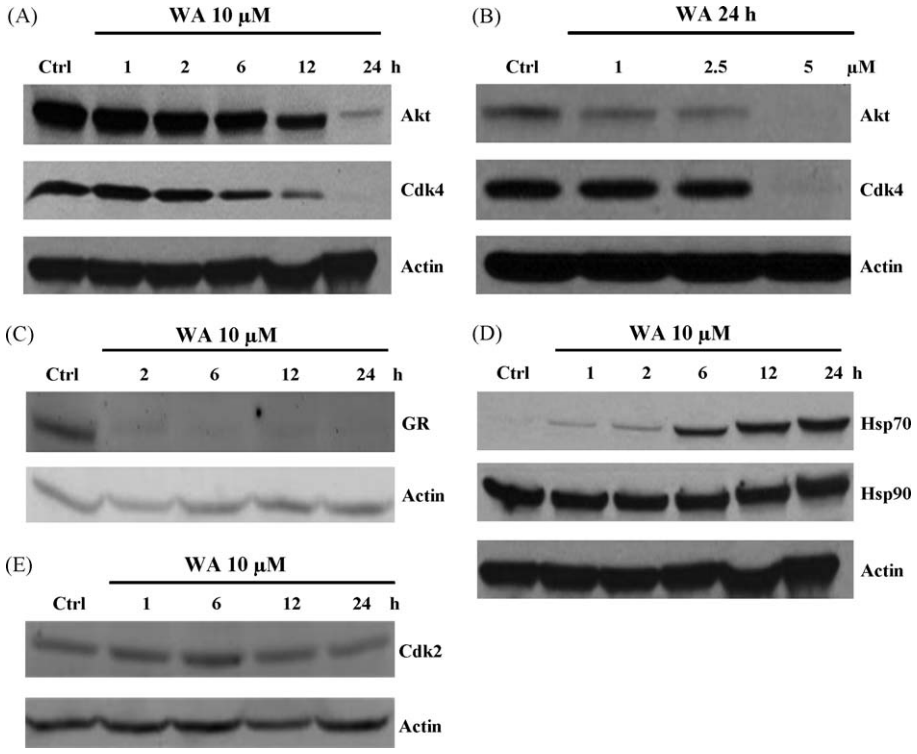

Fig. 3. Protein level changes after WA treatment in Panc-1 cells. (A and B) WA induces Hsp90 client protein Akt and Cdk4 degradation in Panc-1 cells in a time- and dose-dependent manner. Panc-1 cells were treated with different concentrations of WA for different times. Cell lysates (50 μg protein in each lane) were analyzed by Western blot analysis with specific antibodies to Akt, Cdk4 and Actin. Actin was served as internal standard. (C) WA induces Hsp90 client protein GR degradation. Panc-1 cells were treated with 10 μM WA for different times. Cell lysates (50 μg protein in each lane) were analyzed by Western blot analysis with specific antibodies to GR and Actin. Actin was served as internal standard. (D) WA induces expression of Hsp70. Panc-1 cells were treated with 10 μM WA for different times. Cell lysates (50 μg protein in each lane) were analyzed by Western blot analysis with specific antibodies to Hsp70 and Actin. Actin was served as internal standard. (E) WA does not change the Cdk2 protein level. Panc-1 cells were treated with 10 μM WA for different times. Cell lysates (50 μg protein in each lane) were analyzed by Western blot analysis with specific antibodies to Cdk2 and Actin. Actin was served as internal standard.

