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Sulforaphane Potentiates the Efficacy of 17-Allylamino 17-Demethoxygeldanamycin Against Pancreatic Cancer Through Enhanced Abrogation of Hsp90 Chaperone Function

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Abstract

Heat shock protein 90 (Hsp90), an essential molecular chaperone that regulates the stability of a wide range of oncogenic proteins, is a promising target for cancer therapeutics. We investigated the combination efficacy and potential mechanisms of sulforaphane, a dietary component from broccoli and broccoli sprouts, and 17-allylamino 17-demethoxygeldanamycin (17-AAG), an Hsp90 inhibitor, in pancreatic cancer. MTS assay demonstrated that sulforaphane sensitized pancreatic cancer cells to 17-AAG in vitro. Caspase-3 was activated to 6.4-fold in response to simultaneous treatment with sulforaphane and 17-AAG, whereas 17-AAG alone induced caspase-3 activity to 2-fold compared to control. ATP binding assay and coimmunoprecipitation revealed that sulforaphane disrupted Hsp90-p50^{Cdc37} interaction, whereas 17-AAG inhibited ATP binding to Hsp90. Concomitant use of sulforaphane and 17-AAG synergistically downregulated Hsp90 client proteins in Mia Paca-2 cells. Co-administration of sulforaphane and 17-AAG in pancreatic cancer xenograft model led to more than 70% inhibition of the tumor growth, whereas 17-AAG alone only suppressed the tumor growth by 50%. Our data suggest that sulforaphane potentiates the efficacy of 17-AAG against pancreatic cancer through enhanced abrogation of Hsp90 function. These findings provide a rationale for further evaluation of broccoli/broccoli sprout preparations combined with 17-AAG for better efficacy and lower dose-limiting toxicity in pancreatic cancer.

INTRODUCTION

Pancreatic cancer, an aggressive malignancy, is the 4th leading cause of cancer death in the United States (1), and the overall 5-yr survival rate after diagnosis for pancreatic cancer patients is below 5% (2). Currently available therapeutics such as surgery, chemotherapy, and radiotherapy have shown very limited success on treatment of this aggressive disease (3). Since a large number of epidemiological studies have demonstrated an association between the reduced risk of various cancers and consumption of fruits and vegetables, naturally occurring dietary compounds have been tested for cancer chemoprevention. For example, a recent study found that curcumin potentiates anti-cancer activity of gemcitabine in pancreatic cancer mouse model through inhibition of NF- κ B target genes, cell proliferation, and angiogenesis (4).

Numerous studies have substantiated the protective effect of high consumption of cruciferous vegetables, especially broccoli/broccoli sprouts, against carcinogenesis and cancer progression (5,6). These effects have been attributed to the activity of isothiocyanates that are converted from glucosinolates (5,6). In particular, sulforaphane was found to be derived from glucoraphanin, a major glucosinolate in broccoli/broccoli sprouts (7). Previous studies suggest that sulforaphane modulates multiple targets, such as NF- κ B, Chk2, p21, MAPK, death receptor, histone deacetylase, Stat3, Nrf2, and β -catenin (6,8–10), which regulates diverse cellular activities including oxidative stress, apoptosis induction, cell cycle arrest, angiogenesis and metastasis suppression (6,11). In addition, the chemo-prevention efficacy of sulforaphane is also linked to the induction of phase II metabolism enzymes and inhibition of phase I metabolism enzymes, which enhances the detoxification of carcinogens (6,9). Sulforaphane was shown to inhibit cell viability in Panc-1 cell line (12). Very recently, sulforaphane has been demonstrated to enhance the effect of sorafenib against pancreatic cancer (13).

