A Novel HSP90 Inhibitor Delays Castrate-Resistant Prostate Cancer without Altering Serum PSA Levels and Inhibits Osteoclastogenesis

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Abstract

Purpose: Prostate cancer responds initially to antiandrogen therapies; however, progression to castration-resistant disease frequently occurs. Therefore, there is an urgent need for novel therapeutic agents that can prevent the emergence of castrate-resistant prostate cancer (CRPC). HSP90 is a molecular chaperone involved in the stability of many client proteins including Akt and androgen receptor (AR). 17-Allylamino-17-demethoxy-geldanamycin (17-AAG) has been reported to inhibit tumor growth in various cancers; however, it induces tumor progression in the bone microenvironment.

Methods: Cell growth, apoptosis, and AR transactivation were examined by crystal violet assay, flow cytometric, and luciferase assays, respectively. The consequence of HSP90 therapy in vivo was evaluated in LNCaP xenograft model. The consequence of PF-04928473 therapy on bone metastasis was studied using an osteoclastogenesis in vitro assay.

Results: PF-04928473 inhibits cell growth in a panel of prostate cancer cells, induces cell-cycle arrest at sub-G1 and leads to apoptosis and increased caspase-3 activity. These biological events were accompanied by decreased activation of Akt and Erk as well as decreased expression of Her2, and decreased AR expression and activation in vitro. In contrast to 17-AAG, PF-04928473 abrogates RANKL-induced osteoclast differentiation by affecting NF-κB activation and Src phosphorylation. Finally, PF-04929113 inhibited tumor growth and prolonged survival compared with controls. Surprisingly, PF-04929113 did not reduce serum prostate-specific antigen (PSA) levels in vivo; in parallel, these decrease in tumor volume.

Conclusion: These data identify significant anticancer activity of PF-04929113 in CRPC but suggest that serum PSA may not prove useful as pharmacodynamic tool for this drug. Clin Cancer Res; 17(8); 2301–13. ©2011 AACR.

Introduction

Prostate cancer is the most common cancer and the third most common cause of cancer-related mortality in men in the United States (1). Hormonal therapy remains the most effective therapy for patients with advanced prostate cancer, inhibiting proliferation and inducing apoptosis in tumor cells (2). Unfortunately, after short-term remissions (18–24 months), surviving tumor cells recur with castrate-resistant prostate cancer (CRPC), with inevitable progression even in CRPC. HSP90 inhibition has been shown to disrupt nuclear localization of the AR (12). Consequently, HSP90 inhibition represents an exciting strategy as a new treatment for CRPC. To date, many HSP90 inhibitors targeting the ATPase pocket have been developed, including natural compounds like geldanamycin and its analogue 17-allylamino-17-demethoxy-geldanamycin (17-AAG), inducing apoptosis in preclinical
Heat shock protein 90 (HSP90) is a central “node” for several signaling pathways that are known to modulate prostate cancer progression to castrate-resistant prostate cancer (CRPC). Although HSP90 inhibitor, 17-allylamino-17-demethoxy-geldanamycin (17-AAG), is currently in clinical trials, it induces osteoclastogenesis and cannot be used in metastatic disease. In this study, we tested the activity of a novel HSP90 inhibitor, PF-04928473, and found that it is more potent than 17-AAG, inhibits prostate cancer cell growth, and induces apoptosis. PF-04928473 inhibits both AR and AKT pathways via its ability to induce their degradation. In contrast to 17-AAG, PF-04928473 inhibits NF-kB and Src activities thereby abrogating osteoclastogenesis. PF-04928473 delays progression to CRPC in \textit{vivo} without altering circulating prostate-specific antigen (PSA) levels. These results suggest that PSA is not a good surrogate marker for anticancer activity of PF-04928473. Together, data support that targeting HSP90 using PF-04928473 could be considered as a therapeutic strategy for CRPC with bone metastasis.

In vivo experiments were designed to study the effects of PF-04929113 on tumor progression and animal survival in LNCaP CRPC model. Furthermore, we investigated the effects of PF-04928473 on osteoclastogenesis in \textit{vivo} in preosteoclast cells.

