

Sulforaphane inhibits pancreatic cancer through disrupting Hsp90–p50^{Cdc37} complex and direct interactions with amino acids residues of Hsp90[☆]

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Abstract

Sulforaphane [1-isothiocyanato-4-(methyl-sulfinyl) butane], an isothiocyanate derived from cruciferous vegetables, has been shown to possess potent chemopreventive activity. We analyzed the effect of sulforaphane on the proliferation of pancreatic cancer cells. Sulforaphane inhibited pancreatic cancer cell growth *in vitro* with IC₅₀s of around 10–15 μ M and induced apoptosis. In pancreatic cancer xenograft mouse model, administration of sulforaphane showed remarkable inhibition of tumor growth without apparent toxicity noticed. We found that sulforaphane induced the degradation of heat shock protein 90 (Hsp90) client proteins and blocked the interaction of Hsp90 with its cochaperone p50^{Cdc37} in pancreatic cancer cells. Using nuclear magnetic resonance spectroscopy (NMR) with an isoleucine-specific labeling strategy, we overcame the protein size limit of conventional NMR and studied the interaction of sulforaphane with full-length Hsp90 dimer (170 kDa) in solution. NMR revealed multiple chemical shifts in sheet 2 and the adjacent loop in Hsp90 N-terminal domain after incubation of Hsp90 with sulforaphane. Liquid chromatography coupled to mass spectrometry further mapped a short peptide in this region that was tagged with sulforaphane. These data suggest a new mechanism of sulforaphane that disrupts protein–protein interaction in Hsp90 complex for its chemopreventive activity.

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1. Introduction

Numerous studies have shown the chemoprevention efficacy of high consumption of broccoli and broccoli sprouts against various cancers [1]. Sulforaphane is a major compound from broccoli/broccoli sprouts to possess chemopreventive activity [2,3]. Previous

studies suggest that sulforaphane modulates multiple targets [1,4,5], which regulate many cellular activities including oxidative stress, apoptosis induction, cell cycle arrest, angiogenesis and metastasis suppression, and detoxification of carcinogens [1,6].

The regulation of multiple targets by sulforaphane may be through direct inhibition of each individual target or through a common modulator that regulates multiple targets. Previous studies have shown that sulforaphane down-regulates a group of signaling proteins, such as Akt, cyclin-dependent kinase 4 (Cdk4), androgen receptor (AR) and hypoxia-inducible factor-1 α (HIF-1 α), while these proteins are also client proteins of heat shock protein 90 (Hsp90). For instance, sulforaphane decreased Cdk4 protein level in ovarian cancer cells, leading to cell cycle arrest and apoptosis [7]. Sulforaphane also down-regulated Akt, phosphorylated Akt and phosphatidylinositol 3-kinase (PI3K) in ovarian cancer cells with a concomitant inhibition of Akt kinase activity [7]. Sulforaphane reduced AR in prostate cancer cells [8,9] and suppressed HIF-1 α in human tongue squamous cancer cells and prostate cancer cells [10]. A recent study reported that sulforaphane enhanced acetylation of Hsp90 by inactivating histone deacetylase 6 (HDAC6) in prostate cancer cells, thereby inhibiting its association with AR, leading to destabilization of AR protein and

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disruption of AR signaling [8]. Hsp90, an essential molecular chaperone, regulates the stability and maturation of a wide range of oncogenic client proteins [11,12]. To date, more than 200 of Hsp90 client proteins have been identified, which include mutated signaling proteins (Raf, p53), transmembrane tyrosine kinases (epidermal growth factor receptor 2, epidermal growth factor), signaling proteins (Akt, HIF-1 α), cell cycle regulators (Cdk4) and hormone receptors (AR) [12,13]. It has become a promising target for cancer therapy.

In this study, we examined the anticancer effect of sulforaphane against pancreatic cancer cells and investigated the mechanism of Hsp90 modulation by sulforaphane. We did not observe hyperacetylation of Hsp90 in pancreatic cancer cells. We characterized the impact of sulforaphane on Hsp90-cochaperone interactions and adenosine triphosphate (ATP) binding to Hsp90. Our data showed that sulforaphane inhibits Hsp90 through an ATP-binding independent manner. We revealed mechanistic insights in the regulation of Hsp90 by sulforaphane with nuclear magnetic resonance