Given that pancreatic carcinogenesis is characterized by complex molecular basis that are involved with many oncogenic proteins, heat shock protein 90 (Hsp90), an essential molecular chaperone that regulates the stability and maturation of a wide range of oncogenic client proteins, has recently emerged as a promising target for pancreatic cancer therapeutics (14,15). Inhibition of Hsp90 may result in simultaneous degradation of multiple oncogenic client proteins, such as kinases, hormone receptors, and transcription factors in cancers (15,16). Among these proteins, phosphoinositide 3-OH kinase (PI3K)/Akt pathway and p53 mutant are important molecular targets in pancreatic cancer (17–21). PI3K/Akt pathway is constitutively active in the majority of pancreatic cancer (18,21); and amplification or activation of Akt2 occurred in 60% of pancreatic cancer (18–20). Moreover, PI3K/Akt is a critical effector pathway of activated K-Ras (18,22,23). K-Ras mutation is known to occur in approximately 90% of human pancreatic ductal adenocarcinomas (18,24). In addition, overexpression or activation of p53 mutant is an important event in human pancreatic carcinogenesis (17). Thus, interference with Hsp90 function and subsequent down-regulation of these client proteins provides a rational strategy to abrogate signaling against pancreatic cancer.

Since Hsp90 chaperone function depends on the conformational changes driven by its ATPase activity (25), an array of Hsp90 inhibitors have been developed to inhibit its chaperone function by interacting with the N-terminal ATP binding domain (26). 17-Allylamino 17-demethoxygeldanamycin (17-AAG), one of the most well-known Hsp90 inhibitors, competitively binds to N-terminal ATP pocket of Hsp90, and induces a conformational change in the Hsp90 molecule, leading to pro-teasomal degradation of its client proteins (26). 17-AAG has been evaluated in a great deal of preclinical studies and clinical trials; however, hepatotoxicity seems to be the most significant dose-limiting factor

for its application as a single agent (27). Thus, a more appropriate strategy is to combine 17-AAG with other agent(s) to lower 17-AAG dose and to achieve better anticancer effect (27). Indeed, 17-AAG is currently evaluated in clinical trials as part of combination therapy for solid tumors and hematological malignancies (27).

The formation of Hsp90-p50^{Cdc37} complex is required for Hsp90 activity (14,15). As a cochaperone, p50^{Cdc37} acts as a critical molecular adaptor to load protein kinases to the Hsp90 chaperone machinery (14,28). Most of the Hsp90 clients that are associated with p50^{Cdc37} are crucial components in signal transduction, cell proliferation and survival (14). Our study revealed that sulforaphane was able to disrupt Hsp90-p50^{Cdc37} complex, thereby inhibiting Hsp90 chaperone function and inducing degradation of Hsp90 client proteins in pancreatic cancer cells. We further demonstrated that sulforaphane could enhance 17-AAG-induced inhibition of Hsp90 chaperone function, thereby potentiating the therapeutic efficacy of 17-AAG against pancreatic cancer.

MATERIALS AND METHODS

Cell Culture

Human pancreatic cancer cell line Mia Paca-2 was maintained in DMEM medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (Fisher Scientific, Pittsburgh, PA) and 1% penicillin-streptomycin-glutamine (Invitrogen, Carlsbad, CA). Human pancreatic cancer cell line Panc-1 was maintained in RPMI1640 medium (Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin (Invitrogen, Carlsbad, CA).

Reagents

Sulforaphane was purchased from LKT Laboratories (St. Paul, MN), and 17-AAG was obtained from LC Laboratories (Woburn, MA). Drugs were dissolved in DMSO as a stock solution. The following antibodies were used for immunoblotting: Akt (Cell Signaling, Beverly, MA), p23 (Abcam, Cambridge, MA), Raf-1, p53 mutant, Cdk4, p50^{Cdc37}, Hsp90, and β -actin (Santa Cruz Biotechnology, Santa Cruz, CA).

MTS Cell Proliferation Assay

Cells were seeded in 96-well microplates at a density of 3,000 to 5,000 cells per well. Cells were treated with increasing concentrations of 17-AAG with or without 5 μ M sulforaphane as indicated. After 48-h incubation, cell viability was assessed by MTS assay (Promega, Madison, WI) according to the manufacturer's instructions. The number of living cells in the culture is directly proportional to the absorbance at 490 nm of a formazan product reduced from MTS by living cells.