Materials and Methods

Tumor cell lines and reagents

The human prostate cancer cell lines PC-3, PC-3-M, and DU145, were purchased from the American Type Culture Collection (2008 and 1989, ATCC authentication by iso-enzymes analysis) and maintained in Dulbecco’s Modified Eagle’s Medium (DMEM; Invitrogen) supplemented with 5% FBS and 2 mmol/L L-glutamine. LNCaP and C4-2 cells were kindly supplied by Dr. Leland W.K. Chung (1992, MD Anderson Cancer Center, Houston, TX) and tested and authenticated by whole-genome and whole-transcriptome sequencing on Illumina Genome Analyzer IIx platform in July 2009. C4-2 and LNCaP cells were maintained in RPMI 1640 (Invitrogen) supplemented with 5% FBS and 2 mmol/L L-glutamine. All cell lines were cultured in a humidified 5% CO2/air atmosphere at 37°C. All cell lines were passaged for less than 3 months after resurrection. Western blotting and/or real-time PCR was performed for AR and PSA each time when LNCaP or C4-2 cells were resuscitated.

HSP90 inhibitors

HSP90 inhibitors, PF-04928473 (4-(6,6-dimethyl-4-oxo-3-trifluoromethyl-4,5,6,7-tetrahydro-indazol-1-yl)-2-(4-hydroxy-cyclohexylamino)-benzamide) and its prodrug PF-04929113, orally bioavailable, were kindly provided from Pfizer and used for \textit{in vitro} and \textit{in vivo} studies, respectively. These compounds are synthetic small molecule inhibitors that bind the N-terminal ATP-binding site of HSP90. For the \textit{in vitro} studies, PF-04928473 was dissolved in dimethyl sulfoxide (DMSO) at 10 mmol/L stock solutions and stored at −20°C. For the \textit{in vivo} studies, PF-04929113 was dissolved in PBS 1% carboxymethylcellulose (CMC) and 0.5% Tween 80 (Invitrogen) at 15 mg/mL and stored at 4°C.

Cell proliferation and apoptosis assays

Prostate cancer cell lines were plated in media DMEM or RPMI with 5% of FBS and treated with PF-04928473 at indicated concentration and time. After time course exposure, cell growth was measured using the crystal violet assay, as described previously (21). Detection and quantitation of apoptotic cells were done by flow cytometry (described below) and Western blotting analysis. Each assay was repeated in triplicate.

Caspase-3 activity was assessed 3 days after treatment using the Fluorometric CaspACE Assay System (Promega). Fifty micrograms of total cell lysate were incubated with caspase-3 substrate AC-DEVD-AMC at room temperature for 4 hours. Caspase-3 activity was quantified in a fluorometer with excitation at 360 nm and emission at 460 nm.

Cell-cycle analysis

Prostate cancer cell lines were incubated with or without 1 μmol/L PF-04928473 for 24, 48, 72, or 96 hours, trypsinized, washed twice, and incubated in PBS containing 0.12% Triton X-100, 0.12 mmol/L EDTA, and 100 μg/ml ribonuclease A. Propidium iodide (50 μg/mL) was then added to each sample for 20 minutes at 4°C. Cell-cycle distribution was analyzed by flow cytometry (Beckman Coulter Epics Elite, Beckman, Inc.) on the basis of 2N and 4N DNA content. Each assay was done in triplicate.

Western blotting analysis

Samples containing equal amounts of protein (depending on the antibody, 5–50 μg) from lysates of cultured tumor prostate cell lines underwent electrophoresis on SDS-PAGE and were transferred to nitrocellulose filters.
The filters were blocked in Odyssey Blocking Buffer (LI-COR Biosciences) at room temperature for 1 hour. And then, blots were probed overnight at 4°C with primary antibodies (Supplementary Materials) to detect proteins of interest. After incubation, the filters were washed 3 times with washing buffer (PBS containing 0.1% Tween) for 5 minutes. Filters were then incubated for 1 hour with 1:5,000 diluted Alexa Fluor secondary antibodies (Invitrogen) at room temperature. Specific proteins were detected using ODYSSEY IR imaging system (LI-COR Biosciences) after washing, as described above.