3.4. Withaferin A directly binds to Hsp90

Previous study reported that Hsp90 is sensitive to cellular redox conditions and tend to form disulfide bond under oxidative stress [37,40]. In addition, susceptible cysteine residues in C-terminal Hsp90 were revealed including Cys521, Cys589/590 and Cys597 [41,42]. WA was demonstrated to be highly reactive with cysteine residues in proteins [43,44], such as Annexin II [19] and vimentin [18] to form covalent bonds. Therefore, we tested whether WA will bind to Hsp90 using WA-biotin pull down assay. The results show that WA-biotin successfully pulled down Hsp90 both from the cell lysate and purified full length human hsp90 (Fig. 4A). To further illustrate the binding domains of Hsp90 with WA-biotin, we performed the pull down assay against Hsp90 fragments N-terminus Hsp90 (without cysteine residue), C-terminus Hsp90 (with cysteine residue), and full length yeast Hsp90 (without cysteine residues). As shown in Fig. 4A, WA-biotin can only pull down C-terminus Hsp90 (with cysteine residues), but not N-terminus Hsp90 nor yeast Hsp90. Hence, WA-biotin binding to Hsp90 is dependent on cysteine residues on Hsp90. In addition, the WA-biotin binding to Hsp90 was in a competitive manner since 100 μM unlabeled WA preincubation for 1 h would significantly decrease the WA-biotin binding to Hsp90 both in cell lysate and purified full length Hsp90 as well as C-terminus Hsp90 (Fig. 4B).

Next we examined the aggregation of Hsp90 after WA treatment. Non-reducing gel electrophoresis was performed to detect the formation of Hsp90 aggregation, which would exhibit a slower migration pattern and appeared as higher molecular weight bands. As shown in Fig. 4C, WA induced Hsp90 aggregation in a dose-dependent manner.

3.5. Withaferin A induces Hsp90 client protein degradation through proteasome

Since classical Hsp90 inhibitor induced Hsp90 client protein degradation was proteasome mediated, we further investigated whether WA induced Hsp90 client protein degradation was also proteasome-dependent. Two proteasome inhibitors Bortezomib and MG132 were used to reverse the protein degradation. As shown in Fig. 5A, preincubation with 10 μM Bortezomib and MG132 could rescue the degradation of Hsp90 client proteins. All of the three Hsp90 client proteins (Akt, Cdk4, and GR) under investigation were found to accumulate in the triton-insoluble fraction after combination treatment of WA and proteasomal inhibitors, while WA alone decreased the levels of Akt, Cdk4 and GR. In addition, we carried out RT-PCR to examine whether WA affects the mRNA levels of these three genes. Fig. 5B shows that WA treatment did not change the mRNA levels of these three genes. These data suggest that WA induced Hsp90 client protein degradation is proteasome-dependent and WA did not affect the transcriptional level of Hsp90 client proteins.

3.6. Withaferin A does not block ATP binding to Hsp90

Most of current Hsp90 inhibitors, including geldanamycin, 17-AAG, IPI-504 and 17-DMAG, bind to the Hsp90 ATP binding pocket which prevents client protein refolding [23,27]. To investigate whether WA also change the ATP binding to Hsp90, an ATP-sepharose beads pull down assay was performed. As shown in Fig. 6, 5 μM 17-AAG completely blocked ATP beads binding to Hsp90; in contrast, 5, 10, and 20 μM WA did not block ATP beads