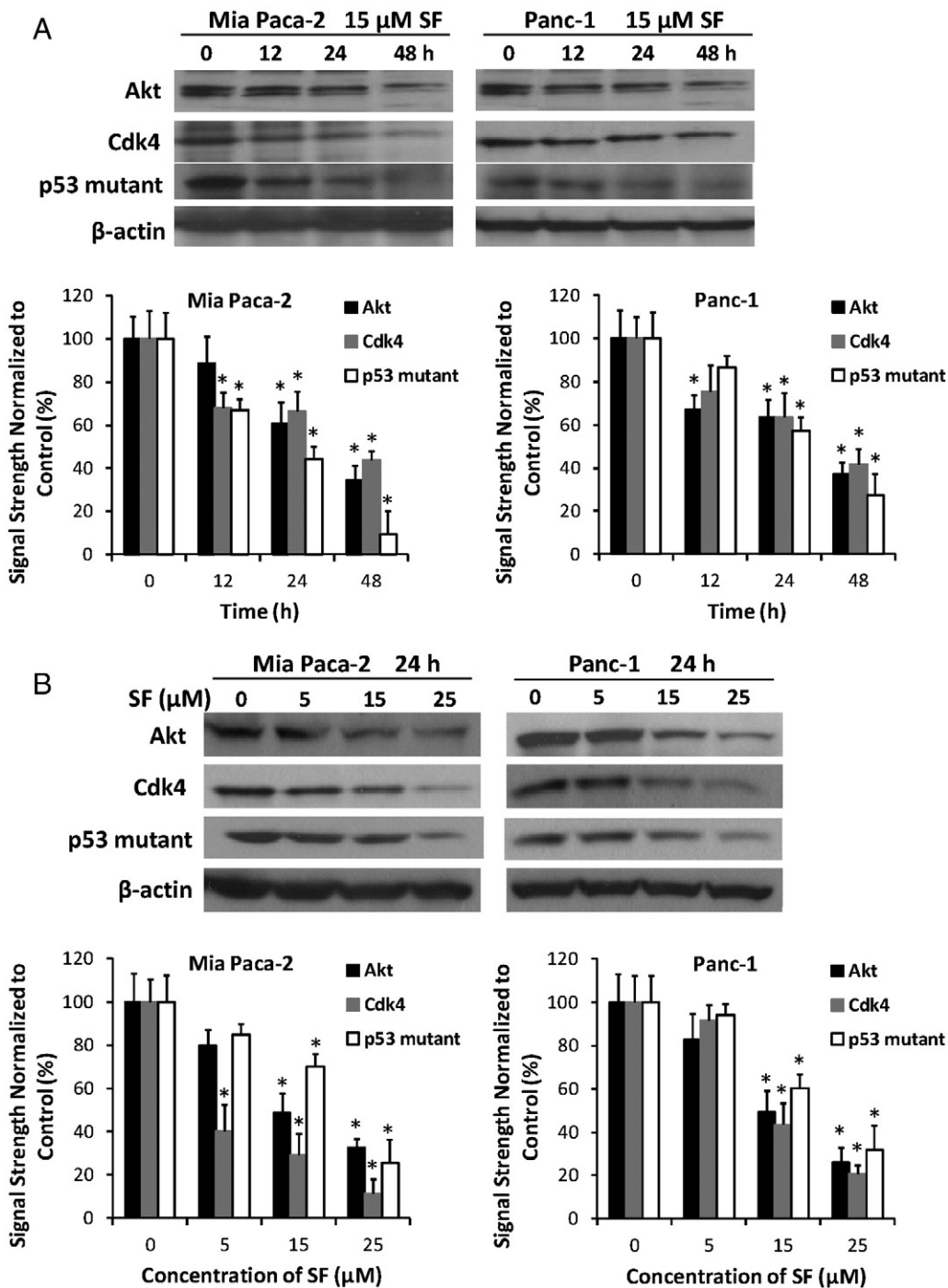


Fig. 1. Sulforaphane induces proteasomal degradation of Hsp90 client proteins. (A) Mia Paca-2 and Panc-1 cells were treated with 15 μ M sulforaphane for different time periods. Sulforaphane induced a time-dependent down-regulation of Akt, Cdk4 and p53 mutant. Densitometry data are presented as mean $\pm$ S.D. ($n=3$, $P<.05$). (B) Cells were treated with 5, 15, 25 μ M sulforaphane. Sulforaphane induced a concentration-dependent down-regulation of these proteins. Densitometry data are presented as mean $\pm$ S.D. ($n=3$, $P<.05$). (C) Cells were preincubated with 10 μ M MG132 before sulforaphane treatment. Sulforaphane-induced down-regulation of Hsp90 client proteins was proteasome mediated. SF, sulforaphane.

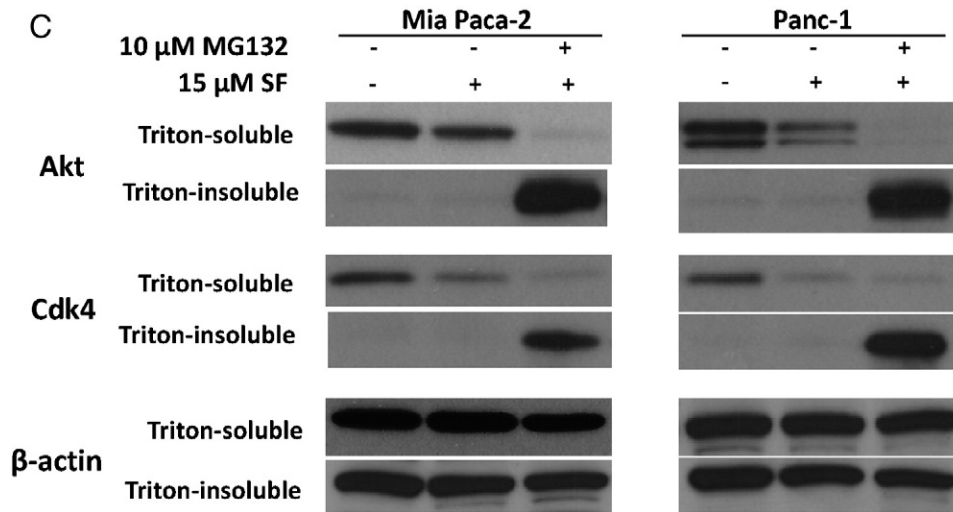


Fig. 1 (continued).

spectroscopy (NMR) technique and liquid chromatography coupled to mass spectrometry (LC-MS) peptide mapping.

2. Materials and methods

2.1. Cell culture

Human pancreatic cancer cell lines Mia Paca-2, Panc-1, AsPc-1 and BxPc-3 were obtained from American Type Culture Collection. Authentication of these cell lines in their origin sources included morphology analysis, growth curve analysis, isoenzyme analysis, short tandem repeat analysis and mycoplasma detection. Panc-1, AsPc-1 and BxPc-3 were maintained in RPMI1640 medium (Invitrogen) supplemented with 10% fetal bovine serum (Fisher Scientific) and 1% penicillin–streptomycin (Invitrogen). Mia Paca-2 was maintained in Dulbecco's modified Eagle's medium (Invitrogen) supplemented with 10% fetal bovine serum and 1% penicillin–streptomycin–glutamine.

2.2. Reagents

Sulforaphane was purchased from LKT Laboratories. The following antibodies were used for immunoblotting: Akt (Cell Signaling), p23^{Sba1} (Abcam), p53 mutant, Cdk4, p50^{Cdc37}, Hsp90, β-actin (Santa Cruz Biotechnology).

2.3. Western blotting analysis

After treatment, cells were harvested by washing twice with ice-cold phosphate-buffered saline (PBS) and lysed in radio-immunoprecipitation assay lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM sodium orthovanadate, pH 7.5) supplemented with protease inhibitors (Pierce) on ice for 20 min. After centrifugation at 14,000 rpm for 15 min at 4 °C, the supernatant was recovered. Protein concentration was determined with BCA Protein Assay Reagents (Pierce). Equal amounts of protein were subject to sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene fluoride membrane (BioRad). The membrane was then incubated with appropriate primary antibodies at 4 °C overnight, followed by 2-h incubation with secondary antibodies at room temperature.

2.4. Triton-soluble and triton-insoluble protein fraction

Sulforaphane (15 μM) was added after preincubation cells with 10 μM MG132. After 24-h treatment, cells were collected and lysed in a lysis buffer (50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1 mM EGTA, 1% Triton X-100, 2 mM sodium orthovanadate, 5 mM sodium fluoride, 1 mM phenylmethylsulfonyl fluoride and 5000 U/ml aprotinin) on ice for 10 min. The samples were centrifuged at 16,000g for 15 min at 4 °C, and the supernatant (triton-soluble fraction) was collected. The pellets (triton-insoluble fraction) were further lysed in 2% SDS in 50 mM Tris-HCl and boiled for 15 min.

2.5. MTS assay

Cells were seeded in 96-well microplates at a density of 3000–5000 cells per well. After 72-h treatment with sulforaphane, cell viability was assessed by 3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay (Promega) according to the manufacturer's instruction. The number of living cells is proportional to the absorbance at 490 nm.