**Quantitative real-time PCR**

Total RNA was extracted from cultured cells after 48 hours of treatment using TRizol reagent (Invitrogen). Two micrograms of total RNA was reverse transcribed using the Transcriptor First Strand cDNA Synthesis Kit (Roche Applied Science). Real-time monitoring of PCR amplification of cDNA was performed using DNA primers (Supplementary Table) on ABI PRISM 7900 HT Sequence Detection System (Applied Biosystems) with SYBR PCR Master Mix (Applied Biosystems). Target gene expression was normalized to glyceraldehyde-3-phosphate dehydrogenase (GAPDH) levels in respective samples as an internal standard, and the comparative cycle threshold (Ct) method was used to calculate relative quantification of target mRNAs. Each assay was performed in triplicate.

**Transfection and luciferase assay**

LNCaP and C4-2 cells (2.5 × 10^5) were plated on 6-well plates and transfected using lipofectin (6 μL per well; Invitrogen). The total amount of PSA or NF-κB plasmids DNA used were normalized to 1 μg per well by the addition of a control plasmid. Media were replaced by charcoal-stripped serum (CSS) ± PF-04928473 for 24 hours ± 0.1 nmol/L R1881 for 12 hours to activate AR or ± 20 ng/mL TNF-α to activate NF-κB. PSA–luciferase or NF-κB–luciferase activities were measured using Dual–Luciferase Reporter Assay System (Promega) with the aid of a microplate luminometer (EGG&G Berthold). All experiments were carried out in triplicate wells and repeated 3 times using different preparations of plasmids.

**Immunofluorescence**

LNCaP and C4-2 cells were grown on coverslips and treated with different concentrations of PF-04928473 for 48 hours and then treated ± 1 nmol/L R1881 for 6 hours. After treatment, cells were fixed in ice-cold methanol completed with 3% aceton for 10 minutes at −20°C. Cells were then washed three times with PBS and incubated with 0.2% Triton/PBS for 10 minutes, followed by washing, and 30-minute blocking in 3% nonfat milk before the addition of antibody overnight to detect AR (1:250). Antigens were visualized using anti-mouse antibody coupled with FITC (fluorescein isothiocyanate; 1:500; 30 minutes). Photomicrographs were taken at 20× magnification using Zeiss Axioplan II fluorescence microscope, followed by analysis with imaging software (Northern Eclipse, Empix Imaging, Inc.).

**Cell culture and osteoclast differentiation assays**

Murine RAW 264.7 monocytic cells (ATCC) were cultured in phenol red–free alpha-MEM (Invitrogen) supplemented with 10% FBS and 1% nonessential amino acids (Invitrogen). To induce osteoclast formation, RAW 264.7 cells were scraped and incubated at 37°C for 2 minutes to allow adherence of the more differentiated cells. Nonadherent cells were then seeded in fresh medium at 3 × 10^5 or 10 × 10^5 cells in 96- or 24-well plates. After 2 hours, the cells were treated with 100 ng/mL recombinant human RANKL (R&D) with or without PF-04928473 or 17-AAG at indicated concentration, for 5 days. Multinucleated cells were counted under a light microscope after May Grünwald/Giems (Sigma) or tartrate-resistant acid phosphatase (TRAP) staining (Leukocyte Acid Phosphatase Assay kit; Sigma). All experiments were performed in triplicate at least 3 times.

