The HSP90 inhibitor 17-PAG effectively inhibits the proliferation and migration of androgen-independent prostate cancer cells

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Abstract: Castration-resistant prostate cancer (CRPC) ultimately occurs after a period of treatment with androgen deprivation therapy. Furthermore, CRPC patients can only derive limited survival benefits from traditional cytotoxic drugs. HSP90, which is a molecular chaperone, plays a vital role in client protein processing and maintaining the function of cells. HSP90 is usually overexpressed in prostate cancer tissues, which makes it a potential target for managing prostate cancer. Geldanamycin (GA), which was recognized as the first natural HSP90 inhibitor, has demonstrated potent anti-tumor efficacy in large-scale pre-clinical studies, but its application in the clinic is not permitted due to its liver toxicity and unstable physical properties. In this study, we report a new GA derivative, 17-PAG (17-(propynylamino)-17-demethoxygeldanamycin), which demonstrates highly effective anti-tumor activity against androgen-independent prostate cancer cells. Treating cells with 17-PAG dose-dependently suppressed proliferation, reduced colony formation and induced apoptosis of DU-145/C4-2B cells. Moreover, 17-PAG suppressed the migration and invasion of DU-145/C4-2B cells by regulating epithelial mesenchymal transition (EMT). 17-PAG also downregulated the HSP90 client proteins, including Her2, EGFR, C-Raf, AKT, p-AKT, and CDK4. Animal assays confirmed that 17-PAG shows strong anti-tumor effects with no obvious organ toxicity in DU-145 cell xenografted nude mice. These results provide us with a potential target for treating androgen-independent prostate cancer in a safe and effective manner.

Keywords: Geldanamycin derivative, hsp90, androgen independent, prostate cancer, chemotherapy

Introduction

Prostate cancer is the most common tumor among males in the United States, with 233,000 newly diagnosed cases and 29,490 deaths reported in 2014 [1]. Androgen ablation therapy alone or in combination with prostatectomy or radiation has been applied in the clinic to improve patient survival. However, the median overall survival (OS) is only 8-16 months [2, 3], and resistance to androgen deprivation therapy ultimately occurs. Therefore, cytotoxic chemotherapy was introduced as a promising therapy for preventing the progression of prostate cancer. However, several phase III clinical trials of taxanes have shown that the survival benefit was marginal, with the increased median OS ranging from 12.7-19.2 months [4-8]. In view of such an unsatisfactory prognosis, new agents are urgently needed to improve patient management in the clinic.

Heat shock protein 90 (HSP90) is an evolutionarily conserved and abundantly expressed molecular chaperone, exerting pivotal housekeeping functions, such as controlling the folding, stability and activation of various proteins, which are generally termed ‘clients’ [9, 10]. Many of these HSP90 clients, including HER-2, Akt, Raf-1, CDK4, H1F1-α, Bcr-Abl, and mutant p53, are often activated, mutated, or overexpressed in cancer cells [11], which makes HSP90 a promising targeting for cancer therapy. In recent years, substantial numbers of HSP90 inhibitors underwent pre-clinical and clinical evaluations [12]. Corresponding results
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Figure 1. 17-PAG effectively inhibits proliferation and induces apoptosis in DU-145 and C4-2B cells. A. Chemical structure of 17-PAG. B. Cell viability of RWPE-1, DU-145 and C4-2B cells treated with various concentrations of 17-PAG for 72 h. The inhibition rate was calculated by MTT assay. The data are represented as the mean ± S.D of three independent experiments. C. Colony-forming capability of DU-145 and C4-2B cells was measured after treatment with various 17-PAG concentrations for 7 days. D. Flow cytometric analysis of 17-PAG-induced cell death at indicated concentrations in C4-2B and DU-145 cells for 48 h. Each value represents the mean ± S.D. of three independent experiments, *P<0.05 vs the control; E. Flow cytometric analysis of 17-PAG-induced cell death at defined concentrations in C4-2B and DU-145 cells for 24 and 48 h. Each value represents the mean ± S.D. of three independent experiments, *P<0.05 vs the control. F. Detection of apoptotic morphological changes in DU-145 cells treated with
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17-PAG at different concentrations (1 μm, 2 μm, 4 μm). Nuclei were stained with Hoechst 33,258 and examined by fluorescence microscopy. G. FACS analysis of DU-145 cells treated with (2 μM) or without 17-PAG and then labeled with Annexin-V FITC and PI as markers for apoptosis. H. Western blot analysis of apoptosis-related protein, Bcl-2, or anti-apoptotic protein, Bax, in DU-145 cells treated with 17-PAG for 0, 4, 8, 12, 24 or 36 h. The relative expression levels of proteins were analyzed using Image J software. Intensity values are expressed as fold changes compared with control. The data were collected from 3 independent experiments, and the mean ± S.E. (error bars) was calculated, *P<0.05 vs the control.