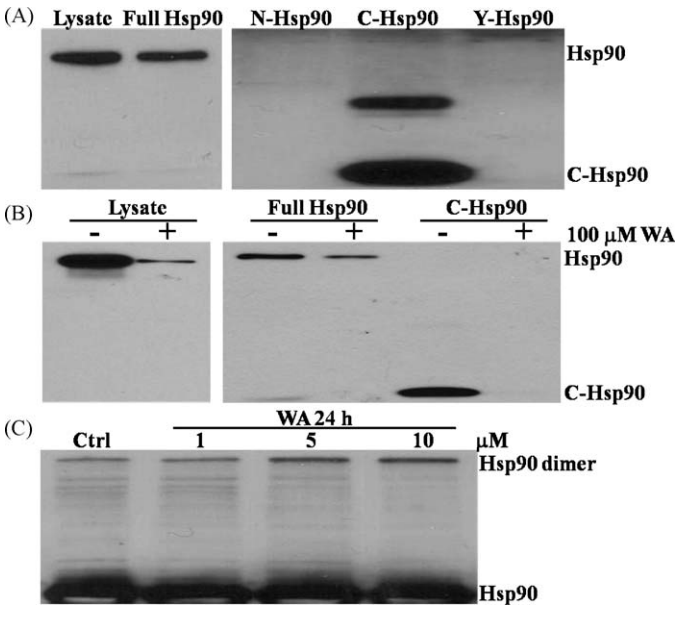

Fig. 4. (A) WA-biotin Hsp90 pull down assay. 1 mg of Panc-1 cell lysates (lysate), 5 μg of purified full length human Hsp90β (Full Hsp90), 5 μg of N-terminus human Hsp90β (N-Hsp90), 5 μg of C-terminus human Hsp90β (C-Hsp90) and 5 μg of yeast Hsp90 (Y-Hsp90) were used to carry out the WA-biotin pull down assay. The WA-biotin pull down protein was subjected to Western blot analysis with specific antibodies to Hsp90. (B) WA competes with WA-biotin binding to Hsp90. The samples (Lysate, Full Hsp90 and C-Hsp90) were preincubated with 100 μM WA for 1 h before subject to WA-biotin binding assay. The WA-biotin pull down protein were subjected to Western blot analysis with specific antibodies to Hsp90. (C) WA induces Hsp90 aggregation in a dose-dependent manner. Panc-1 cells were treated with 1, 5 and 10 μM WA for 24 h. Cell lysates (50 μg protein in each lane) were subjected to non-reducing gel electrophoresis and then analyzed by Western blot with specific antibodies to Hsp90.

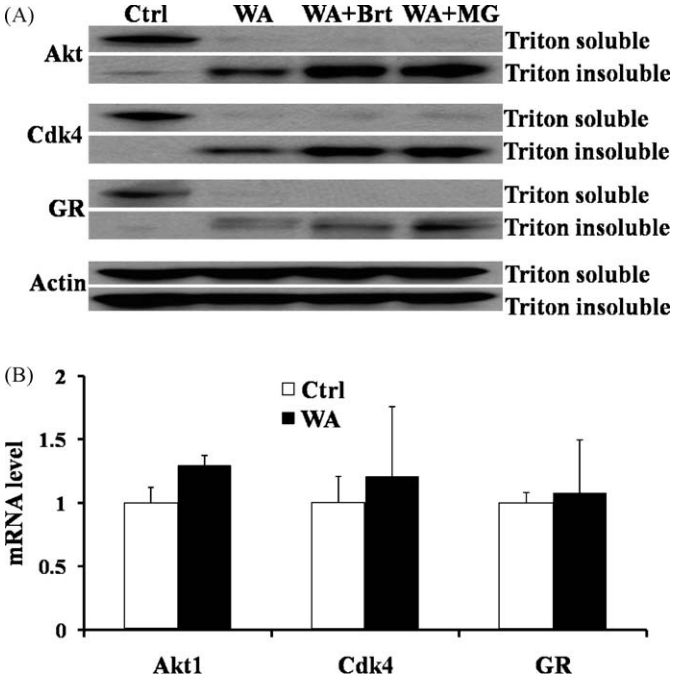

Fig. 5. (A) WA induced Hsp90 client protein degradation is proteasome-dependent. Panc-1 cells were preincubated with 10 μM Bortezomib (Brt) or MG132 (MG) for 1 h, and then were treated with 5 μM WA for another 12 h. Cells were collected and proteins were isolated as triton-soluble part and triton-insoluble part. Proteins (both triton-soluble and triton-insoluble parts) were subjected to Western blot analysis with specific antibodies to Akt, Cdk4, GR and Actin. Actin was served as internal standard. (B) mRNA levels of Hsp90 client protein in Panc-1 cells after WA treatment. Panc-1 cells were treated with 5 μM WA for 12 h, and the total mRNAs were isolated. RT-PCR was carried out to examine the mRNA levels of Akt, Cdk4 and GR using the specific primers of Akt, Cdk4, GR and Actin. Actin was served as internal standard.