Caspase-3 Activity Assay

Mia Paca-2 cells were treated with 10 μ M sulforaphane, 0.1 μ M 17-AAG, or combination of the two drugs and collected after 24 h. The caspase-3 activity assay was based on the manufacturer's instructions of Caspase-3/CPP32 Fluorometric Assay Kit (Biovision Research Products, Mountain View, CA). Cellular protein was extracted with the supplied lysis buffer, followed by determination of protein concentration using BCA Protein Assay Reagents (Pierce, Rockford, IL). The cleavage of DEVD-AFC, a substrate of caspase-3, was quantified by using a fluorescence microtiter plate reader with a 400-nm excitation filter and a 505-nm emission filter.

Western Blotting Analysis

The procedure for Western blotting analysis was briefly described below. After treated with 10 μ M sulforaphane, 0.1 μ M 17-AAG, or combination of the two drugs for 24 h, cells were harvested by washing twice with ice-cold PBS, collected in RIPA lysis buffer (20 mM Tris-HCl, 150 mM NaCl, 1% NP-40, 5 mM EDTA, 1 mM Na_3VO_4 , pH 7.5) supplemented with a protease inhibitor cocktail (Pierce, Rockford, IL), and incubated on ice for 20–30 min. Afterwards, cell lysate was centrifuged at 14,000 rpm for 15 min at 4°C, and the supernatant was recovered. Protein concentration was determined with BCA Protein Assay Reagents (Pierce, Rockford, IL). Equal amounts of protein were subject to SDS-PAGE and transferred to PVDF membrane (BioRad, Richmond, CA). The membrane was then incubated with appropriate primary antibodies at 4°C overnight, followed by 2-h incubation with secondary antibodies at room temperature.

ATP-Sepharose Binding Assay

The ATP-Sepharose binding assay was similar to that in a previous report (29). Cells were treated with 15 μ M sulforaphane or 5 μ M 17-AAG for 24 h, and then lysed in TNESV buffer (50 mM Tris, 2 mM EDTA, 100 mM NaCl, 1 mM Na_3VO_4 , 25 mM NaF, 1% Triton X-100, pH 7.5) supplemented with protease inhibitors for 30 min. After centrifugation, supernatant was recovered and protein concentrations were determined with BCA Protein Assay Reagents. Protein (200 μ g) was incubated with 25 μ l pre-equilibrated γ -phosphate-linked ATP-sepharose (Jena Bioscience GmbH, Jena, Germany) in 200 μ l incubation buffer (10 mM Tris-HCl, 50 mM KCl, 5 mM MgCl_2 , 20 mM Na_2MoO_4 , 0.01% NP-40, pH 7.5) overnight at 4°C. The sepharose beads were washed 4 times with incubation buffer, and the bound proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Hsp90 Coimmunoprecipitation

The Hsp90 coimmunoprecipitation was similar to that in a previous report (29). Cells were treated with 15 μ M sulforaphane or 5 μ M 17-AAG for 24 h, and then lysed in 20 mM Tris-HCl (pH 7.4), 25 mM NaCl, 2 mM DTT, 20 mM Na_2MoO_4 , 0.1% NP-40, and protease inhibitors. After centrifugation, supernatant was recovered and protein concentrations were determined with BCA Protein Assay Reagents. Protein (500 μ g) was first incubated with H9010 antibody (Axxora, San Diego, CA) for 1 h at 4°C, followed by overnight incubation with protein A/G agarose (Santa Cruz Biotechnology, Santa Cruz, CA). The bound proteins were resolved by SDS-PAGE and analyzed by Western blotting.