demonstrated that they can significantly inhibit tumor growth in vivo or in vitro, which provides support for its consideration as a new approach for cancer therapy.

Geldanamycin (GA), which was first extracted from the fermentation broth of Streptomyces hygroscopicus in 1970, was the earliest natural HSP90 inhibitor [13]. It was reported that GA can competitively bind to the N-terminal ATP pocket of HSP90, thereby sequentially inhibiting its chaperone function and inducing an ubiquitin-mediated degradation of its client proteins [14-16]. Further investigations demonstrated that GA had potential for in vivo or in vitro anti-tumor efficiency in pre-clinical studies [17, 18]. However, despite its potent anti-cancer activities, GA failed to enter clinical trials due to poor solubility in water, limited in vivo stability and severe hepatotoxicity in animal models [19]. To improve these defects, we designed and synthesized a series of new derivatives of GA in our early research [20, 21]. In this study, we report on another new GA derivative, 17-PAG (as shown in Figure 1A), which demonstrates potent anti-tumor activities in vivo and in vitro.

Materials and methods

Cells and reagents

The human prostate cancer cell lines DU-145, C4-2B and RWPE-1 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). The cells were cultured in RPMI-1640 medium (HyClone) supplemented with 10% fetal bovine serum (HyClone) and 1% penicillin/streptomycin in a humidified incubator at 37°C in an atmosphere of 5% CO₂. Anti-β-actin, 3-(4,5)-dimethylthiazol (2-yl)-3,5-diphenyltetrazolium bromide (MTT), Hoechst 33,258 and RNaseA were purchased from Sigma-Aldrich (Sigma-Aldrich Corp, St. Louis, MO, USA). Annexin V, FITC Apoptosis Detection Kit, and Matrigel were purchased from BD Pharmingen (Becton, Dickinson and Company, Franklin Lakes, NJ, USA). Anti-AKT, anti-phospho-AKT (ser 473), anti-Her2, anti-EGFR, anti-CDk4, anti-C-Raf, anti-Bcl-2, anti-Bax, anti-Vimentin, anti-Zeb1 and anti-Slug were purchased from Cell Signaling Technology (Cell Signaling Technology, Inc. Boston, MA, USA). Anti-Snail, anti-E-cadherin, and anti-HSP70 were purchased from Santa Cruz Biotechnology (Santa Cruz Biotechnology, Inc. Santa Cruz, CA, USA).

Cell viability assay

Cells (5,000/well) were seeded in sterile 96-well flat-bottomed plates in a final volume of 200 µl and incubated overnight. Then, 17-PAG, which was dissolved in dimethyl sulfoxide (DMSO) at various final concentrations, was added to the wells. Every concentration was tested in triplicate. After incubating for 72 h, 20 µL of 5 mg/mL MTT solution was added to each well, and the plates were incubated for 4 h. Next, the reaction was stopped by the addition of 150 µL of DMSO to each well. After shaking the wells for 10 min, the formazan crystals were completely dissolved. The plates were then read for absorbance (OD) at 490 nm on a microplate reader. Finally, the cell inhibition rate (%) at different drug concentrations was calculated as follows: [(OD of control-OD of drug treated)/(OD of control-OD of blank treated)] ×100. The 50% inhibitory concentration of 17-PAG was defined as the concentration that caused 50% growth inhibition of cells.