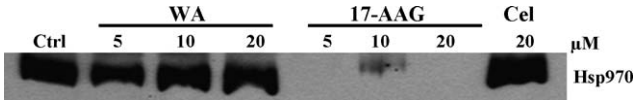

Fig. 6. WA does not inhibit ATP binding to Hsp90. 5 μg of purified human Hsp90β was incubated with WA, 17-AAG and Celastrol (Cel) for 30 min. 25 μl ATP-sepharose beads were added to the samples to pull down Hsp90. Western blot was used to detect Hsp90 using specific antibody to Hsp90.

binding to Hsp90. As a negative control, 20 μM Celastrol did not block ATP beads binding to Hsp90, which is consistent with previous study [35].

3.7. Withaferin A dissociates Hsp90–Cdc37 complex in pancreatic cancer cells

Since Hsp90 forms a superchaperone complex with other cochaporones, including Hsp70, P23, Cdc37, Hop and immunophilins, we further tested the alteration of Hsp90 superchaperone complexes in response to WA treatment by using co-immunoprecipitation (coIP) assay in Panc-1 cells. Panc-1 cells were treated with 10 μM WA for 1, 6, 12, and 24 h, coIP Hsp90 was carried out. The coIP samples were then immunobloted with anti-Cdc37 antibody. Fig. 7A shows that 10 μM WA completely disrupted the Hsp90–Cdc37 complex as early as 1 h post-treatment. In contrast, the Hsp90–Hop complex was not decreased by WA treatment. Fig. 7B shows the dose-dependence of WA on disrupting the Hsp90–Cdc37 complex. After exposure to WA for 24 h, 1 μM WA decreased the amount of Cdc37 by 2.04-fold, which was pulled down by Hsp90. WA (5 μM) completely blocked the Hsp90–Cdc37 complex. To investigate whether the decreased levels of Cdc37 in the coIP results were due to the expression level alteration of Cdc37, Western blotting was carried out to examine the protein level of Cdc37 without coIP. Fig. 7C shows that WA did not change the Cdc37 protein level. These data further confirmed that the decreased level of Cdc37 by WA in coIP Hsp90 samples was due to the dissociation of Hsp90–Cdc37 complex.

P23 has been demonstrated to bind directly to Hsp90 when it is in the ATP bound conformation [45]. Classical Hsp90 inhibitors like geldanamycin (GA) and PU24FCl bind to the ATP binding pocket and lock the Hsp90 in the intermediate superchaperone complex [46,47], hence Hsp90 will no longer be available to bind to P23. As shown in Fig. 7D, coIP with P23 showed that WA did not change Hsp90–P23 association, resulting in equal amount of Hsp90 pulled down by P23. In contrast, 17-AAG decreased the Hsp90–P23 interaction dramatically, which was consistent with ATP binding assay (Fig. 7D).

3.8. Withaferin A exhibits anticancer activity in pancreatic cancer xenografts

The data described above showed that WA is an Hsp90 inhibitor by binding to Hsp90, which resulted in Hsp90 client protein degradation and apoptosis in pancreatic cancer cells *in vitro*. We next examined WA's therapeutic efficacy *in vivo*. Pancreatic cancer (Panc-1) xenografts were generated in female nude mice. When the tumors reached 100 mm^3, the mice were randomly divided into three groups ($n = 6$). The mice were treated i.p. with either vehicle control or WA at 3.0 or 6.0 mg/kg. After 70 days, control tumors grew to an average size of 1014 ± 176 mm^3. In contrast, tumors from 3.0 to 6.0 mg/kg WA treatment group grew to an average size of 701 ± 268 and 422 ± 95 mm^3, corresponding to 30% and 58% inhibition, respectively ($P < 0.001$; Fig. 8A). Systemic toxicity of WA was analyzed by measuring the animal weights. As shown in Fig. 8B, the higher dose (6 mg/kg) WA treatment group had negligible weight loss (<10%) during the first week treatment, regained their weight from the second week, and had a 12% increase in body weight increase after 70 days. Meanwhile, mice in control group and the 3 mg/kg WA treatment group did not display weight loss. These data demonstrated WA shows potential anticancer effect against pancreatic cancer *in vivo* without significant toxicity.

