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MEK Inhibition Potentiates the Activity of Hsp90 Inhibitor 17-AAG against Pancreatic Cancer Cells

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Abstract: The Ras/Raf/MEK/ERK signaling has been implicated in uncontrolled cell proliferation and tumor progression in pancreatic cancer. The purpose of this study is to evaluate the antitumor activity of MEK inhibitor U0126 in combination with Hsp90 inhibitor 17-allylamino-17-demethoxy-geldanamycin (17-AAG) in pancreatic cancer cells. Western blotting showed that 17-AAG caused a 2- to 3-fold transient activation of MEK/ERK signaling in pancreatic cancer cells. The activation sustained for 6 h before phospho-ERK (p-ERK) destabilization. The selective MEK inhibitor U0126 completely abolished 17-AAG induced ERK1/2 activation and resulted in more than 80% of phospho-ERK degradation after only 15 min treatment. Moreover, U0126 had complementary effect on 17-AAG regulated oncogenic and cell cycle related proteins. Although 17-AAG downregulated cyclin D1, cyclin E, CDK4 and CDK6, it led to cyclin A and CDK2 accumulation, which was reversed by the addition of U0126. Antiproliferation assay showed that combination of U0126 and 17-AAG resulted in synergistic cytotoxic effect. More importantly, 17-AAG alone only exhibited moderate inhibition of cell migration *in vitro*, while addition of U0126 dramatically enhanced the inhibitory effect by 2- to 5-fold. Taken together, these data demonstrate that MEK inhibitor U0126 potentiates the activity of Hsp90 inhibitor 17-AAG against pancreatic cancer cells. The combination of Hsp90 and MEK inhibition could provide a promising avenue for the treatment of pancreatic cancer.

Keywords: Hsp90; MEK; ERK; 17-AAG

Introduction

Pancreatic cancer is the fourth leading cause of cancer death in the United States.[1] It is an aggressive and devastating disease, and most patients diagnosed with pancreatic cancer die of their disease within months.[2] Despite efforts in the past several decades, conventional treatments such as surgery, radiation, chemotherapy, or the combination of these have had little impact on the progression of this disease.[3] The average one-year and five-year survival rates of pancreatic cancer patients are merely 24% and 5%, respectively.[4] The low survival rate of pancreatic cancer patients is attributable to the fact that less than 20% of patients have confined tumors in pancreas at the time of diagnosis; a majority of patients (>80%) are present with advanced or distant metastases which cannot be treated with surgical removal.[2] Even within the 15−20% of patients who are able to undergo potentially curative resection, the 5-year survival is only 20%.[5]

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The advances in molecular biology have dramatically improved our knowledge of pancreatic cancer pathogenesis and provided the foundation for targeted therapy. Some of the important genetic and epigenetic changes have been targeted for therapy of various solid tumors, such as cetuximab against EGFR, Herceptin against HER2/Neu and tyrosine kinase inhibitors.[6] However, the clinical results for those single targeted agents have not shown impressive efficacy for pancreatic cancer therapy.[6] Considering that pancreatic cancer has multiple biochemical and genetic abnormalities, targeting a single pathway is unlikely to be effective. Thus, discovery of molecular targets that modulate multiple signaling pathways, such as molecular chaperone Hsp90, has emerged as a promising strategy.

Hsp90 is a highly abundant molecular chaperone in eukaryotic cells.[7] It simultaneously regulates the conformation, activation, maturation, and stability of a wide range of client proteins, many of which are mutated and/or overexpressed signaling proteins in cancers.[8−10] Hsp90 inhibition results in ubiquitination and proteasomal degradation of client proteins, thus rendering potent anticancer activity.[11] Currently, several Hsp90 inhibitors, such as 17-allylamino-17-demethoxygeldanamycin (17-AAG) and 17-allylamino-17-demethoxy-geldanamycin hydroquinone hydrochloride (IPI-504), are in clinical trials to treat various types of cancer.[12,13] However, the detailed kinetics of Hsp90 client protein activation and degradation and their impact on cancer development have not yet been fully understood. It is generally accepted that Hsp90 inhibition shows anticancer activity via degradation of various oncogenic proteins. However, Price et al have reported that 17-AAG and other Hsp90 inhibitors can enhance osteoclast formation and may potentiate bone metastasis of a human breast cancer cell line MDA-Mb-231SA.[14] Hsp90 inhibition also promotes growth of prostate carcinoma cells (PC-3M) in bone in the xenograft model, which may be due to transient activation of ERK, Akt and Src kinase.[15]

Therefore, preventing the short-lived kinase activation induced by Hsp90 inhibition may further enhance the therapeutic efficacy of 17-AAG. In the present study, we found that 17-AAG induced the transient activation of ERK and Src kinase in pancreatic cancer cells. U0126, a potent and selective MEK inhibitor,[16] abolished the transient ERK activation induced by 17-AAG and potentiated the growth inhibitory effect of 17-AAG against pancreatic cancer cells. Combination of U0126 with 17-AAG also suppressed pancreatic cancer cell migration. These data support the combinational use of Hsp90 inhibitors with specific Raf/MEK/ERK signaling inhibitors in pancreatic cancer treatment.