2.6. Caspase-3 activity assay

Cells were treated with sulforaphane and collected after 24 h. Caspase-3 activity assay was based on the manufacturer's instruction on the Caspase-3/CPP32 Fluorometric Assay Kit (Biovision Research Products). The cleavage of a caspase-3 substrate was quantified by using a fluorescence microtiter plate reader with a 400-nm excitation filter and a 505-nm emission filter.

2.7. Pancreatic cancer xenograft

The animal study protocol was approved by the University Committee on Use and Care of Animals at University of Michigan. Four- to six-week-old athymic (nu/nu) female mice were obtained from NCI. Mia Paca-2 cells (5×10^5 – 10×10^6) mixed with Matrigel were subcutaneously implanted into the right and left flanks of the mice. Tumor volume was calculated with $V = 1/2(\text{width}^2 \times \text{length})$. After tumor volume reached 100–150 mm³, mice were randomized into three groups for treatment. The mice were intraperitoneally injected with vehicle (saline), 25 mg/kg sulforaphane or 50 mg/kg sulforaphane (five times per week) for 4 weeks. Tumor size was monitored twice a week and normalized to the initial volumes.

2.8. Protein cloning, expression and purification

The plasmid pET28a(+)-hHsp90β(529–723) for expression of human Hsp90βC (Hsp90β-G529-D723) was kindly provided by Dr. Thomas Ratajczak (University of Western Australia). The purified Hsp90βN protein (Hsp90β-P1-E245) was kindly provided by Dr. Dan Bolon (University of Massachusetts Medical School). The plasmid was transformed into *Escherichia coli* strain Rosetta 2(DE3) (EMD Biosciences Inc.) according to the protocol provided by the manufacturer. Protein expression was induced by 0.2 mM IPTG. His-tagged protein was purified by affinity chromatography through mixing with HisPur™ Cobalt Resin (Pierce). Purified protein was dialyzed against PBS, and the purity was assessed by SDS-PAGE. Protein was stored at –70 °C after adding glycerol to 10%.

2.9. ATP-Sepharose binding assay

Cells were treated with sulforaphane for 24 h and then lysed in TNE SV buffer (50 mM Tris, 2 mM EDTA, 100 mM NaCl, 1 mM Na₂VO₄, 25 mM NaF, 1% Triton X-100, pH 7.5) supplemented with protease inhibitors at 4 °C for 30 min. Protein (200 μg) was incubated with 25 μl preequilibrated γ-phosphate-linked ATP-Sepharose (Jena Bioscience GmbH) in 200-μl incubation buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 20 mM Na₂MoO₄, 0.01% NP-40, pH 7.5) overnight at 4 °C. The protein bound to Sepharose beads was analyzed by Western blotting.

2.10. Hsp90 co-immunoprecipitation

Cells were treated with sulforaphane for 24 h and lysed in 20 mM Tris-HCl (pH 7.4), 25 mM NaCl, 2 mM dithiothreitol (DTT), 20 mM Na₂MoO₄, 0.1% NP-40 and

protease inhibitors. Protein (500 µg) was incubated with H9010 antibody (Axxora) for 1 h at 4°C, followed by overnight incubation with protein A/G agarose at 4°C. The bound proteins were analyzed by Western blotting.

2.11. Proteolytic fingerprinting assay

Purified protein (0.5 µg) was preincubated with dimethyl sulfoxide (DMSO) or sulforaphane in assay buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl₂, 0.1 mM EDTA, pH 7.4) on ice for 1 h and was digested with trypsin for 6 min. The reaction was terminated by adding SDS buffer and boiling for 3–5 min. The digested products from Hsp90βN (Hsp90β-P1-E245) and Hsp90βC (Hsp90β-G529-D723) were analyzed by Western blotting with Hsp90 antibody (N-17, Santa Cruz Biotechnology) and Hsp90 (AC88) antibody (Assay Designs, Inc., Ann Arbor, MI, USA), respectively.

2.12. LC-MS

Hsp90βN (Hsp90β-P1-E245, 80 µM) was incubated with DMSO or sulforaphane in PBS at 37°C for 30 min. Small molecules were removed by gel filtration using P-30 micro Bio-Spin columns. Samples were analyzed on Shimadzu LCMS-2010EV after separation on a PLRP-S polymeric reverse phase high performance liquid chromatography column (50×2.1 mm, 1000 Å, 5 µm) with a gradient of 5% to 35% B in 30 min (A: 0.1% formic acid in H₂O; B: 0.1% formic acid in CH₃CN) at a flow rate of 0.2 ml/min. For peptide mapping, Hsp90βN (80 µM) was incubated with DMSO or sulforaphane (2 mM) for 30 min and was then treated with DTT (10 mM) for 1 h, followed by Sequencing Grade Modified Trypsin (Promega) for 24 h at 37°C. Samples were analyzed on a Vydac Everest reverse-phase C18 monomeric column (250×2.1 mm, 300 Å, 5 µm) with a gradient of 5% to 65% B in 60 min at a flow rate of 0.2 ml/min.

2.13. NMR spectroscopy

Hsp90 was recombinantly produced with N-terminal hexa-histidine tag and specifically methyl labeled at Ile side chains as described [14]. The NMR experiments were performed with 150 µM of Hsp90 in 100% D₂O, 25 mM sodium phosphate, pH 7.2, 300 mM NaCl and 1 mM tris(2-carboxyethyl)phosphine, and sulforaphane concentration was varied (dissolved in deuterated DMSO, final concentration 1% at 1.5 mM sulforaphane) for NMR measurements. ¹³C-¹H-methyl transfer relaxation-optimized spectroscopy (TROSY) spectra [15] were recorded at 25°C on a Bruker Avance II 900-MHz spectrometer equipped with a Triple Resonance CryoProbe. Reported chemical shifts are referenced against 2,2-dimethylsilapentane-5-sulfonic acid, and processing and peak position plots were done as described [14]. The cross peaks of the methyl-transfer relaxation-optimized spectroscopy (TROSY) spectra were 2D-line shape fitted using mixed Lorentzian/Gaussian deconvolution parameters (TopSpin). The rainbow spectra indicate increasing intensity from blue to red over 14 contour levels. All contours above the intensity of the cross peak at 0.72 ppm (¹H) and 10.8 ppm (¹³C) are red. Comparisons with Hsp90 in the absence of sulforaphane were corrected for DMSO effects.

2.14. Statistical analysis

Statistical analysis was performed using Student's *t* test. Data are presented as mean±S.D. (*n*≥3, *P*<.01 or <.05).