4. Discussion

In the present study, we evaluated the anticancer efficacy of WA against pancreatic cancer. WA treatment significantly induced antiproliferative effects against Panc-1, BxPc3 and MiaPaCa2 pancreatic cancer cells in cell culture and exhibited potent tumor

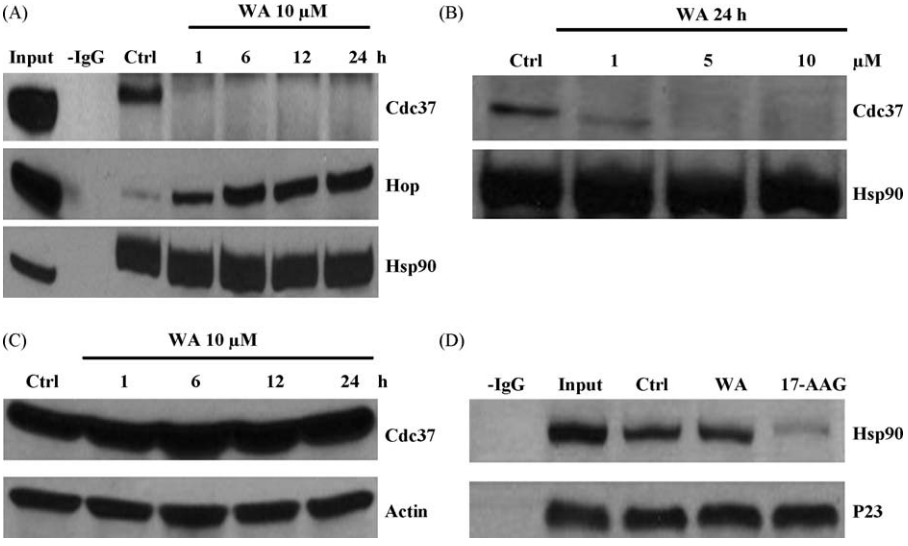

Fig. 7. WA disrupts Hsp90–Cdc37 complex in Panc-1 cells. (A and B) Co-immunoprecipitation (coIP) Hsp90. Cell lysates (500 μg total protein) were immunoprecipitated with Hsp90 antibody. Western blot was performed to detect Cdc37, Hop and Hsp90 using specific antibodies to Cdc37, Hop and Hsp90. (A) Panc-1 cells were treated with 10 μM WA for 0 to 24 h. (B) Panc-1 cells were treated with 1, 5, and 10 μM WA for 24 h. Input, total cell lysate; IgG, without adding antibody. (C) Western blot analysis of Cdc37 expression level. Panc-1 cells were treated with 10 μM WA for different times. Cell lysates (50 μg protein in each lane) were analyzed by Western blot with specific antibodies to Cdc37 and Actin. Actin was served as internal standard. (D) CoIP P23. Cell lysates (500 μg total protein) were immunoprecipitated with P23 antibody, then Western blot was performed to detect Hsp90 and P23 using specific antibodies to Hsp90 and P23.