Materials and Methods

Cell Culture and Reagents. Human pancreatic cancer cell lines PANC-1, MIA PaCa-2, BxPC-3 and AsPC-1 were obtained from the American Type Culture Collection (Rockville, MD). PANC-1, BxPC-3 and AsPC-1 were maintained in RPMI-1640, and MIA PaCa-2 in Dulbecco's modified Eagle's medium (DMEM) (Gibco, Grand Island, NY). All growth media contain 10% fetal bovine serum, 100 units/mL penicillin, and 100 μg/mL streptomycin. Hsp90 inhibitors 17-AAG and radicicol were purchased from LC Laboratories (Woburn, MA). MEK1/2 inhibitor U0126 was obtained from Cell Signaling Technologies Inc. (Beverly, MA). The compounds were dissolved in dimethyl sulfoxide (DMSO)/ethanol, and 20 mM stock aliquots were stored at −20 °C.

Western Blotting. Cells were seeded in 100 mm tissue culture dishes and grown to 80% confluence in medium supplemented with 10% FBS. Drug treated or control cells were washed twice in cold phosphate-buffered saline (PBS), scraped off the plate, and lysed in RIPA lysis buffer containing 25 mM Tris-HCl pH 7.6, 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS, and protease inhibitors. After incubation on ice for 20 min, cell extracts were clarified by centrifugation at 14000*g* for 10 min at 4 °C, and protein concentrations were determined by BCA

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method (Pierce, Rockford, IL). Equal amount of protein was separated by SDS−PAGE and transferred onto PVDF membrane (Biorad, Hercules, CA). To detect pERK and pSrc, cells were starved in serum-free medium for 20 h before drug treatment. Antibodies to Akt, phospho-Src (Tyr-416), Src, phospho-ERK1/2, ERK1/2 were purchased from Cell Signaling Technology (Beverly, MA); antibodies to Hsp90, Hsp70, Raf-1, cyclin A, cyclin D1, cyclin E, CDK2, CDK4, CDK6, p27 were from Santa Cruz Biotechnology (Santa Cruz, CA). Immunodetection was performed with corresponding horseradish peroxide-conjugated secondary antibodies and detected by enhanced chemiluminescence assay (ECl, Amersham, Piscataway, NJ). Quantification of Western blotting bands was performed by densitomery analysis with Image J software (NIH, Bethesda, MD).

Cell Proliferation Assay. Cells were seeded in 96-well plates at a density of 3000−5000 cells per well. After an overnight attachment period, cells were exposed to various concentrations of 17-AAG and U0126, alone or in combination for 48 h. Control cells received DMSO only. The percentage of viable cells was assessed with a colorimetric MTS (3,4-(5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium salt) cell proliferation assay (Promega, Madison, WI). The IC_{50} values for cytotoxicity were calculated with WinNonlin software (Pharsight, Mountain View, CA). All experiments were carried out in hexaplicate and repeated at least twice independently.

Cell Migration Assay. Cell migration assays were performed using 24-well Costar Transwell chambers with 8.0 μm pore polycarbonate membranes (Corning Inc., Corning, NY). The bottom chambers were filled with 600 μL of cell culture medium supplemented with 10% FBS. Pancreatic cancer cells (Mia PaCa-2, AsPC-1) were detached with 0.05% trypsin/EDTA, and resuspended at 1×10^6 cells/mL in serum-free medium. A 100 μL aliquot of cell suspension was added to the upper chamber. After 2 h incubation to allow cell attachment, inhibitors or DMSO was added to the upper chambers and cells were allowed to migrate for 24 h at 37 °C. Cells on the upper side of the membrane were gently removed with cotton swabs, while those that had penetrated to the bottom side of the membrane were fixed with ice-cold methanol for 10 min and stained with 0.5% crystal violet solution. After rinsing with water, the dye was extracted by the addition of 10% acetic acid. The absorbance at 570 nm, which corresponds to the number of live adherent cells, was measured with a microplate reader.

Wound Healing Assay. Cells were seeded into 6-well plates to obtain 80%−90% confluence. After serum starvation, wounds were made by dragging a sterile pipet tip through the monolayer. Cells were washed to remove debris and 17-AAG and/or U0126 was added with 25 ng/mL mitomycin C, which was used to inhibit proliferation. Images were taken under an inverted microscope, and migration index was calculated as follows: migration index = [(width of wound at time 0 h − width of wound at time *t* h)/(width of wound at time 0 h)] × 100%.

Statistics. All values are expressed as means ± SD. When appropriate, statistical significance (defined as $p < 0.05$) was determined by Student's *t* test.