Pancreatic Tumor Xenograft

The animal study protocol was approved by the University Committee on Use and Care of Animals (UCUCA) at the University of Michigan. Four- to six-week-old athymic (nu/nu) female mice were obtained from NCI (National Cancer Institute at Frederick). Mia-Paca-2 cells (5×10^6 – 10×10^6) mixed with Matrigel (BD Biosciences, San Jose, CA) were implanted subcutaneously into the right and left flanks of the mice. Tumor volumes were calculated with $V = 1/2 (\text{width}^2 \times \text{length})$. After 2 wk, tumor volumes reached 100–150 mm^3 . Mice were randomized into 4 groups for treatment, with 6 animals each group. The mice were i.p. injected with vehicle, 25 mg/kg 17-AAG (3 times per wk), 25 mg/kg sulforaphane (5 times per wk), or combination of 2 for 4 wk. Sulforaphane was dissolved in saline whereas 17-AAG in 10% DMSO, 70% cremophor/ethanol (3:1), and 20% PBS (30). Tumor growth was monitored twice a week and normalized to the initial volumes. Mice were humanely euthanized at the end of drug treatment.

Evaluation of Combination Effect

The combination effect of sulforaphane and 17-AAG was determined by combination index (CI) according to the literature (31). The CI value was calculated using the equation: $CI_{50} = D_{1,comb}/D_1 + D_{2,comb}/D_2$; in which D_1 and D_2 are drug concentrations that produce 50% of cell growth inhibition when used alone; $D_{1,comb}$ and $D_{2,comb}$ are drug concentrations that produce 50% of effect when used in combination. The synergism, additivity, and antagonism of the combination will be shown when CI is less than, equal to, or greater than 1, respectively.

Statistical Analysis

All experiments were performed independently at least 3 times. Statistical analysis was performed using Student *t*-test. Data are presented as mean $\pm$ SD ($n = 3$, $P < 0.01$ or 0.05).

RESULTS

Sulforaphane Sensitizes Pancreatic Cancer Cells to 17-AAG In Vitro

In order to examine the anticancer effect of the combined treatment of sulforaphane and 17-AAG in pancreatic cancer cells, we incubated Mia Paca-2 and Panc-1 cells with these drugs alone or in combination. As shown in Fig. 1A, sulforaphane inhibited the cell proliferation of Mia Paca-2 with an IC_{50} approximately $13 \mu M$. Similarly, this compound suppressed Panc-1 growth with an IC_{50} around $14 \mu M$ (Fig. 1B). Thus, the concentration of sulforaphane chosen for combination treatment was $5 \mu M$ at which below 10% of pancreatic cancer cells were eliminated. Sulforaphane significantly potentiated the antiproliferative effect of 17-AAG in both cell lines, with the CI values approximately 0.62 and 0.87 for Mia Paca-2 and Panc-1 cells, respectively (Table 1). In Mia Paca-2 cells, the IC_{50} of 17-AAG ($0.07 \pm 0.03 \mu M$) when combined with $5 \mu M$ of sulforaphane was more than 4-fold lower than the IC_{50} of 17-AAG alone ($0.31 \pm 0.15 \mu M$). Panc-1 cell line was resistant to 17-AAG with IC_{50} of $10.59 \pm 5.81 \mu M$. The resistance was attenuated in the presence of sulforaphane; the IC_{50} of 17-AAG ($5.47 \pm 0.83 \mu M$) when combined with $5 \mu M$ of sulforaphane was about 2-fold lower than the IC_{50} of 17-AAG alone in Panc-1 cells. These data suggest that sulforaphane significantly enhanced the antiproliferative effect of 17-AAG.

To further confirm the enhanced effect of combination of sulforaphane and 17-AAG against pancreatic cancer cells, we measured the apoptosis by caspase-3 activity in Mia Paca-2 cells. While $0.1 \mu M$ of 17-AAG alone and $10 \mu M$ of sulforaphane alone induced caspase-3 activity to only 2-fold and 3.4-fold, the combination of the 2 drugs achieved a dramatic increase of caspase-3 activity to 6.4-fold (Fig. 2). These results demonstrated that sulforaphane sensitized pancreatic cancer cells to 17-AAG in vitro.

Sulforaphane Blocks Hsp90-p50^{Cdc37} Interaction While 17-AAG Inhibits ATP Binding to Hsp90

17-AAG is well known to inhibit Hsp90 activity by blocking N-terminal ATP binding pocket of Hsp90. Our preliminary studies suggest that sulforaphane can inhibit Hsp90 through an ATP-binding independent manner and may directly interact with Hsp90 (unpublished data) (32). Therefore, we performed ATP-sepharose binding assay and Hsp90 co-immunoprecipitation to further confirm that sulforaphane and 17-AAG interfere with Hsp90 chaperone function through different mechanisms.