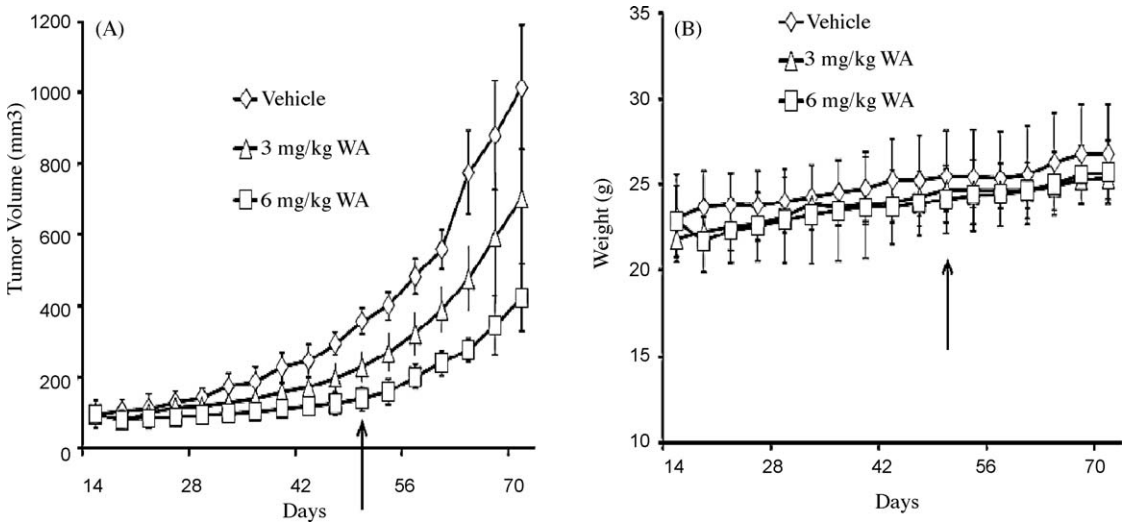

Fig. 8. Antitumor effect of WA in Panc-1 xenografts. (A) Tumor growth curves. The pancreatic tumor xenograft mouse model was generated by injecting the Panc-1 cancer cells s.c. to the right and left flanks of the nude mice. When the tumors reached 100 mm^3, mice were divided randomly into three groups (n = 6/group) to receive vehicle, 3 or 6 mg/kg WA treatment as scheduled. Tumor sizes and body weights were measured twice a week. Drug treatment was stopped after 30 days' treatment, and tumor sizes and body weights were monitored until 70 days. *Arrows* indicate the date that stops treatment. (B) Body weight of mice. Body weights of mice were monitored twice per week.

growth inhibition of pancreatic cancer xenografts. Our data showed that WA directly binds to Hsp90 and leads to the degradation of Hsp90 client proteins through an ATP-independent mechanism.

Hsp90 is crucial for maintaining the native conformation of proteins. It was found to be highly expressed in various cancerous tissues compared to the non-cancerous tissue [48,49], which provides cancer cells selectivity by Hsp90 inhibitor [22,50]. For instance, 17-AAG binding affinity to Hsp90 in cancer cells is 100-fold higher than that to Hsp90 in normal cells [22]. Therefore, inhibiting the Hsp90 chaperone activity emerged as a new molecular target for developing anticancer agents because of its high selectivity and simultaneous knockdown of various oncogenic proteins. Several Hsp90 inhibitors have been developed and tested in preclinical and clinical models for their anticancer activity including 17-AAG, 17-DMAG and IPI-504 [23–26].

In addition to ATP binding blockage of Hsp90, researchers have also identified other Hsp90 inhibition mechanisms. For example, the histone deacetylase inhibitors (hydroxamic acid analogue, LAQ 824 and LBH589) were shown to induce the hyperacetylation of Hsp90, resulting in inhibition of ATP binding and attenuation of chaperone activity [51]. In this study, we demonstrated that WA inhibited Hsp90 chaperone activity to induce Hsp90 client protein degradation. However, unlike the classical Hsp90 inhibitor, WA directly binds to Hsp90 C-terminus. These were evidenced by the pull down assay of WA-biotin to C-terminus Hsp90 containing cysteine residues but not N-terminus Hsp90 or yeast Hsp90 (without cysteine residues). In addition, the binding of WA-biotin to Hsp90 could be competitively inhibited by unlabeled WA. Furthermore, ATP-sepharose beads pull down assay did not show any inhibition of WA to ATP binding to Hsp90. These data also further suggest that WA inhibition of Hsp90 is not through an ATP-dependent mechanism, which is different from the classical Hsp90 inhibitors.

To further confirm that WA binds to Hsp90 reactive cysteine residues, we examined whether exogenous thiols in cell culture would rescue the WA inhibition to Hsp90. N-acetylcysteine (NAC) is a natural sulfur-containing amino acid derivative and is a thiol antioxidant [52]. Preincubation of Panc-1 cells with NAC reversed WA induced Hsp90 aggregation (data not shown), whereas NAC failed to reverse a mild oxidant, hydrogen peroxide (H_2O_2) induced Hsp90 aggregation. Although geldanamycin and its derivatives were shown to be able to produce ROS [53], 17-AAG was unable to induce Hsp90 aggregation. In addition, WA induced Hsp90 client protein degradation and Hsp70 induction were also rescued by NAC pretreatment (Data not shown). Regardless the mechanism of NAC in reverse WA effects (chemical reaction or cellular response), these data suggest that WA may inhibit Hsp90 function through cysteine of the C-terminal Hsp90. However, the specific cysteine residues that WA binds to need to be further elucidated.