Colonies formation assay

Cells were treated with 17-PAG at different concentrations. The plates were further incubated at 37°C with 5% CO₂ for 7 days. On the last day, the medium was removed, and the cells were washed twice with PBS, fixed with 4% paraformaldehyde fixative for 20 min, stained with crystal violet for 30 min at room temperature, washed, and photographed.
Apoptosis analysis

17-PAG-induced apoptosis was assessed using a Hoechst 33,258 nuclear staining kit according to the manufacturer’s instructions. Cell apoptosis was also determined by flow cytometry using Annexin V and PI staining following the manufacturer’s protocol (Annexin V FITC Apoptosis Detection Kit; BD Pharmingen, USA). Briefly, the cells were cultured in a 6-cm dish for one day. After 17-PAG treatment for 24 h, the cells were collected, washed with PBS and resuspended. Then, Annexin V and PI (propidium iodide) were added, and the cells were incubated at room temperature for 20 min and analyzed by flow cytometry.

Wound healing assay

Cells (2×10⁵) were seeded in a 6-well plate in complete medium and incubated overnight. Next, wounds were created using a sterile 10-μL pipette tip. The cells were then rinsed with PBS and covered with fresh medium with 2% FBS supplemented with 4 μM 17-PAG. After incubation for 24, 48 and 72 h, the wounds were photographed under an inverted microscope.

Transwell assay

The invasion assay was performed using coated Matrigel in the upper chamber, and the migration assay was performed using Transwell chambers (8-μm, Corning, NY, USA) only. Cells (5×10⁴) were starved for 24 h prior to the experiment and were then seeded into the upper chamber with 150 μL serum-free medium. A total of 500 μL of medium containing 20% FBS was placed in the lower chamber. After incubation at 37°C for 36 or 48 h, non-invading cells were removed mechanically using cotton swabs. The inserts were fixed in 4% paraformaldehyde for 20 min, stained with 0.1% crystal violet for 30 min, counted in three random fields by microscopy, and photographed. All experiments were performed at least three times.

Western blot analysis

Samples were harvested, washed with PBS twice and lysed in a lysis buffer containing 1% phenylmethylsulfonyl fluoride (PMSF) for 10 min at 4°C. The proteins were extracted, subjected to electrophoresis on 8-12% SDS-polyacrylamide gels and subsequently transferred to nitrocellulose membranes. Then, the membranes were blocked with 5% skim milk in phosphate-buffered saline at room temperature for 1.5 h. Next, they were incubated overnight with the corresponding primary antibodies in blocking solution at 4°C. After incubation, the membranes were washed 5 times with washing buffer (PBS containing 0.1% Tween) for 6 min. Filters were then incubated with diluted secondary antibodies at room temperature for 1.5 hour. After washing, specific proteins were detected using the FluorChem E system (Protein Simple, USA).

Immunohistochemistry

The formalin-fixed and paraffin-embedded tumor-bearing mouse tissues were sectioned into 5-μm-thick sections. After the sections were deparaffinised in xylene, they were incubated in hydrogen peroxide to abolish endogenous peroxidase activity. Next, they were subjected to microwave pretreatment with citrate buffer (pH 6.0). The sections were stained with hematoxylin and eosin (HE) or peroxidase (DAB) immunohistochemistry (IHC) staining, which was performed following the manufacturer’s protocol (EnVision™ Detection Kit Dako Diagnostics, Zug, Switzerland). All images were obtained at 200× or 100× magnification.

Tumor xenograft model

The presence of tumors, tumor volume, and body weight were determined as previously described [25]. All of the animal protocols in the present study were approved by the Shanghai Medical Experimental Animal Care Commission.

Statistical analysis

Student’s t-tests were used to determine the significance of differences between the treatment and the control groups, and P≤0.05 was considered to be statistically significant. All of the experiments were conducted at least three times.