As shown in Fig. 3A, $5 \mu M$ of 17-AAG dramatically blocked ATP binding to Hsp90, which was evidenced by the decreased amount of Hsp90 in ATP-sepharose binding assay. In contrast, $15 \mu M$ of sulforaphane did not alter the amount of Hsp90 pulled down by ATP sepharose beads.

On the other hand, sulforaphane significantly abrogated the interaction between Hsp90 and p50^{Cdc37}, whereas 17-AAG had no effect on Hsp90-p50^{Cdc37} complex formation (Fig. 3B). In Fig. 3B, immunoprecipitation (IP) of Hsp90 by its antibody also pulled down cochaperones that were associated with Hsp90. Sulforaphane (15 μ M) largely eliminated p50^{Cdc37} in the immunoprecipitated complex, whereas 17-AAG (5 μ M) did not change the p50^{Cdc37} level in the IP assay. Another cochaperone, p23, has been demonstrated to associate with ATP-bound conformation of Hsp90 (33). 17-AAG binds to the ATP pocket and locks the Hsp90 molecule in the intermediate multichaperone complex (29,34), hence Hsp90 will no longer be available for p23 binding (35,36). As shown in Fig. 3B, 17-AAG decreased the interaction between Hsp90 and p23, whereas sulforaphane did not affect Hsp90-p23 complex formation. These were inconsistent with the results of ATP-Sepharose binding assay. These data suggest that sulforaphane may enhance 17-AAG-induced inhibition of Hsp90 chaperone function through disruption of Hsp90-p50^{Cdc37} interaction.

17-AAG Induces More Degradation of Hsp90 Client Proteins in the Presence of Sulforaphane in Pancreatic Cancer Cells

In order to further investigate the combination effect of sulforaphane and 17-AAG on Hsp90 chaperone function, we tested low concentrations of sulforaphane and 17-AAG for their impact on the levels of Hsp90 oncogenic client proteins using Western blotting. Here, the concentration of sulforaphane we chose was 10 μ M, which showed a moderate effect on the destabilization of these proteins compared to control (29%, 43%, 14%, and 14% for Akt, p53 mutant, Raf-1, and Cdk4, respectively) (Fig. 4). As shown in Fig. 4, in comparison with 17-AAG alone, concomitant use of sulforaphane and 17-AAG further downregulated Akt, p53 mutant, Raf-1, and Cdk4 in Mia Paca-2 cells by 78%, 87%, 93%, and 44%, respectively. These data indicate that 17-AAG induced more degradation of Hsp90 client proteins that are critical to pancreatic carcinogenesis in the presence of sulforaphane, which may contribute to the enhanced effect against pancreatic cancer cells in vitro.

Sulforaphane Potentiates the Therapeutic Efficacy of 17-AAG in Pancreatic Cancer Xenograft Model In Vivo

To test the combination anticancer efficacy of sulforaphane and 17-AAG in vivo, we evaluated them in a pancreatic cancer xenograft model. It has been reported in the literatures that 17-AAG (50–100 mg/kg) (37,38) and sulforaphane (50–100 mg/kg) (39) exhibited anticancer activity against various cancers. In order to examine the combined effect, we selected relatively low doses of sulforaphane and 17-AAG that exhibit only moderate effects when they are used alone. Two weeks after subcutaneous implantation of Mia Paca-2 cells, we injected 25 mg/kg 17-AAG 3 times per wk or 25 mg/kg sulforaphane 5 times per wk for 4 wk. In addition, we administered both drugs in a group of mice with the same dose regimen. The tumor growth rates were compared across different treatment groups. As shown in Fig. 5A, individual treatment with sulforaphane and 17-AAG suppressed the tumor growth by approximately 45% and 50%, respectively, compared to control group. In contrast, combination treatment with sulforaphane and 17-AAG led to about 70% inhibition of the tumor growth, compared to control group (Fig. 5A). The final tumor volume of the combination group was significantly smaller than that of the individual treatment groups. Meanwhile, neither sulforaphane nor 17-AAG at the administered dose regimen had apparent toxicity as determined by body weight measurement (Fig. 5B). These data indicate that combination of sulforaphane and 17-AAG enhanced their anticancer efficacy.