One interesting phenomenon we observed is that WA disrupts Hsp90–Cdc37 complex. Cdc37 is believed to play a central role in regulating kinase client proteins in the intermediate Hsp90 superchaperone complex. A client protein first binds the Hsp70/Hsp40 chaperone complex and then interacts with Cdc37. Hsp90 is subsequently recruited to the complex via p60/Hop [54]. Similar to Hsp90, Cdc37 is also upregulated in cancer cells. Cdc37 is highly expressed in all prostate tumors and absent from normal prostate epithelium. Transgenic mice expressing Cdc37 in the prostate epithelium have displayed dramatic proliferative disorders in the prostate, including epithelial hyperplasia and dysplasia [55]. Hepatocellular carcinoma also over-expresses Cdc37 and Hsp90 compared to normal and surrounding tissues [56]. Previous study showed that Celastrol inhibited Hsp90 chaperone activity by blocking Hsp90–Cdc37 interactions and led to Hsp90 client protein degradation for its anticancer activity [35]. Celastrol also binds to C-terminus of Hsp90 (Zhang et al., unpublished data). In the present study, we found that WA disrupts Hsp90–Cdc37 complex. WA blockage of Hsp90–Cdc37 complex might be a result of WA binding-induced conformational change of Hsp90 or other unknown mechanisms. Further study is warranted to elucidate these mechanisms. It is worth noting that WA inhibition of Hsp90 chaperone activity should not be solely due to the disruption of Hsp90–Cdc37 association. As described earlier, Cdc37 helps load its client proteins onto the Hsp90 chaperone complex. The client proteins of Cdc37 include many kinases, such as Raf-1, Akt and Src family kinases, and steroid receptors such as androgen receptor but not the closely related glucocorticoid receptor (GR) [57]. However, our data showed that WA treatment could also induce the degradation of GR. These data suggest that WA inhibits Hsp90 chaperone activity might be also due to the direct binding of WA to Hsp90 [42].

Furthermore, P23 and Cdc37 were demonstrated to bind to the same sites on the N-terminus of Hsp90, and binding of P23 and Cdc37 to Hsp90 is mutually exclusive [35,58]. Cdc37 and P23 bind to Hsp90 at different stages of the chaperone cycle: Cdc37 binds to Hsp90 in the intermediate complex; whereas P23 binds to Hsp90 in the mature complex in which Hsp90 adopts a different conformation from the intermediate complex. Classical Hsp90 inhibitors such as geldanamycin and 17-AAG lock Hsp90 in the intermediate complex, hence preventing P23 binding to Hsp90 [35]. Our data showed that WA did not interfere with the Hsp90–P23 interaction, while 17-AAG induced Hsp90–P23 dissociation. There are two possible mechanisms for WA induced Hsp90–Cdc37 dissociation but not Hsp90–P23 dissociation: one is that WA binds directly to the cysteine residues on the C-terminus of Hsp90, resulting in a conformational change of Hsp90 which preventing Cdc37 but not P23 from binding; another is that besides Hsp90, WA also reacts with cysteines in Cdc37 which contributes to the disruption of Hsp90–Cdc37 interaction, however, although cysteine residues are present in P23, they are not accessible to WA, thus providing a selectivity of action. It is important to note that this is rather speculative and needs to be further confirmed.