Results

Src and ERK Activation after 17-AAG Treatment. We investigated the ability of 17-AAG to activate ERK by Western blotting (Figure 1). Mia PaCa-2 cells were exposed to 17-AAG for 24 h. We observed a slight increase of p-ERK1/2 level upon 0.03 μM (∼IC50, shown later in Figure 3) 17-AAG treatment, which peaked at 3 h with a 2-fold increase (Figure 1A). Thereafter, it progressively declined to basal level. At 24 h, there was no discernible difference in p-ERK level compared with nontreatment control at 0 h. The transient increase of p-ERK was dose-dependent, which reached maximum level upon 0.5−1 μM 17-AAG treatment (Figure 1B). After treatment with 1 μM 17-AAG, p-ERK protein level increased about 3-fold at 1 h followed by gradual degradation (Figure 1C). At 24 h, the p-ERK protein level reduced to approximately 60% of control. There was no obvious change in total ERK protein. Similar ERK activation was also observed in another Hsp90 inhibitor radicicol (Figure 1D).

It has been reported that in T24 cell, a cell line derived from transitional cancers of human bladder, Src activation accounts for the transient increase of p-ERK caused by GA treatment.[17] To examine the possible involvement of Src in 17-AAG induced ERK activation in pancreatic cancer cells, we tested Src phosphorylation on Tyr-418, which indicates Src activation. We noticed a similar trend of p-Src increase, with slightly stronger magnitude and longer duration compared with p-ERK transient increase (Figure 1E). The accumulation of p-Src started after 15 min treatment and reached peak at 1 h with a 4-fold increase. The protein level of total Src had no change. These data suggest that the activation of Src and ERK may correlate with each other in pancreatic cancer cells after 17-AAG treatment.

MEK Inhibitor Abolished 17-AAG Induced Src and ERK Activation. The activation of MEK/ERK signal transduction cascade has been implicated in human pancreatic cancer.[18] We used selective MEK inhibitor U0126 to test whether it inhibits 17-AAG stimulated MEK/ERK activation. MTS assay has shown that 10−20 μM of U0126 had very little antiproliferative effect, therefore, we treated Mia PaCa-2 cells with 10 μM of U0126 and collected cells at various time points to monitor the protein levels of p-ERK and p-Src. Surprisingly, U0126 (10 μM) exhibited dramatic inhibition on p-ERK (Figure 2A). P-ERK decreased to under detection level after only 15 min treatment, and the inhibition sustained

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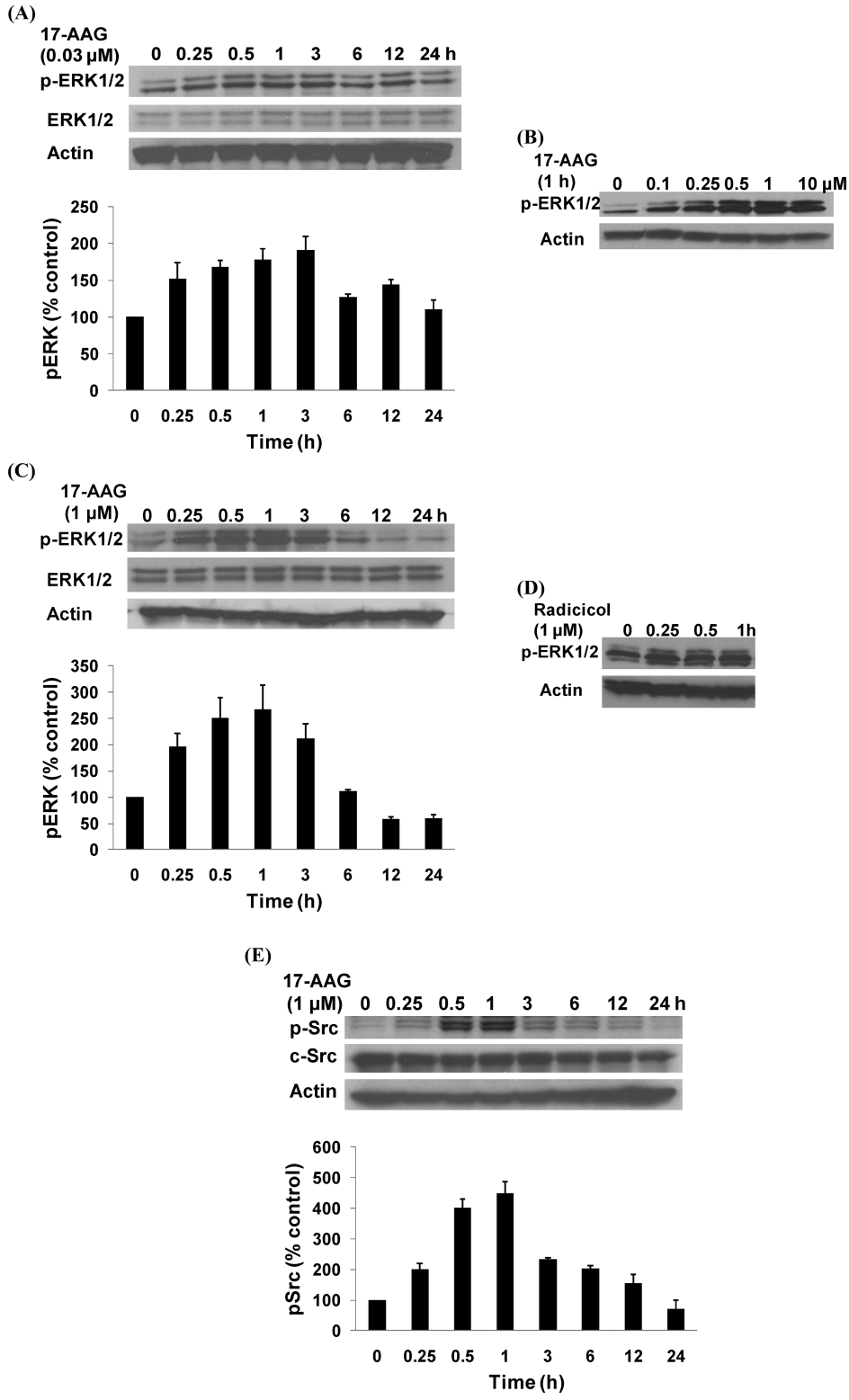