Results

17-PAG inhibited proliferation in DU-145 and C4-2B cell lines

To investigate the anti-proliferative activity of 17-PAG, the MTT assay was performed in RWPE-
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**Figure 2.** 17-PAG inhibits the migration and invasion of prostate cancer cells. A. Effect on wound healing. DU-145 and C4-2B cells were wounded using a 10-μL micropipette tip, then incubated with or without 17-PAG (4 μM) for 24, 48, or 72 h. B. DU-145 and C4-2B cells were placed in transwell chambers covered with or without Matrigel and incubated with or without 17-PAG (0.5 μM) for 48 h. Representative images of cells stained with crystal violet are shown at 200× magnification (left panel). Quantitative data are presented as the mean ± SD of three independents experiments (right panel). *P<0.05 indicates a significant difference compared with the controls. C. Western blot results of Zeb1, Slug, Snail1, Vimentin, and E-cadherin in DU-145 and C4-2B cells treated with 17-PAG for 48 h. The relative expression levels of Zeb1, Slug, Snail1, Vimentin, and E-cadherin were analyzed using Image J software. Intensity values are expressed as fold changes compared with control. The data were collected from 3 independent experiments, and the mean ± S.E. (error bars) was calculated, *P<0.05 vs the control.
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1, DU-145, and C4-2B cells treated with various concentrations of 17-PAG for 72 hours (Figure 1B). Cell growth was significantly inhibited in a dose-dependent manner after treatment with 17-PAG. The IC50 concentrations of 17-PAG at 72 h were 0.182 μM, 0.087 μM, and 0.098 μM for RWPE-1, DU-145, and C4-2B cells, respectively. In addition, cell death was evaluated in DU-145 and C4-2B cells by flow cytometry. The results showed that the rate of cell survival decreased with increased drug concentrations and exposure time (Figure 1D and 1E). A colony formation assay was also performed. As shown in Figure 1C, 17-PAG resulted in an obvious decrease in colony formation in the groups treated with 100 nm/ml of 17-PAG compared with the DMSO-treated control group. This result indicates that 17-PAG has a robust anti-proliferation effect on prostate cancer cells.

17-PAG induced apoptosis in DU-145 cell lines

To determine whether 17-PAG induces apoptosis, flow cytometry and Hoechst 33,325 staining were performed. As shown in Figure 1F, apoptotic characteristics, such as cell shrinkage and nuclear fragmentation were clearly exhibited in DU-145 cells after 17-PAG treatment. Furthermore, the apoptotic characteristics were altered in a dose-dependent manner. The flow cytometry assay also demonstrated that 17-PAG can induce apoptosis compared with the control group (Figure 1G). The results were also confirmed by western blot, as shown in Figure 1H, the expression of anti-apoptotic protein Bcl-2 was gradually decreased, whereas the level of apoptosis-related protein, Bax, increased steadily over time.

17-PAG inhibited the migration and invasion in DU-145 and C4-2B cell lines

Migration and invasion are crucial for cancer metastasis. To further explore the effect of 17-PAG on migration and invasion of prostate cancer cells, wound healing and transwell assays were performed. As shown in Figure 2A, compared with the control group, 17-PAG significantly inhibited the gap closure in a time-dependent manner in the experimental group. The results were also demonstrated by transwell assay, in which the number of cells migrating to the bottom of the insert were significantly decreased after treatment with 17-PAG for 48 h (Figure 2B). Epithelial mesenchymal transition (EMT) is an important feature of cells with increased migration and invasion and is accompanied by the loss of epithelial markers, such as E-cadherin, and the gain of mesenchymal markers, such as vimentin. To test the changes in epithelial and mesenchymal markers, western blot analysis was used to determine the effect of 17-PAG on E-cadherin and vimentin in C4-2B and DU-145 cells at 48 h. As shown in Figure 2C, the expression of E-cadherin was upregulated, but that of vimentin was downregulated with increased concentrations of 17-PAG. Furthermore, EMT-inducing transcription factors, such as Snail, Slug and Zeb1, which can increase the expression of vimentin and inhibit the expression of E-cadherin, were also increased at the indicated concentration. These results indicate that treatment with 17-PAG represses an EMT phenotype in these cells and inhibits the migration and invasion of prostate cancer cells.