3. Results

3.1. Sulforaphane induces proteasomal degradation of Hsp90 client proteins in pancreatic cancer cells

We first examined whether sulforaphane could reduce the levels of Hsp90 client proteins, Akt, Cdk4 and p53 mutant, in Mia Paca-2 and

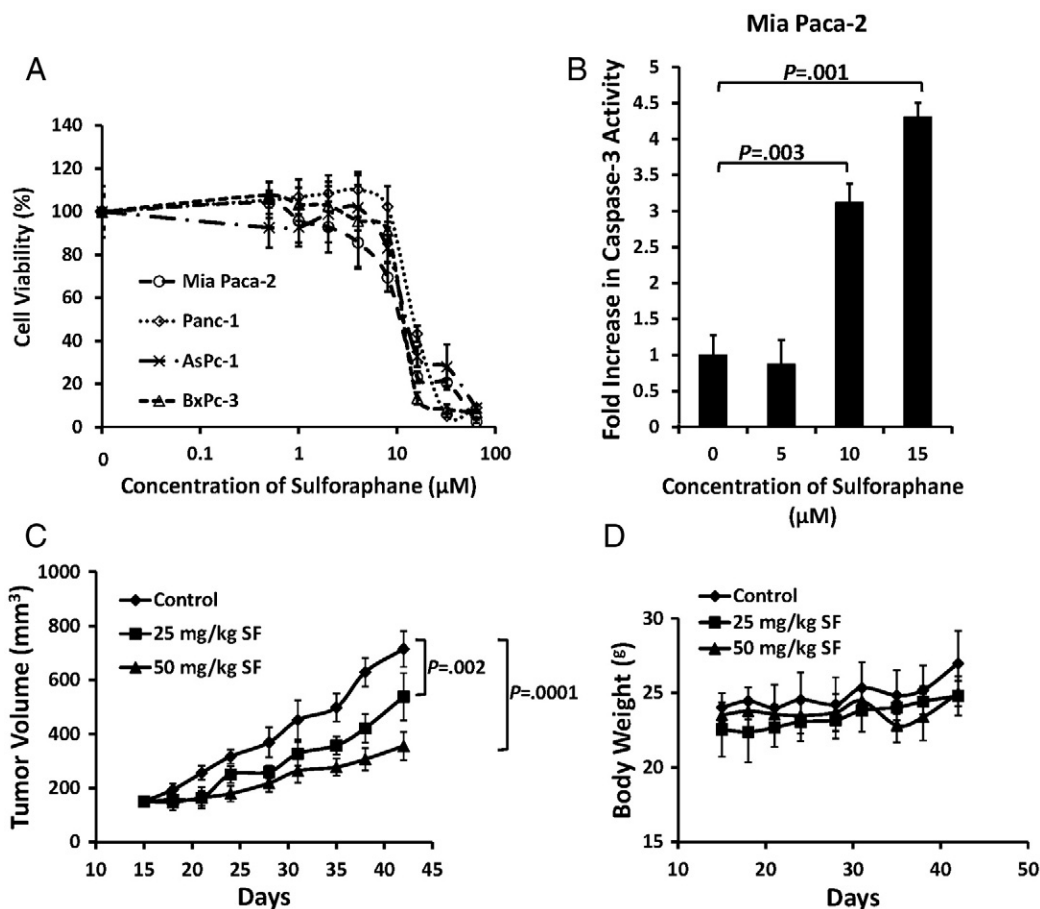


Fig. 2. Sulforaphane exhibits anticancer activity *in vitro* and *in vivo*. (A) Sulforaphane inhibited proliferation of Mia Paca-2, Panc-1, AsPc-1 and BxPc-3 cells. (B) Sulforaphane induced caspase-3 activity in Mia Paca-2 cells. Data are presented as means±S.D. (*n*=3, *P*<.01). (C) Antitumor effect of sulforaphane in xenografts. The pancreatic tumor xenograft model was generated by inoculating Mia Paca-2 cancer cells subcutaneously to the right and left flanks of nude mice. When the tumors reached 100–150 mm³, mice were randomly divided into three groups (*n*=6) to receive vehicle or 25- or 50-mg/kg sulforaphane treatment (five times/week) for 4 weeks. Data are presented as means±S.D. (*n*=6, *P*<.01). (D) Mouse body weight was measured twice a week.

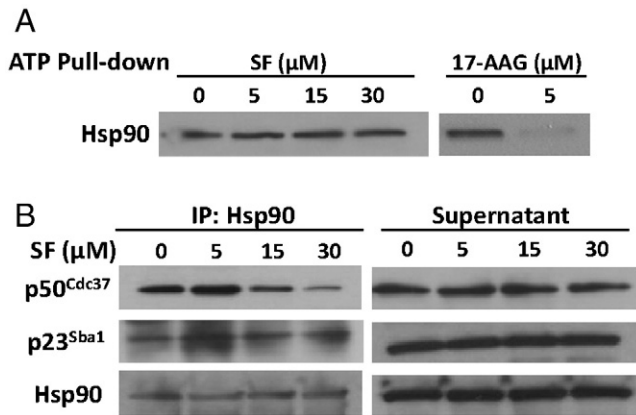


Fig. 3. Influence of sulforaphane on ATP binding of Hsp90 and Hsp90-cochaperone association in Mia Paca-2 cells. (A) Sulforaphane (5, 15, 30 μM) did not affect ATP binding, while 17-AAG (5 μM) decreased ATP binding to Hsp90. (B) Sulforaphane reduced the amount of p50^{Cdc37} bound with Hsp90, while it showed no effect on p23^{Sba1}.

Panc-1. Both cell lines contain p53 mutant and activated Ras [16–18]. As shown in Fig. 1A and B, the levels of these proteins declined in a time- and concentration-dependent manner in response to sulforaphane treatment. Sulforaphane (15 μM) down-regulated Akt, Cdk4 and p53 mutant by 70%–95% after 48 h, and 25 μM of sulforaphane decreased these client proteins by 80%–95% after 24 h (Fig. 1A & B).

We further investigated whether sulforaphane-induced Hsp90 client protein down-regulation was through proteasomal degradation. MG132, a well-known proteasome inhibitor, was preincubated

with pancreatic cancer cells to block proteasome function. Inhibition of proteasome leads to accumulation of unfolded proteins, which form insoluble “aggresomes” in the triton-insoluble fraction of cell lysates. As shown in Fig. 1C, Akt and Cdk4 were found to accumulate in the triton-insoluble fraction after sulforaphane treatment in the presence of MG132, while they were undetectable in triton-insoluble fraction after sulforaphane treatment alone. These data indicate that sulforaphane induced proteasomal degradation of Hsp90 client proteins in pancreatic cancer cells.