Figure 1. Transient activation of ERK and Src by Hsp90 inhibition. (A) Mia PaCa-2 cells were treated with 0.03 μM 17-AAG for the indicated time periods. Protein levels of phosphorylated ERK1/2 (p-ERK1/2), total ERK1/2 and actin in cell lysates were detected by Western blotting with specific antibodies. The density of the protein bands were measured by Image J and normalized to actin. Results were given as a percentage of the control (0 h) and plotted in the bar graph below. (B) Mia PaCa-2 cells were treated with indicated concentrations of 17-AAG for an hour. (C) Mia PaCa-2 cells were treated with 1 μM 17-AAG for the indicated time periods. (D) Mia PaCa-2 cells were treated with 1 μM radicicol for the indicated time periods. (E) Mia PaCa-2 cells were treated with 1 μM 17-AAG, and the protein levels of Src and phosphorylated Src (p-Src) were detected by Western blotting.

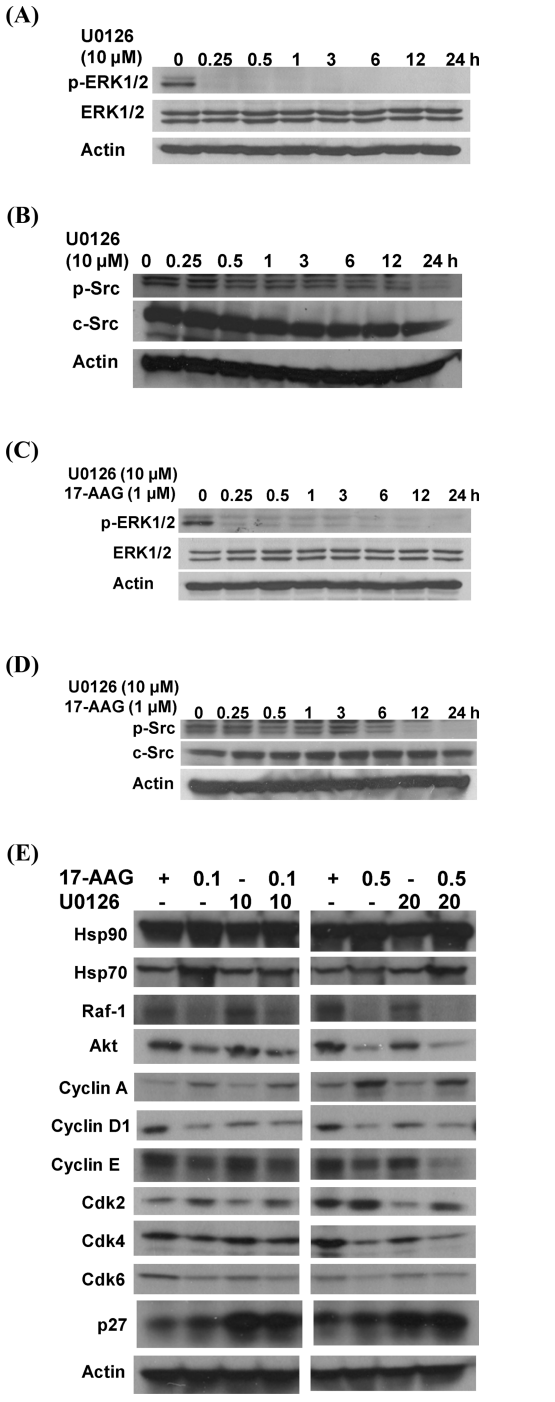

Figure 2. MEK inhibitor U0126 blocked 17-AAG induced ERK activation. (A) Mia PaCa-2 cells were treated with 10 μM U0126 for the indicated time periods. Protein levels of p-ERK1/2, total ERK1/2 and actin in cell lysates were detected by Western blotting using specific antibodies. (B) Mia PaCa-2 cells were treated with 10 μM U0126, and protein levels of p-Src and Src were detected by Western blotting. (C, D) Mia PaCa-2 cells were treated with the combination of 17-AAG (1 μM) and U0126 (10 μM), and the protein levels of p-ERK1/2, p-Src, total ERK1/2 and Src were detected by Western blotting. (E) Mia PaCa-2 cells were incubated with DMSO, 17-AAG (0.1 or 0.5 μM), U0126 (10 or 20 μM), or the combination of 17-AAG and U0126 for 24 h. The indicated protein levels were determined by Western blotting with specific antibodies.

for at least 24 h. Although p-Src degradation did not occur immediately upon U0126 treatment, p-Src did decrease progressively and we never noticed any increase as that after 17-AAG treatment (Figure 2B). Next, we treated cells with the combination of 17-AAG and U0126 (Figure 2C,D). The data showed that U0126 completely abrogated 17-AAG induced p-ERK activation, and the effect is comparable with that treated with U0126 alone (Figure 2C). Similarly, 17-AAG stimulated Src activation was also blocked by U0126 (Figure 2D). Thus, MEK inhibitor efficiently suppresses the transient MEK/ERK activation caused by 17-AAG.