17-PAG downregulates HSP90 client proteins but upregulates HSP70 in DU-145 cell lines

GA is considered a HSP90 inhibitor, and it suppresses tumor growth and migration by down-regulating HSP90 client proteins. To determine whether 17-PAG can regulate HSP90, western blot analysis was used to assess the expression of HSP90 client proteins. As shown in Figure 3, the levels of client proteins in DU-145 cells treated with 17-PAG, including Her2, EGFR, C-Raf, AKT, p-AKT, and CDK4, were downregulated significantly in a dose-and time-dependent manner. Further, the expression of HSP70 contributed to tumor cell survival and resistance to therapy and was gradually upregulated. These data suggest that the biological behavioral changes of prostate cancer cells treated with 17-PAG may, to some extent, be attributed to the inhibition of HSP90 client proteins.

17-PAG inhibits xenograft tumor growth in mice and no obvious organ toxicities were observed

Considering the inhibitory effect of 17-PAG on prostate cancer cells in vitro, we next evaluated whether 17-PAG suppressed the tumor growth of xenografts with DU-145 cells in mice. As shown in Figure 4A, 4C and 4D, the tumor volume was significantly decreased in all of the mice treated with 17-PAG after 21 days com-
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pared with control mice. However, there was no obvious body weight loss in either the control mice or the treatment group (Figure 4B). These data suggest that 17-PAG can effectively suppress tumor growth of xenografts in vivo. Moreover, immunohistochemical analysis showed a decrease in Ki67 expression and in that of the client proteins of HSP90, such as AKT and EGFR (Figure 4E). In addition, no obvious organ toxicities were observed by immunohistochemical analysis (Figure 4F). The results demonstrate that 17-PAG indeed inhibited tumor growth, downregulated HSP90 client protein expression, and did not induce significant side effects.

Discussion

Prostate cancer is highly heterogeneous, and castration-resistant and progressive disease inevitably develops, despite castrate levels of testosterone [22]. HSPs are essential for normal cell viability and growth via its molecular chaperone function. HSP90 interacts with cell signaling proteins that are involved in essential processes such as proliferation, cell cycle control, angiogenesis and apoptosis [23]. It has been reported that HSPs are often highly expressed in various types of cancers [24]; in addition, HSP90 is usually overexpressed in prostate cancer cells compared with normal tissues [25]. GA and its analogs, which have been identified as HSP90 inhibitors, have been examined widely to identify new treatment strategies against various carcinomas in preclinical studies [26-29]. Some derivatives, such as 17-AAG and 17-DMAG, have been evaluated in clinical trials [30, 31]. However, in a phase II trial, further evaluation of 17-AAG as a single
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Figure 4. 17-PAG inhibits the tumor growth of DU-145 xenografts in mice. A. Photographs of tumor-bearing mice with or without 17-PAG treatment. B. The mean tumor body weight treated mice were compared with the control. C. Photographs of dissected DU-145 tumor tissues with or without 17-PAG treatment. D. The mean tumor volume of treated mice was compared with the control. E. Different tumor sections from DU-145 xenografts mice were subjected to H&E staining and immunohistological analysis with antibodies against Ki-67, AKT, EGFR (original magnification: 200×). Scale bar is 100 μm. F. Representative H&E-stained sections of the heart, liver, spleen, lung, and kidney from the mice after treatment (original magnification: 100×). Scale bar is 100 μm.
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agent in patients with metastases was not warranted because 60% of the patients experienced a grade 3 adverse event and because the median time to disease progression was only 1.8 months [30].

In fact, without evaluation in clinical trials, we cannot readily understand the exact efficiency and side effects of the agent through pre-clinical studies alone. However, it is worth noting that many agents have the underlying possibility of becoming a potent anti-tumor drug before they are tested in the clinic. In this study, we have stated that 17-PAG, a new GA derivative, efficiently suppressed proliferation and migration and induced cell apoptosis in androgen-independent prostate cancer cells.