3.2. Sulforaphane inhibits pancreatic cancer cells in vitro and exhibits anticancer activity in pancreatic cancer xenograft

We evaluated the antiproliferative activity of sulforaphane in human pancreatic cancer cell lines Mia Paca-2, Panc-1, AsPc-1 and BxPc-3 by MTS cell proliferation assay. After treatment with increasing concentrations of sulforaphane for 72 h, the percentage of viable cells relative to untreated cells is plotted in Fig. 2A. Cell viability decreased as the concentration of sulforaphane increased in a similar pattern among all the four cell lines, with IC₅₀s of around 10–15 μM. Then we used Mia Paca-2 to illustrate the effect of sulforaphane on caspase-3 activation. The results of caspase-3 assay showed that sulforaphane induced activation of caspase-3 in a concentration-dependent manner (Fig. 2B). In comparison with control cells, 10 μM sulforaphane was able to enhance the caspase-3 activity to more than threefold ($P=0.003$), and more than a fourfold increase was observed when cells were exposed to 15 μM sulforaphane ($P=0.001$). These data suggest that sulforaphane inhibits pancreatic cancer cell growth and induces apoptosis *in vitro*.

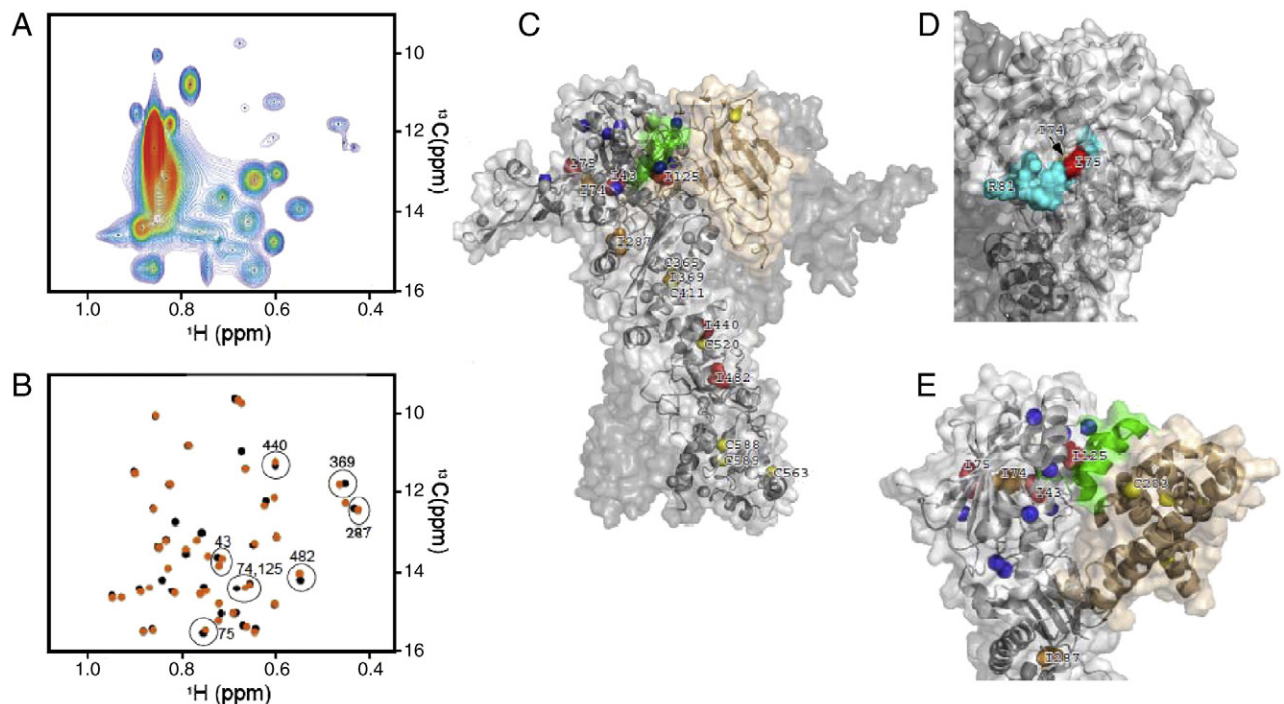


Fig. 4. Sulforaphane binding to Hsp90 mapped by NMR. (A) Rainbow representation of ¹H-¹³C-Ile methyl TROSY cross peaks of full-length Hsp90 bound to sulforaphane (1.5 mM) (blue to red gradient indicates increasing intensities; peak centers, black dots). (B) Overlaid peak position plots of Hsp90 Ile methyl TROSY spectra in absence (black) and presence of sulforaphane (orange). Significant shifts are encircled [combined chemical shift difference $\Delta\nu=(0.25\Delta\nu_C^2 + \Delta\nu_H^2)^{1/2} > 0.01$ ppm]. (C) Homology model of human Hsp90 bound to p23^{Sba1}. (Hsp90 protomers, gray; p23^{Sba1}, light orange). The shifting pattern upon sulforaphane binding is indicated as space fillings in one monomer (chemical shifts $\Delta\nu > 0.01$ ppm, orange side chains; $\Delta\nu > 0.015$, red side chains; no shifts, blue δ -methyl groups; no assignments or inconclusive results, grey δ -methyl groups; lid of the ATP pocket, green backbone; cysteines, yellow). (D) Surface representation of Hsp90, zoomed into peptide stretch IDIIPNPQER [Hsp90, gray; IDIIPNPQER stretch, cyan; isoleucines that shift upon sulforaphane binding, red (Ile 75) and orange (Ile 74)]. (E) The Hsp90 N-terminal domain in complex with p50^{Cdc37} [Hsp90, grey; p50^{Cdc37}, light orange; isoleucines and cysteines are colored as in (C)].

We next evaluated the efficacy of sulforaphane in Mia Paca-2 xenograft model. Two weeks after cancer cell implantation, the mice were injected (intraperitoneally) with either vehicle or sulforaphane at 25 mg/kg or 50 mg/kg for 1 month. At the end of treatment, control tumors reached an average size of $715 \pm 66 \text{ mm}^3$, while 25 mg/kg and 50 mg/kg sulforaphane treatment limited the tumor volumes to $538 \pm 88 \text{ mm}^3$ and $354 \pm 52 \text{ mm}^3$, respectively (Fig. 2C). These correspond to 25% ($P=.002$) and 50% ($P=.0001$) inhibition of tumor growth. Meanwhile, sulforaphane showed no apparent toxicity as determined by body weight measurement (Fig. 2D).