Combination Effect of Hsp90 and MEK Inhibition on the Signaling Proteins. Because the impact of transient Src and ERK activation has not been fully studied, we next evaluated the changes of various signaling proteins in pancreatic cancer cells when treated with 17-AAG and U0126, alone or in combination (Figure 2E). 17-AAG exerted broad effects on various oncogenic proteins. For instance, 0.5 μM 17-AAG resulted in 50% decrease of Raf-1 and over 70% reduction of Akt. As a molecular signature of Hsp90 inhibition, 17-AAG induced the expression of Hsp70. In contrast, U0126 treatment had no effect on Hsp70, little impact on Raf which is upstream of MEK, and minor influence on Akt which has cross-talk with MEK/ERK pathway.

In addition, different response patterns were observed on the expression of cell cycle regulatory proteins after 17-AAG or U0126 treatment (Figure 2E). 17-AAG (0.5 μM) treatment decreased cyclin D1 by 70%, cyclin E by 40%, CDK4 by 62% and CDK6 by 53%. Meanwhile, 17-AAG caused marked induction of cyclin A by 3-fold and CDK2 by 1.4-fold. In comparison, U0126 (20 μM) either did not change or only caused a little decrease in the levels of cyclin D1, cyclin E, CDK4, and CDK6, but it downregulated cyclin A by 40% and CDK2 by 80%.

Furthermore, the expression of p27, a critical cell cycle regulator, increased 2- to 3-fold upon U0126 treatment (Figure 2E). In contrast, 17-AAG (0.5 μM) exhibited little effect on the expression of p27.

Combination of the two inhibitors exhibited complementary inhibitory effect on various proteins (Figure 2E). Using both 17-AAG and U0126 decreased cyclin E levels by more than 67%. In addition, U0126 also reversed the induction of cyclin A and CDK2 caused by 17-AAG.

U0126 Potentiated 17-AAG Induced Cytotoxicity. 17-AAG has shown antiproliferation effect in many cancers including pancreatic carcinoma. Since U0126 blocked 17-AAG induced transient activation and accumulation of signaling proteins, we examined whether this inhibition can sensitize pancreatic cancer cells to 17-AAG. Four pancreatic cancer cell lines (Mia PaCa-2, AsPC-1, BxPC-3, PANC-1) were treated with 17-AAG or U0126 alone. 17-AAG exhibited potent antiproliferation effect with IC50 less than 0.05 μM in AsPC-1, BxPC-3 and Mia PaCa-2 (Figure 3). In contrast, the efficacy of U0126 was much weaker. Up to 40 μM of U0126 was not able to inhibit 50% of cell growth. This is consistent with the previous studies that U0126 does