DISCUSSION

Concomitant use of chemoprevention agents with no or low toxicity has been suggested to be a potential strategy to enhance chemotherapy effect (40,41). Sulforaphane has shown

promise in this regard with several therapeutic agents. Coadministration of sulforaphane and doxorubicin in mouse fibroblasts enhanced the efficacy of doxorubicin (41). Shankar et al. demonstrated that sulforaphane enhanced the therapeutic potential of tumor necrosis factor-related apoptosis-inducing ligand TRAIL against prostate cancer (42). Moreover, as a chemo-prevention agent, sulforaphane possesses many advantages, including high bioavailability and low toxicity (11). Sulforaphane from broccoli extracts is efficiently and rapidly absorbed in human small intestine, reaching the plasma concentration peak 0.94–2.27 μM in humans, and quickly distributed throughout the body (6,43,44). Treatment of lymphocytes with 0.01 to 100 μM of sulforaphane for 24 h did not significantly affect cell viability (45). Sulforaphane was shown to be nontoxic to human primary skin fibroblasts, umbilical vein endothelial cells, and embryonic kidney cells (46). Sulforaphane at concentrations below 10 μM did not show significant effect on cell apoptosis induction of human non-transformed T-lymphocytes (47). The growth of normal breast cells (MCF10A) was not affected by up to 10 μM sulforaphane (48). Higher concentrations of sulforaphane may affect the growth of normal cells; however, we do not expect to see significant toxicity in humans because sulforaphane usually cannot reach such high concentrations in vivo. Indeed, clinical trials have shown that oral administration of 25 μmol isothiocyanates (primarily sulforaphane) at 8-hr intervals for 7 days or consumption of broccoli sprout solution containing 400 μmol glucoraphanin (precursor of sulforaphane) nightly for 2 wk caused no significant toxicity in human subjects (49,50).

Although 17-AAG has been evaluated in a great deal of pre-clinical studies and clinical trials, several drawbacks of 17-AAG including toxicity, low water solubility, instability in solution, and low oral bioavailability prevent its use as a single agent (51). In particular, hepatotoxicity is the most significant dose-limiting factor of 17-AAG (27). Given that different Phase I and II clinical trials have reported a significant inconsistency in the adequacy of Hsp90 inhibition by 17-AAG as a single agent (27), it is more effective to combine 17-AAG with other agent(s) to elicit an improved anticancer response while reducing the dose-limiting toxicity (27). For example, the combination of rapamycin and 17-AAG abolished Akt activation and potentiated mTOR blockade in breast cancer cells (52). Administration of 17-AAG and carboplatin in specific sequences produced a more pronounced growth inhibitory effect in human ovarian cancer models (53).

Collectively, these studies provide a strong basis for investigating the combinatorial efficacy of sulforaphane and 17-AAG against pancreatic cancer. Herein, utilizing in vitro pancreatic cancer cells and in vivo pancreatic cancer xenograft model, we have shown that sulforaphane potentiates the therapeutic efficacy of 17-AAG against pancreatic cancer. This finding provides a rationale for further preclinical and clinical investigation of sulforaphane or broccoli/broccoli sprout preparations as a supplement to 17-AAG to sensitize pancreatic cancer and elicit a more pronounced clinical response.