Previous studies have shown that the 4β-hydroxy-5β, 6β-epoxy-2-en-1-one moiety and unsaturated lactone are critical for WA's biological function [59]. The epoxide within B ring and the unsaturated lactone ring were demonstrated to be involved in Michael addition thioalkylation reactions [43,44,60]. In addition, the ketone containing unsaturated A ring could also react with thiol-nucleophiles and act as Michael acceptor [59]. Thus these three functional groups could be crucial for the interaction between WA and Hsp90. Gedunin and aforementioned Celastrol are two natural products exhibiting Hsp90 inhibitory activity. They modulate Hsp90 activity by a mechanism different from classical Hsp90 inhibitors such as GA, since they do not competitively bind to the ATP binding pocket of Hsp90 [47]. The exact mechanism how Gedunin inhibits Hsp90 is still unknown. Gedunin also possesses a ketone containing unsaturated A ring and an additional epoxide which are similar to WA. Although the preliminary structure–activity studies of Gedunin revealed that the α,β-unsaturated ketone within the A ring of Gedunin does not behave as a Michael acceptor [61], the epoxide on Gedunin might act as a Michael acceptor and thus Gedunin might also inhibit Hsp90 in a way similar to WA. On the other hand, Celastrol also has electrophilic sites within the unsaturated A and B ring and was shown to react with thiols in proteins [62,63]. Our unpublished data by Zhang et al. indicate that Celastrol binds to C-terminus Hsp90; hence, it would not be surprised if Celastrol inhibits Hsp90 via binding to reactive cysteine residues of Hsp90.

Withaferin A belongs to a large family of natural products steroidal lactone triterpenoids, the withanolides, which are major constituents purified from medicinal plant *W. somnifera* and its related Solanaceae species such as *Physalis*, *Nicandra*, *Dunalia*, *Datura*, *Jaborosa*, and *Acnistus* [64]. The withanolides are demonstrated to have antitumor, antibacterial, anti-inflammatory, antidepressant, antioxidant, antiulcer, cytotoxic, quinone reductase induction, antileishmanial, antitrypanosomal, immunosuppressive, cognition-enhancing and memory-improving effects, as well as hypotensive, bradycardic and respiratory-stimulant action [65–67]. As a prototype of the withanolides, Withaferin A has been studied extensively. Up to date, over 130 withanolides are known and more than 40 withanolides are isolated [68]. The individual withanolides were purified and evaluated for their biological functions. For instance, some newly isolated withanolides have been shown to have cytotoxic activity, including Withangulatin B, Withangulatin C, Withangulatin G, Withangulatin H, and Withangulatin I [66,67]. The structure–activity studies further confirmed that the unsaturated A ring and epoxide are important for the cytotoxic activity of withanolides [66]. Another Withanolide, Withangulatin A was shown to inhibit topoisomerase II and induce heat shock response [69,70]. Although DNA damage mediated by topoisomerase II inhibitors has been shown to induce heat shock response [71], another topoisomerase II inhibitor VM-26 could not induce heat shock response in the same cell line as Withangulatin A [70]. Therefore, Withangulatin A induces heat shock response through a way other than topoisomerase II inhibition mediated DNA damage. Considering the quasi-identical structure (the same unsaturated ketone containing A ring, epoxide containing B ring and unsaturated lactone ring) of Withangulatin A to Withaferin A, Withangulatin A might induce heat shock response by inhibition of Hsp90 [72]. In addition, Tubocapsenolide A (TA), another withanolide possessing the three key functional groups, showed high cytotoxicity against cancer cells and exhibited Hsp90 inhibitory activity [37]. TA induces Hsp90 client protein degradation and induces Hsp90 dimer formation. Although there is no direct evidence that TA binds to Hsp90, TA could inhibit Hsp90 through binding to the reactive cysteine residues of Hsp90 like WA.

In conclusion, our data suggest that Withaferin A represents a new type of Hsp90 inhibitor. It directly binds Hsp90, inhibits Hsp90 with an ATP-independent mechanism, induces Hsp90 client protein degradation and disrupts the Hsp90–Cdc37 interaction. These Hsp90 inhibition mechanisms of Withaferin A may partially contribute to its anticancer activity *in vitro* pancreatic cancer cell lines and in vivo pancreatic cancer xenografts. These data provide a potential of Withaferin A as a novel Hsp90 inhibitor for use against pancreatic cancers.

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