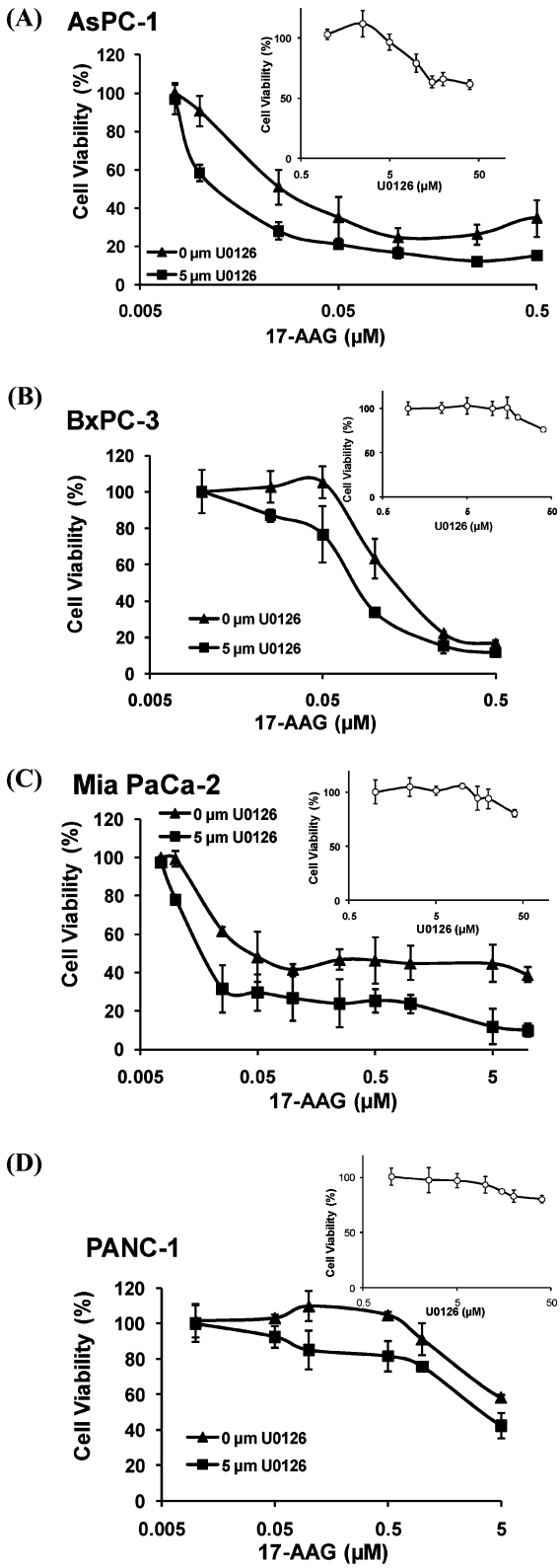

Figure 3. MEK inhibitor U0126 enhanced the antiproliferation activity of 17-AAG. Four pancreatic cancer cell lines AsPC-1, BxPC-3, Mia PaCa-2 and PANC-1 were treated with increasing concentrations of U0126 (insert) or 17-AAG, and grow inhibition was determined by MTS assay. The effects of combining varying concentrations of 17-AAG with a noninhibitory concentration of U0126 (5 μM) were also shown. Points represent mean $\pm$ standard deviation.

not possess strong antiproliferative activity.[19] Next, we examined the combination effect of 17-AAG and U0126 (Figure 3). Cells were treated with various concentrations of 17-AAG and 5 μM of U0126. At this concentration, U0126 had produced little antiproliferative effect. However, this low concentration of U0126 potentiated dose-dependent inhibitory effect of 17-AAG as shown in Figure 3. The IC50 of 17-AAG decreased by 50−80% when combined with 17-AAG.

MEK Inhibition Prevented Pancreatic Cancer Cell Migration. It is unknown whether the transient activation of MEK/ERK and Src caused by Hsp90 inhibition undermines the anticancer effect of Hsp90 inhibitors because Hsp90 inhibition will eventually result in the degradation of those kinases and lead to cell death. A few studies suggest that transient activation of AKT and Src in MDA-Mb-231SA breast cancer cells and PC-3 M prostate cancer cells may involve in cell migration and metastasis.[14,15] Since we also observed that 17-AAG induced Src and ERK activation in pancreatic cancer cells, we tested the effect of 17-AAG on pancreatic cell motility by two assays. In wound healing assay, an injury line was marked with a pipet tip and cells were incubated with 17-AAG, U0126 or the combination. In this assay, the concentrations used for 17-AAG and U0126 were 0.02 and 10 μM, because these concentrations exhibited little/minor effect on cell survival determined by MTS assay (Figure 3A). The data showed that cell migration index from wound edge to empty surface in control Mia PaCa-2 and AsPC-1 cells was 76% and 64%, respectively (Figure 4A,B). Incubation with 0.02 μM 17-AAG did not show much inhibitory effect on cell migration, with migration index of 67% and 57% in these two pancreatic cancer cell lines. In contrast, 10 μM of U0126 significantly decreased cell migration index, the value of which was 43% and 20% in Mia PaCa-2 and AsPc-1, respectively. The combination of the two inhibitors resulted in maximum inhibitory effect with migration index of 27% and 13% in Mia PaCa-2 and AsPC-1, respectively.

Next, we tested cell migration with Transwell chambers. Pancreatic cancer cells were plated on Transwell membranes in the presence of drug, and experiments were conducted as described in Materials and Methods. As shown in Figure 4C,D, 0.02 μM 17-AAG decreased the number of cells penetrating through the membrane to lower surface by 18% and 11% in Mia PaCa-2 and AsPC-1, respectively. Higher concentration of 17-AAG (0.05 μM) inhibited cell penetration by 30% and 17% in these two cell lines. Combination of 0.02 μM 17-AAG and 5 μM U0126 inhibited cell penetration by 47% and 55% in Mia PaCa-2 and AsPC-1, while 0.05 μM 17-AAG and 10 μM U0126 inhibited by 66% and 80% in Mia PaCa-2 and AsPC-1, respectively.