The chaperoning cycle of Hsp90 depends on an ordered assembly and disassembly of co-chaperones that preferentially bind to a specific conformation of Hsp90 to modulate its ATPase activity (15). As revealed by the crystal structure, p50^{Cdc37} facilitates the client loading to Hsp90 by inserting its C-terminal side chain into the nucleotide binding pocket of Hsp90; binding of ATP to Hsp90 would have to eject this side chain of p50^{Cdc37} out of ATP pocket (15). Our previous study has demonstrated that p50^{Cdc37} can only bind ADP-bound, open conformation of Hsp90 but not ATP-bound Hsp90 (36). Hence, it is possible that p50^{Cdc37} is removed from the early Hsp90 complex after the binding of ATP to Hsp90 (36). On the other hand, it was demonstrated that p23 is recruited to the ATP-bound, closed conformation of Hsp90 and locks it in an ATP-dependent conformational state that has high affinity for client proteins (54). The unaltered level of Hsp90-p23 complex indicates that either the chaperone cycle still proceeds to later stages without client folding or some client proteins

are loaded onto Hsp90 without the help of p50^{Cdc37} but require p23 for maturation. The latter hypothesis may hold true because the majority of p50^{Cdc37}-associated clients are kinases (28). Given that 17-AAG inhibits binding of ATP to Hsp90, it is reasonable to expect sulforaphane and 17-AAG to collaborate to achieve a more pronounced inhibition of Hsp90 chaperone function.

Our previous studies revealed that sulforaphane could block Hsp90-p50^{Cdc37} association through direct binding to Hsp90 and p50^{Cdc37} (unpublished data) (32). The present study extends these observations by demonstrating that sulforaphane-induced Hsp90-p50^{Cdc37} disruption improves 17-AAG-induced inhibition of Hsp90 function, leading to synergistic depletion of Hsp90 client proteins. Moreover, sulforaphane may be able to enhance the metabolism of 17-AAG to 17-AAGH₂ through induction of NQO1; 17-AAGH₂ is a more potent Hsp90 inhibitor compared to 17-AAG (55,56). In addition, sulforaphane modulates a plethora of cellular activities of cancer cells by regulating a variety of molecules such as NF- κ B, Chk2, p21, MAPK, death receptor, histone deacetylase, Stat3, Nrf2, and β -catenin (6,8–10). All of them may contribute to the potentiated therapeutic efficacy of 17-AAG against pancreatic cancer. A very recent study found that sulforaphane-induced hyperacetylation of Hsp90 by inhibiting HDAC6 enzyme activity in prostate cancer cells, thereby leading to destabilization of androgen receptor (AR), a client protein of Hsp90 (57). Although there has been no evidence for this activity of sulforaphane in pancreatic cancer cells, hyperacetylation of Hsp90 could be another potential mechanism for the improved abrogation of Hsp90 function.

In conclusion, we have demonstrated that coadministration of 17-AAG with sulforaphane can enhance 17-AAG-induced inhibition of Hsp90 chaperone function, thereby potentiating anticancer efficacy in pancreatic cancer xenograft model. Our study identified the disruption of Hsp90-p50^{Cdc37} interaction by sulforaphane as a potential mechanism for the combinatorial effect against pancreatic cancer. These findings provide a rationale for further preclinical and clinical evaluation of sulforaphane or broccoli/broccoli sprout preparations combined with 17-AAG for better efficacy and lower dose-limiting toxicity of 17-AAG in pancreatic cancer.

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FIG. 1.

Antiproliferative effect of sulforaphane (SF), 17-allylamino 17-demethoxygeldanamycin (17-AAG), or the combination against pancreatic cancer cells. A: Mia Paca-2 cells growing in log phase were treated with increasing concentrations of sulforaphane (▲: 0, 1, 2, 5, 10, 15, 25, and 50 μ M), 17-AAG (◆: 0, 0.001, 0.005, 0.01, 0.1, and 0.5 μ M), or the combination of 5 μ M sulforaphane plus different concentrations of 17-AAG (■: 0, 0.001, 0.005, 0.01, 0.1, and 0.5 μ M) for 48 h. B: Similarly, Panc-1 cells growing in log phase were treated with increasing concentrations of sulforaphane (▲: 0, 2, 5, 10, 15, 25, and 50 μ M), 17-AAG (◆: 0, 0.01, 0.1, 1, 2, 5, and 10 μ M), or the combination of 5 μ M sulforaphane plus different concentrations of 17-AAG (■: 0, 0.001, 0.005, 0.01, 0.1, and 0.5 μ M) for 48 h. Cell viability was assessed by MTS assay.