3.3. Sulforaphane inhibits Hsp90 by blocking Hsp90–p50^{Cdc37} complex in pancreatic cancer cells

Most Hsp90 inhibitors, e.g., 17-AAG, bind to Hsp90 N-terminal ATP pocket, preventing the maturation of Hsp90 client proteins [19]. However, sulforaphane (5–30 μM) was unable to decrease the amount of ATP-bound Hsp90 (Fig. 3A), which suggests that sulforaphane did not affect the ATP-binding capacity of Hsp90. As positive control, 17-AAG (5 μM) blocked the ATP binding to Hsp90 and decreased the amount of ATP-bound Hsp90 in the ATP-Sepharose binding assay. Since Hsp90 chaperone function depends on multiple cochaperones to form the complexes, we investigated the effect of sulforaphane on Hsp90–cochaperone interaction using co-immunoprecipitation assay. We found that sulforaphane inhibited Hsp90 and p50^{Cdc37} interaction. Sulforaphane (15–30 μM) treatment for 24 h in Mia Paca-2 cells reduced the amount of p50^{Cdc37} co-precipitated with Hsp90 by approximately three- to fivefold (Fig. 3B). However, sulforaphane (15–30 μM) did not change the amount of p23^{Sba1} in precipitated Hsp90 complex. The total protein levels of Hsp90, p50^{Cdc37} and p23^{Sba1} were unchanged after sulforaphane treatment (Fig. 3B). These data suggest that sulforaphane blocked Hsp90–p50^{Cdc37} interaction, but not Hsp90–p23^{Sba1} interaction.

3.4. NMR reveals the sulforaphane binding sites on Hsp90

To investigate whether sulforaphane directly modulates Hsp90, we employed the NMR spectroscopy for full-length Hsp90 [14,15,20,21]. Conventional NMR method are restricted to proteins up to 50 kDa; however, complexes of full-length Hsp90 dimer with cofactors are around 200 kDa, which poses challenges for this study. Recently, the Kay laboratory performed pioneering NMR spectroscopy for large proteins by specific labeling of methyl groups of aliphatic amino acids, including isoleucine (Ile), to overcome the limitations [23,24]. Here we applied a similar strategy.

When comparing the NMR spectra of Hsp90 in the absence and presence of sulforaphane, we found that a subset of peaks shifts (Fig. 4A and B). We assigned the N-terminal and middle domains of Hsp90 [14], which allowed us to map the chemical shifts within those domains (Fig. 4C). Two binding clusters were particularly intriguing. We found chemical shifts in sheet 2 for Ile 74 and Ile 75 in the N-terminal domain (Fig. 4D). The signals of Ile 43 and Ile 125 are also shifting (Fig. 4E). Ile 43 is found in α -helix in the neighborhood of Ile 74, and the change of environment of this residue might have affected Ile 43 indirectly. Ile 125, which belongs to the p50^{Cdc37} interaction interface (Fig. 4E), shifts upon sulforaphane interaction, but the nearby Ile 122 is not affected. This indicates that Ile 125 shifting is likely caused by an allosteric effect, which may disturb the interaction between p50^{Cdc37} and Hsp90.

In the middle domain, we also identified chemical shifts in four isoleucines (Fig. 4C). Three of those (Ile 369, Ile 440 and Ile 482) are sufficiently close to Cys side chains, which in other proteins are known to undergo reaction with sulforaphane. Cys 365 is 6.3 Å away from Ile 369, and Cys 520 is 5.3 Å and 13.6 Å away from Ile 440 and Ile 482, respectively. Ile 287 is found in the loop in the interface of the N-

terminal and middle domain of Hsp90, and this could be shifting due to change in the domain orientation.

At present, no assignment of the C-terminal domain is available. For the three residues we identified in the C-terminal domain, we did not observe sulforaphane-dependent shifts. This indicates that sulforaphane did not have effects on Hsp90 C-terminal domain, despite harboring four out of six cysteines.

Comparing to the spectra of sulforaphane-free Hsp90, we noted line broadening for a subset of signals. Those signals reside in the region of unstructured and dynamic segments, which indicated an effect of sulforaphane on Hsp90 dynamics. As controls, we also compared the sulforaphane-dependent shifts with those of nucleotides and the ATP binding inhibitor geldanamycin [14]. While all of those ligands show a coherent pattern clustering around the nucleotide binding pocket, sulforaphane binds in a different mode. The NMR data further confirm that sulforaphane does not block the nucleotide binding of Hsp90, in agreement with the results of ATP-Sepharose binding assay (Fig. 3A). In addition, we also analyzed whether sulforaphane binding could have any effect on binding of cochaperone p23^{Sba1} (Fig. 4C). The p23^{Sba1} binding site in Hsp90 is not affected by sulforaphane, so direct obstruction of the binding interface for them is unlikely.

3.5. Proteolytic fingerprinting and LC-MS detect sulforaphane binding with Hsp90

We next examined the interaction of Hsp90 with sulforaphane using isolated protein domains. We first performed proteolytic fingerprinting assay to test if sulforaphane can bind to Hsp90 N-terminus. In the absence of sulforaphane, the N-terminal domain of Hsp90 (Hsp90N, Hsp90-P1-E245) was highly sensitive to trypsin digestion. Preincubation of sulforaphane with Hsp90N prevented it from trypsin hydrolysis, with a strong band representing intact Hsp90N even at a high concentration of trypsin (Fig. 5A). However, very slight difference was observed between control and treatment upon trypsin digestion when sulforaphane was incubated with Hsp90 C-terminus (Hsp90 β C, Hsp90 β -G529-D723) (Fig. 5B).

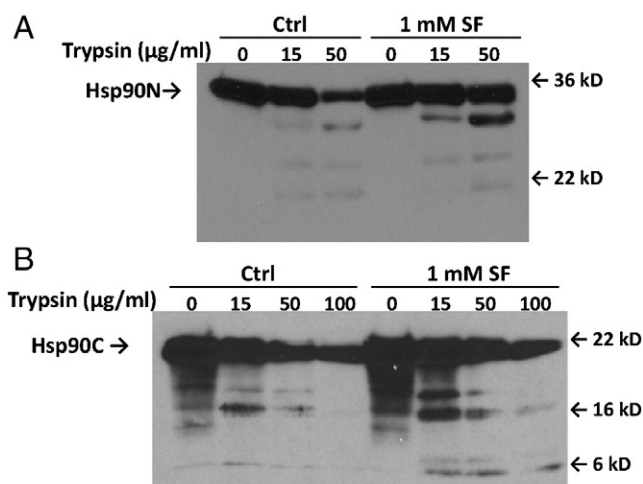


Fig. 5. Proteolytic fingerprinting assay and LC-MS analysis of Hsp90 interaction with sulforaphane. (A) After incubation with DMSO or sulforaphane, protein sample (Hsp90 β N) was digested with the indicated concentrations of trypsin. Hsp90 antibody (N-17), which detects N-terminus epitope of Hsp90, was used for immunoblotting. (B) Similarly, purified Hsp90 β C protein was incubated with DMSO or sulforaphane, followed by trypsin digestion. The Hsp90 (AC88) antibody was used for immunoblotting. (C) Purified Hsp90 β N was incubated with DMSO or sulforaphane (2 mM) for 30 min. Each sample was analyzed by LC-MS. (D) Purified Hsp90 β N protein was incubated with DMSO or sulforaphane (2 mM) for 30 min. The samples were digested with trypsin for 24 h at 37°C and analyzed by LC-MS.