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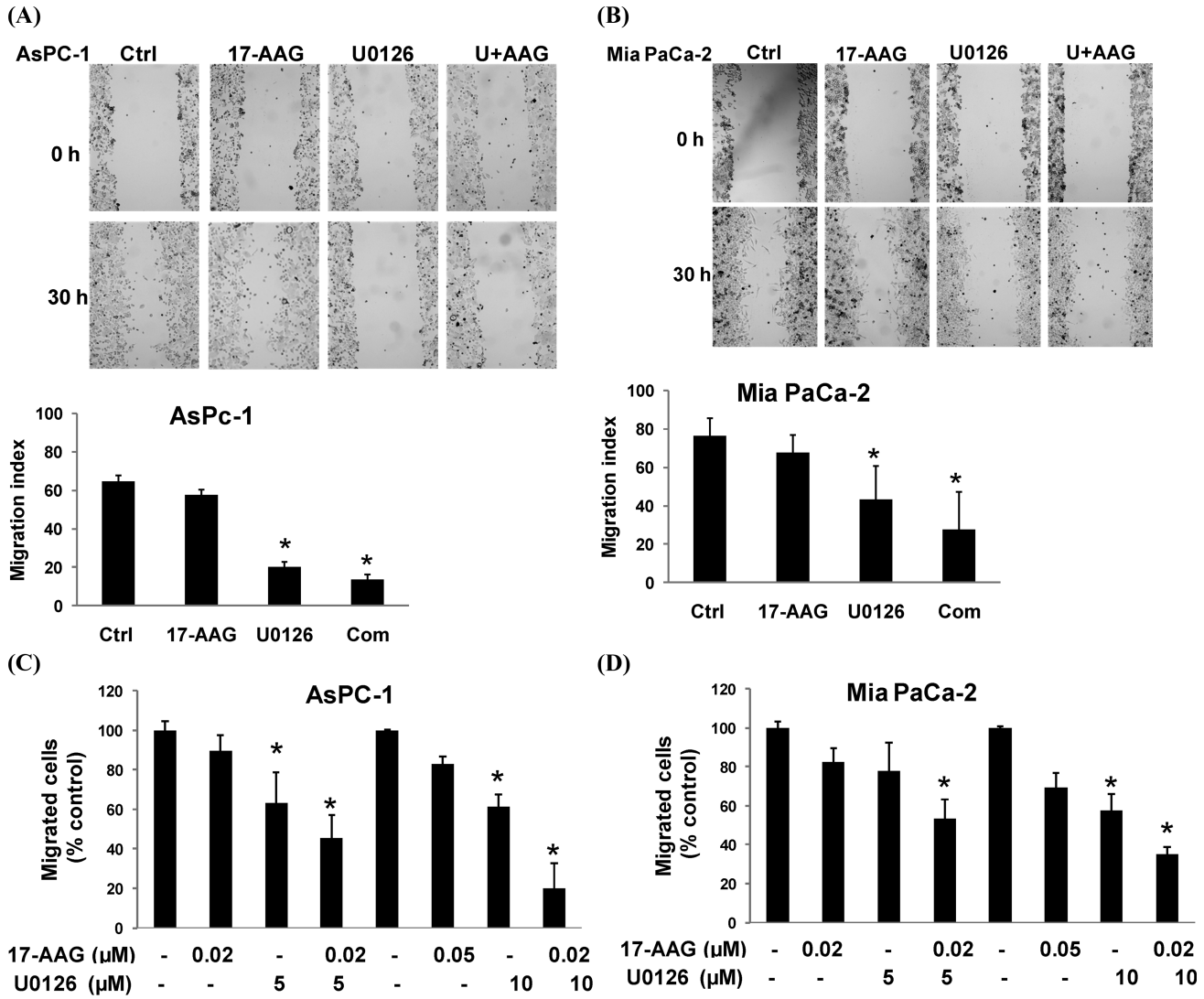

Figure 4. Effects of 17-AAG and U0126 alone or in combination on cell motility and migration. (A, B) AsPC-1 or Mia PaCa-2 cells were treated with DMSO, 17-AAG (0.02 μM), U0126 (10 μM), or the combination, and cell migration was determined with wound healing assay. Cells were allowed to migrate into the cell-free area for 30 h. Photographs were taken with light microscopy. The migration index was calculated as described in Materials and Methods and plotted in bar graphs. (C, D) AsPC-1 and Mia PaCa-2 cells were exposed to different concentrations of 17-AAG, U0126, or the combination for 24 h and tested with migration assay as described in Materials and Methods. Results are expressed as percentage of control and the mean of triplicate; bars, $\pm$ SD; *, $p < 0.05$.

Discussion

Pancreatic cancer is one of the most lethal human cancers, which causes over 30,000 deaths per year in the United States.[20] Most patients diagnosed with pancreatic cancer are present with locally advanced or metastatic disease, and their average survival duration is within 4−10 months due to the aggressive nature of pancreatic carcinoma.[21] There is no effective treatment for this disease. The study on pathogenesis of pancreatic cancer has revealed that pancreatic carcinogenesis is driven by multiple genetic and epigenetic changes.[6] Thus, targeting a single molecule that exerts control over multiple oncogenes involved in tumor development and progression may offer advantages to treat this disease. Hsp90 has emerged as a new therapeutic target to simultaneously regulate numerous oncogenic client proteins, which are pathological hallmarks of malignancy.[22]

The antibiotic benzoquinone ansamycins, represented by geldanamycin (GA), are the first identified Hsp90 inhibi-