Proliferation and apoptosis are widely used biomarkers and are applied for diagnosis and measurement of tumor aggressiveness; therefore, they are used extensively to evaluate tumor responses to new anti-tumor drugs [32]. Herein, we examined the anti-proliferation and apoptosis-inducing effects of 17-PAG on prostate cancer cells in vitro and in vivo. The MTT assay and colony formation assay revealed that 17-PAG significantly inhibited the growth and proliferation of prostate cancer cells in vitro. The Bcl-2 family generally plays a vital role in the regulation of apoptosis [33]. Some pro-apoptotic members of this family, such as Bax and Bak, are largely increased during apoptosis, whereas anti-apoptotic proteins, such as Bcl-2 and Bcl-x, may be significantly decreased. Moreover, when apoptosis was induced, apoptotic morphological features, such as chromatin condensation and nuclear fragmentation, could be detected by nuclear staining [34]. In the present study, 17-PAG induced Bax expression while decreasing Bcl-2 levels, and cell shrinkage and nuclear fragmentation were clearly exhibited after treatment with 17-PAG; in addition, cell apoptosis was widely induced by 17-PAG in vitro.

The invasion and migration of tumor cells are two major risk factors for progression. Accumulating evidence indicates that EMT is implicated in cancer metastasis and invasion because it promotes the detachment of cancer cells from the primary tumor areas [35, 36]. Transcription factors, such as Snail (SNAI1) and Slug (SNAI2), can regulate EMT by increasing the expression of mesenchymal markers, such as N-cadherin and vimentin, and by reducing the level of adhesion-related proteins such as E-cadherin and cytokeratins [37]. In the present study, western blot analysis showed that the expression of E-cadherin was increased markedly with increased concentrations of 17-PAG. Conversely, vimentin expression was downregulated gradually and was accompanied by the downregulation of the transcription factors Zeb1, Snail and Slug. In addition, the inhibition of invasion and migration was confirmed by a wound healing assay and transwell assay in vitro.

HSP70 is a critical co-chaperon for HSP90 and is involved in the delivery of client proteins to HSP90; in turn, HSP90 inhibition is associated with the upregulation of HSP70 [38, 39]. Our research also demonstrated that the expression of HSP70 was upregulated upon treatment with 17-PAG, which is consistent with a previous study [39]. Emerging evidence demonstrates that AKT plays a key role in the development and maintenance of CRPC through the PI3K-AKT-mTOR signaling axis [40]. EGFR and Her2 are implicated in disease progression from localized to metastatic, in the androgen-independent state, and in the survival of androgen-independent prostate cancer cells [41]. Proliferation and growth in cancer are frequently regulated by the activity of CDK4/6 in modulating the cell cycle [42]. In this study, western blot results showed that many of the key client proteins of HSP90, such as Her2, EGFR, C-Raf, AKT and CDK4, were downregulated by 17-PAG in a dose- and time-dependent manner. The mechanism through which the above-described anti-tumor effects are achieved may be attributed to the suppression of HSP90 by 17-PAG and the simultaneous downregulation of several pathways crucial for cell viability and invasion.

To investigate the efficacy of the anti-tumor response and toxicity of 17-PAG in vivo, nude mice xenograft models were established by subcutaneously inoculating mice with DU-145 cells. After the development of visible tumors, 17-PAG was injected into tumor-bearing animals via the tail vein. The results showed that 17-PAG can significantly reduce tumor growth in xenograft models without increasing tissue toxicity, which suggests that it is a promising candidate for an anti-tumor drug.
In conclusion, the present study showed that 17-PAG, a new GA derivative, can effectively inhibit the proliferation and invasion of androgen-independent prostate cancer cells both in vivo and in vitro by modulating the expression of HSP90 client proteins. The results also show that 17-PAG has the potential to become a new anti-tumor agent. However, further pre-clinical and clinical studies are needed before this agent can be applied in the clinic.

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Disclosure of conflict of interest

None.

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