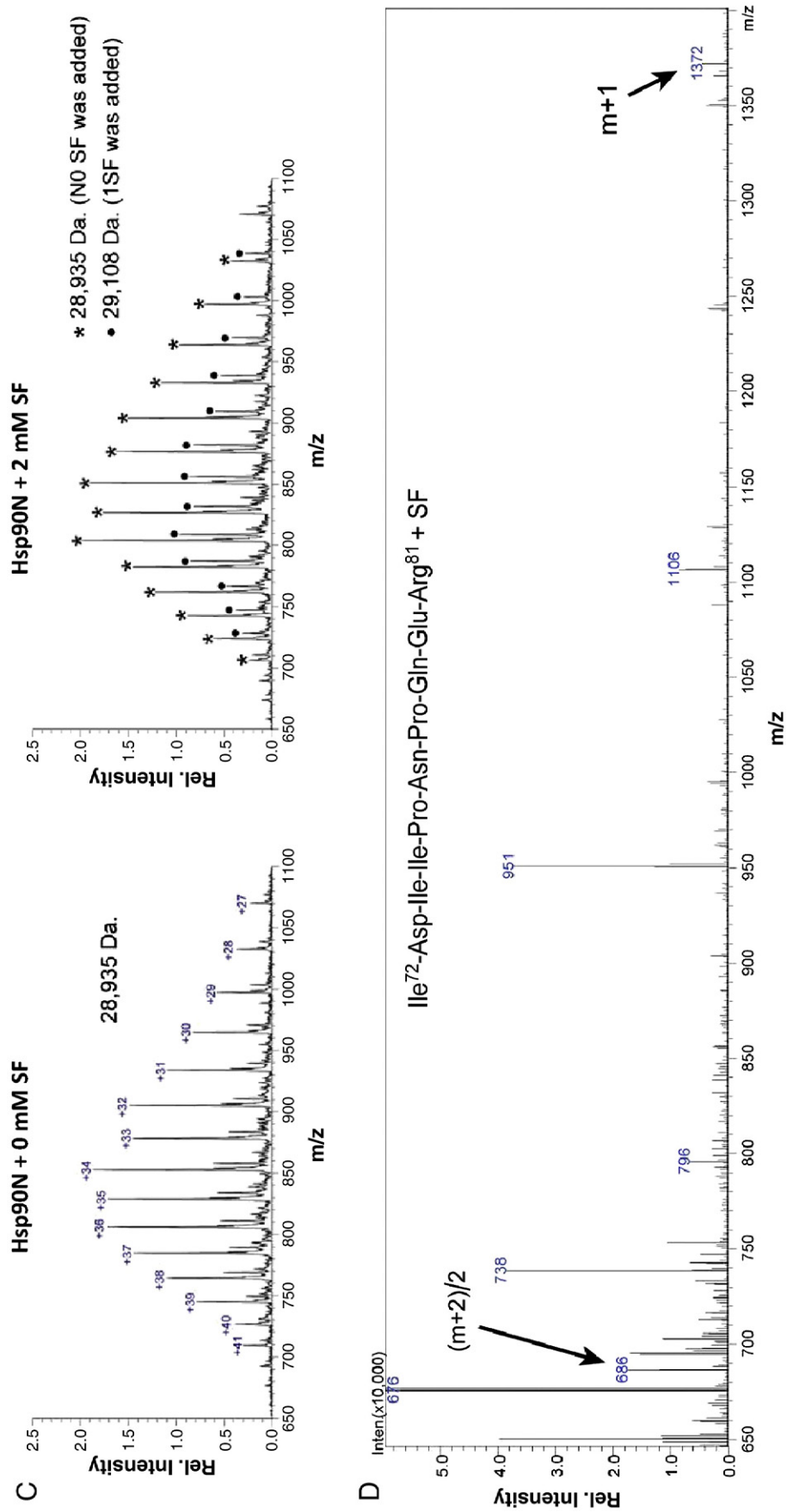


Fig. 5 (continued).

We next conducted LC-MS analysis of Hsp90N domain to characterize the interaction with sulforaphane. After incubation with 2 mM sulforaphane, the mass spectrum of Hsp90N exhibited a mass increase which corresponded to the molecular weight of one sulforaphane molecule (Fig. 5C). Further peptide mapping study indicated that a short peptide Ile 72–Arg 81 (IDIIPNPQER) was tagged with sulforaphane (Fig. 5D). The LC-MS data are in agreement with the NMR findings because chemical shift changes (Ile 74 and Ile 75) identified were located in this peptide. These results suggest that sulforaphane covalently interacts with Hsp90N.

4. Discussion

Pancreatic cancer is one of the leading causes of cancer death in the United States [22]. The currently available therapeutics (such as gemcitabine) have shown very limited success on treatment of this aggressive disease [23]. Given that pancreatic carcinogenesis is characterized by complex molecular basis that involved many oncogenic proteins, Hsp90, an essential molecular chaperone that regulates the stability and maturation of a wide range of oncogenic proteins, may provide advantages as a promising target for pancreatic cancer therapeutics [11,12]. Inhibition of Hsp90 may result in simultaneous degradation of multiple client proteins, such as kinases, hormone receptors and transcription factors, in cancers [12,13]. Among these proteins, phosphoinositide 3-OH kinase (PI3K)/Akt pathway and p53 mutant are important molecular targets in pancreatic cancer [24,25]. K-Ras mutation is known to occur in approximately 90% of human pancreatic ductal adenocarcinomas [22,26], and PI3K/Akt is a critical effector pathway of activated K-Ras [22,27]. Moreover, PI3K/Akt pathway is constitutively active in the majority of pancreatic cancer [22,28], and amplification or activation of Akt2 is encountered in up to 60% of pancreatic cancer [22,25,29]. Overexpression or activation of p53 mutant is a critical event in human pancreatic carcinogenesis as well [24]. Thus, interference with Hsp90 function and subsequent down-regulation of these client proteins provide a rational strategy to abrogate signaling against pancreatic cancer.

In the current study, we show that sulforaphane, a dietary component from broccoli/broccoli sprouts, inhibits Hsp90 chaperone function and promotes proteasomal degradation of Hsp90 client proteins (Fig. 1), resulting in pancreatic cancer cell death (Fig. 2). Sulforaphane has been reported as a potent chemoprevention agent, which provides many advantages over traditional Hsp90 inhibitors, including high bioavailability and low toxicity [6]. Sulforaphane from broccoli extracts is efficiently and rapidly absorbed in human small intestine and quickly distributed throughout the body [1,30]. Although the accumulation of sulforaphane in human pancreas tissue is not clear yet, plasma concentrations of sulforaphane equivalents reached micromolar concentrations in the blood after administration of a single dose of 200 μ mol broccoli sprout isothiocyanates (mainly sulforaphane) [31]. Clinical trials have shown that oral administration of 25 μ mol isothiocyanates (primarily sulforaphane) at 8-h intervals for 7 days or consumption of broccoli sprout solution containing 400 μ mol glucoraphanin (precursor of sulforaphane) daily for 2 weeks is well tolerated with no significant toxicity in human subjects [32,33].