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tors.[23] GA has exhibited potent anticancer effect, but the strong hepatotoxicity prevented its clinical development.[24] 17-AAG is a geldanamycin derivative that has less side effects than its parent compound.[13] As the first-in-class Hsp90 inhibitor, 17-AAG has been studied in a number of phase I trials for both solid tumors, including prostate, breast, and pancreatic cancer, and hematological malignancies.[13] At the given doses, 17-AAG resulted in good pharmacokinetic exposures and showed molecular signatures of Hsp90 inhibition such as degradation of CDK4 and induction of Hsp70.[25,26] Although cancer patients with prolonged stable disease were observed in several phase I trials using 17-AAG, results from three phase II trials were less impressive, which was likely due to the limited potency and high toxicity of 17-AAG.[13] This suggests that combination of differently targeted therapeutic agents may be required to achieve desired antitumor activity. In this context, several studies have shown a synergistic or additive cytotoxicity via combinational treatment with 17-AAG and other targeted agents against a variety of cancer cells.[27−32] In the current study, we also observed the selective MEK inhibitor U0126 can potentiate the efficacy of 17-AAG in pancreatic cancer cells (Figure 3). A low concentration of U0126 (5 μM), which showed no inhibitory effect alone, substantially enhanced the antiproliferation effect of 17-AAG.

The potentiation of 17-AAG activity by U0126 may result from the complete inhibition of the MEK/ERK signaling because U0126 is a MEK-specific inhibitor.[16] The Ras/Raf/MEK/ERK cascade, which has been studied intensively, is the first signal transduction cascade delineated from the cell membrane to the nucleus.[33] It is a key intracellular signaling pathway regulating diverse cellular functions including cell proliferation, survival, and migration.[34] The cascade is generally described as a linear signaling pathway initiated by Ras activation, followed by Raf, MEK and ERK activation.[34] The only known targets of MEK1/2 are ERK protein, which in principle are capable of mediating all the diverse functions attributed to this pathway.[33,34] Active ERK can phosphorylate over 100 possible substrates with diverse functions, either in the nucleus or in the cytoplasm.[35,36] Hsp90 inhibitors and specific MEK inhibitors interfered with MEK/ERK signaling in distinct ways. Hsp90 inhibition by 17-AAG initially caused a transient activation of ERK, which could last several hours, and then led to the destabilization of activated ERK in pancreatic cancer cells (Figure 1A,C). On the contrary, specific MEK inhibitor U0126 resulted in a rapid and complete blockade of the MEK/ERK signaling (Figure 2A). This difference could exert diverse influence on related signaling proteins.

The downstream of MEK/ERK pathway include many cell cycle protein kinases, cyclins and mitotic inhibitors.[37−39]The cyclin/cyclin-dependent kinase (CDK) complexes, together with the flow of information from outside the cell, play a crucial role in regulating cell cycle progression.[40] Various combinations of the complexes include cyclin D1/CDK4, cyclin D1/CDK6, cyclin E/CDK2, cyclin A/CDK1, cyclin A/CDK2, and cyclin B/CDK1.[40,41] Several molecules that inhibit cell cycle kinases have been developed as potential anticancer agents.[40] However, none of these have been approved as an effective strategy to control malignant cell

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proliferation.[40] A major obstacle is that much remains to be determined on the complicated functions of cyclin/CDK complexes. In our study, we found that 17-AAG caused the degradation of cyclin D1, cyclin E, CDK4 and CDK6, while it induced the expression of cyclin A and CDK2 (Figure 2E). Studies have shown that sensitivity to CDK inhibition depends on tumor type, and CDKs may have compensatory roles in cell cycle control.[40] Therefore, the accumulation of some cyclins and CDKs after 17-AAG treatment might in a sense favor tumor progression, correlating with the activation of ERK. The addition of U0126 could reverse 17-AAG induced accumulation of cyclin A and CDK2 (Figure 2E). The activities of cyclin/CDK complexes are regulated by p27, which suppresses the cell cycle by binding to and inhibiting cyclin E/CDK2.[42] 17-AAG did not significantly induce p27 expression, while U0126 dramatically increased p27 expression (Figure 2E). Taken together, the combination of MEK and Hsp90 inhibitor exerts more intensive inhibition on proteins downstream of MEK/ERK.

Although there is no direct evidence that transient activation of MEK/ERK attenuates the antiproliferative effect of Hsp90 inhibitors, there is evidence that it can affect cancer cell motility and promote tumor metastasis under certain circumstances in some breast cancer and prostate cancer cell lines.[14,15,17] In evaluation of pancreatic cancer cell motility, we found that 17-AAG showed a relatively weak inhibitory effect on pancreatic cancer cell migration (Figure 4). It is worth noting that at the same concentrations (0.02 and 0.05 μM) 17-AAG exhibited potent cytotoxity (Figure 3), and thus the inhibition on migration may be overestimated due to the contribution of cell death. On the contrary, U0126, at non-growth-inhibitory concentrations, displayed dramatic inhibition of cell migration (Figure 4). This is consistent with the studies that MEK inhibitor can effectively inhibit cancer cell motility and metastasis.[43−45]

In summary, we have shown that Hsp90 inhibition transiently activates MEK/ERK signaling in pancreatic cancer cells. MEK/ERK inhibition completely suppressed 17-AAG-induced transient ERK activation. Combinational use of Hsp90 and MEK/ERK inhibitors in pancreatic cancer cells may achieve higher efficacy in preventing tumor progression and metastasis.

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