FIG. 2. Effect of combination of sulforaphane (SF) and 17-allylamino 17-demethoxygeldanamycin (17-AAG) on caspase-3 activation in pancreatic cancer cells. Mia Paca-2 cells were treated with sulforaphane (10 μ M), 17-AAG (0.1 μ M), or combination of the two drugs for 24 h. Cell lysates were prepared for caspase-3 activity. Results are expressed as fold increase in caspase-3 activity in comparison to control. * $P < 0.01$ when compared with single treatment.

FIG. 3. Impact of sulforaphane (SF) and 17-allylamino 17-demethoxy geldanamycin (17-AAG) on ATP binding to Hsp90 and Hsp90-cochaperone association in pancreatic cancer cells. Mia Paca-2 cells were treated with sulforaphane (15 μ M), 17-AAG (5 μ M), or combination of the 2 drugs for 24 h. A: The amount of Hsp90 pulled down by the ATP-sepharose beads was determined by ATP binding assay. B: After immunoprecipitation of Hsp90 with H9010 antibody, the amounts of cochaperones interacting with Hsp90 were measured by Western blotting.

FIG. 4. Effect of coadministration of sulforaphane (SF) and 17-allylamino 17-demethoxygeldanamycin (17-AAG) on Hsp90 client proteins in pancreatic cancer cells. Mia Paca-2 cells were treated with sulforaphane (10 μ M), 17-AAG (0.1 μ M), or combination of the 2 drugs for 24 h. The level of oncogenic client proteins was measured with Western blotting and quantified with Image J software (NIH, Bethesda, MD). * $P < 0.01$ when compared with single treatment.

FIG. 5.

Evaluation of sulforaphane (SF) and 17-allylamino 17-demethoxygeldanamycin (17-AAG) combination in pancreatic cancer xenograft model. Mia Paca-2 cells ($5 \times 10^6 - 10 \times 10^6$) mixed with Matrigel were implanted subcutaneously into the right and left flanks of 4- to 6-wk-old athymic (nu/nu) mice. After 2 wk, tumor volumes reached 100–150 mm³. Mice were randomized into 4 groups, with 6 animals each. The mice were i.p. injected with vehicle, 25 mg/kg 17-AAG (3 times per wk), 25 mg/kg SF (5 times per wk), or combination of the 2 drugs for 4 wk. A: Tumor growth was monitored twice a week and normalized to the initial volumes. * $P < 0.01$ when compared with individual treatment of SF. ** $P < 0.05$ when compared with individual treatment of 17-AAG. B: Body weight was measured twice a week and normalized to the initial body weight of control group.

TABLE 1

Synergy between 17-AAG and sulforaphane in pancreatic cancer cells Mia Paca-2 and Panc-1

Cell lines	IC ₅₀ of 17-AAG alone (μM)	IC ₅₀ of SF alone (μM)	IC ₅₀ of 17-AAG when combined with 5 μM SF (μM)	Combination index (CI) [†]
Mia Paca-2	0.31 ± 0.15	13.08 ± 0.91	0.07 ± 0.03 *	0.62
Panc-1	10.59 ± 5.81	14.11 ± 1.24	5.47 ± 0.83 *	0.87

* $P < 0.01$ when compared with single treatment of 17-AAG.

[†] We first determined the IC₅₀ values by fitting the data from MTS cell proliferation assay (Fig. 1) with WinNonlin software, and then calculated the combination index (CI) according to the literature [31]. The CI value was calculated using the equation: $CI_{50} = D_{1,comb}/D_1 + D_{2,comb}/D_2$; in which D₁ and D₂ are drug concentrations that produce 50% of cell growth inhibition when used alone; D_{1,comb} and D_{2,comb} are drug concentrations that produce 50% of effect when used in combination. The synergism, additivity, and antagonism of the combination will be shown when CI is less than, equal to, or greater than 1, respectively.