Hsp90 consists of an N-terminal ATP-binding domain (Hsp90-P1-E245), a middle domain (Hsp90-K246-D528) and a C-terminal dimerization domain (Hsp90-G529-D723) [34,35]. Since Hsp90 chaperone function depends on the conformational changes driven by its ATPase activity [19], numerous Hsp90 inhibitors, represented by geldanamycin and 17-AAG, have been developed to inhibit its chaperone function by interfering with ATP binding [36]. These small molecules competitively bind to

the deep ATP pocket of Hsp90, thereby blocking the ATPase cycle and resulting in proteasomal degradation of its client proteins [37,38]. Although 17-AAG has entered clinical trials, its hepatotoxicity, low water solubility and low oral bioavailability may limit its application [39]. Recently, our group has demonstrated that disruption of Hsp90–p50^{Cdc37} complex inhibits Hsp90 chaperone function and induces degradation of Hsp90 client proteins, leading to anticancer activity [40–42].

In contrast to the classical Hsp90 inhibitors, sulforaphane impaired Hsp90 chaperone function by blocking formation of the Hsp90–p50^{Cdc37} complex (Figs. 1 and 3), and it did not enter the ATP binding pocket (Fig. 3A); therefore, the mechanism of action is clearly different from other drugs such as geldanamycin, 17-AAG or radicicol. Incubation of sulforaphane with full-length Hsp90 dimer induced multiple chemical shifts (Fig. 4). Higher concentration of sulforaphane was used to incubate with Hsp90 because previous studies have indicated that sulforaphane accumulated in cells when added to culture medium and resulted in millimolar concentrations in cellular lysates [43]. The shift of Ile 125, which belongs to the Hsp90–p50^{Cdc37} interface, may affect the interaction (Fig. 4E). Most of the other Ile shifts caused by sulforaphane do not belong to the interface of Hsp90–p50^{Cdc37} (Fig. 4). For example, sulforaphane interacts with the sheet 2 of Hsp90 N-terminal domain and its adjacent loop and covalently modifies the short peptide IDIIPNPQER covering that region (Figs. 4 and 5). This kind of interaction may induce dramatic allosteric effect. Using the isolated N-terminal domain of Hsp90, sulforaphane can protect the entire domain from trypsin digestion (Fig. 5A). Other mechanisms may be involved as well. Literature reported that sulforaphane can mediate chemopreventive effects through its ability to react with thiol groups of cysteine in target proteins [44], such as Keap1 [45] and cFos [44]. We mapped the six cysteine residues on Hsp90 and noted that only Cys 365 is to a minor extent solvent accessible; all others are entirely buried. The Cys 365 is close to the shifted isoleucine 369 in the middle domain identified by NMR (Fig. 4C). Two other shifted isoleucine 440 and 482 are close to Cys 520 (Fig. 4C). At present, no NMR assignment of the C-terminal domain is available, but we could identify three residues that could be allocated to the C-terminal domain. For those three residues, we did not observe sulforaphane-dependent shifts (Fig. 4A, B). The NMR data cannot fully exclude, though, that sulforaphane might interact with flexible areas in the C-terminus. The dynamic segments of Hsp90 correspond to the large area around the cross peak at 0.85 (¹H) and 12.5 (¹³C), which shows line broadening upon sulforaphane binding. This may indicate an effect of sulforaphane on the dynamics in Hsp90.

In comparison, our data suggest that sulforaphane did not affect Hsp90–p23^{Sba1} interaction (Figs. 3B and 4C), although p23^{Sba1} binds to a similar region of Hsp90 as p50^{Cdc37} does. The crystal structures of Hsp90–p50^{Cdc37} and Hsp90–p23^{Sba1} complex may provide some clues for the specificity. The p50^{Cdc37} binding Hsp90 is in an open conformation with small conformational changes compared with nucleotide-free structure [46]. The majority of the interaction involves Met 164, Leu 165, Ala 204 and Leu 205 of p50^{Cdc37}, and Ala 103, Ala 107, Ala 110, Gly 111, Ala 112, Met 116 and Phe 120 of Hsp90 [46]. Results of our recent split Renilla luciferase protein fragment-assisted complementation bioluminescence assay have shown that mutation of a single amino acid can reduce the Hsp90–p50^{Cdc37} interaction by 70%–95% [47]. Thus, it is not surprising that binding of sulforaphane to Hsp90 and p50^{Cdc37} resulted in disruption of the complex. By contrast, extensive interaction interface is involved in Hsp90–p23^{Sba1} complex when p23^{Sba1} binds to the ATP-bound, closed conformation of Hsp90, which may make this interface less sensitive to sulforaphane.

A very recent study found that sulforaphane regulated Hsp90 function by changing its acetylation status. Sulforaphane was shown to inhibit HDAC6 enzyme activity to induce hyperacetylation of Hsp90 in prostate cancer cells, thereby leading to destabilization of AR, a client protein of Hsp90 [8]. The data showed an increased amount of Hsp90 immunoprecipitated with acetyl-lysine. However, we did not observe Hsp90 hyperacetylation with immunoprecipitation of either acetyl-lysine (followed by immunoblotting of Hsp90) or Hsp90 (followed by immunoblotting of acetyl-lysine) in pancreatic cancer cells. This discrepancy could come from different cancer cells or different experimental settings. In addition, previous studies showed that HDAC6 inhibition by HDAC6 knockdown and LBH589 decreased ATP binding to Hsp90 and Hsp90–p23^{Sba1} complex, but not Hsp90–p50^{Cdc37} interaction [48]. Our data suggest that sulforaphane inhibits Hsp90 and disrupts Hsp90–p50^{Cdc37} complex through a different mechanism than HDAC inhibition, in which the direct modification of specific amino acid residues of Hsp90 is one of the plausible mechanisms to block the Hsp90–p50^{Cdc37} interaction.

It is worth noting that sulforaphane may modify other proteins in the Hsp90 complex. For instance, a very recent proteomics study has shown Hsp70 as a potential protein target for sulforaphane modification [49]. These new findings warrant further investigation for the new molecular targets of sulforaphane.

In summary, our study has shown that sulforaphane inhibits pancreatic cancer cell growth *in vitro* and suppresses tumor growth *in vivo* in xenograft mouse models. We revealed a mechanism indicating that sulforaphane directly interacts with specific amino acid residues of Hsp90, disrupts Hsp90–p50^{Cdc37} complex, inhibits Hsp90 chaperone function and induces degradation of Hsp90 client proteins.

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