**Animal treatment**

Male athymic mice (Sprague Dawley; Harlan, Inc.) were injected subcutaneously with 2 × 10^6 LNCaP cells (suspended in 0.1 mL Matrigel; BD Biosciences) and castrated once tumors reach 300 to 500 mm^3 or when serum PSA increased above 50 ng/mL. Once tumors were progressed to castrate resistance, mice were randomly assigned to vehicle or PF-04929113 (50 mg/kg; formulation in 0.5% CMC + 0.5% Tween-80) and treated orally 3 times per week. Each experimental group consisted of 8 mice. Tumor volume and PSA measurements were performed twice and once weekly, respectively. Tumor volume was calculated by the formula: length × width × depth. Serum PSA measurements were performed by enzymatic immunoassay (ClinPro International Co. LLC). Data points were expressed as average tumor volume ± SE or average PSA concentration ± SE. After sacrifice (when the tumor volume reached ≥10% of body weight), tumors were harvested for evaluation of protein expression by Western blotting analyses and immunohistochemistry. All animal procedures were performed according to the guidelines of the Canadian Council on Animal Care and appropriate institutional certification.

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded 4-μm sections of tumor samples. Immunohistochemical staining was conducted using a primary antibody (Supplementary Materials) in the Ventana autostainer Discover XT (Ventana Medical System) with enzyme-labeled biotin streptavidin system and solvent resistant 3,3’-diaminobenzidine Map kit. All comparisons of staining intensities were made at 200× magnifications.

**Statistical analysis**

All in vitro data were assessed using the Student’s t-test and Mann–Whitney test. Tumor volumes of mice were
compared using Kruskal–Wallis test. Overall survival was analyzed using Kaplan–Meier curves. Levels of statistical significance were set at $P < 0.05$.

Results

**PF-04928473 inhibits prostate cancer cell proliferation**

Figure 1A illustrates the chemical structure of PF-04928473 (active molecule) and PF-04929113 (prodrug). We first assessed the effects of PF-04928473 and 17-AAG on cell growth in several prostate cancer cell lines (PC3, PC3-M, DU145, LNCaP, and C4-2) using a crystal violet assay. In all cancer cell lines tested, PF-04928473 inhibited cell growth in dose-dependent manner, with IC$_{50}$ at 72 hours values ranged from 6.5 to 42 nmol/L (LNCaP: 6.5 nmol/L; C4-2: 8.2 nmol/L; DU-145: 16 nmol/L; PC-3: 36 nmol/L; and PC-3-M: 42 nmol/L; Fig. 1B) and IC$_{90}$ values ranged from 13.5 to 95 nmol/L (LNCaP: 19.5 nmol/L; C4-2: 13.5 nmol/L; DU-145: 43 nmol/L; PC-3: 50 nmol/L; and PC-3-M: 95 nmol/L; Fig. 1B). Compared with 17-AAG, PF-04928473 was more potent in all cell lines except C4-2. Moreover, LNCaP and C4-2 cells, which express the AR, are at least 2-fold more sensitive to PF-04928473 than the other AR-negative cell lines (PC-3, PC-3-M, and DU-145). Subsequent experiments focused on LNCaP and C4-2 androgen-sensitive cell lines. Because insulin-like growth factor (IGF) I and interleukin (IL) 6 play important roles in the proliferation of prostate tumor cells, we investigated whether PF-04928473 suppresses the proliferation induced by these cytokines. PF-04928473 significantly inhibits cancer cell proliferation induced by IL-6 or IGF-I (Supplementary Fig. S1B; $P < 0.001$). These results demonstrate that HSP90 inhibition using PF-04928473 potently inhibits
tumor cell proliferation induced by cytokine, IL-6, and IGF-1.

**PF-04928473 induces apoptosis in prostate tumor cell lines**

We next assessed the effects of HSP90 inhibition on prostate cancer cell apoptosis using flow-cytometric and Western blot analyses. The fraction of cells undergoing apoptosis (sub-G1 fraction) significantly increased in a time-dependent manner with PF-04928473 compared with control in both LNCaP (88% after 96 hours vs. 3% in control; \( P < 0.001 \); Fig. 1C, left) and C4-2 cells (Supplementary Fig. S2). Consequently, the G0/G1, S, and G2/M fractions were significantly reduced in a time-dependent manner, accompanied by decreased levels of cell-cycle proteins, CDK4 and cyclin D1 (Fig. 1C, right). Furthermore, PF-04928473 induced caspase-3 activation in a dose-dependent manner as shown by both increased cleaved caspase-3 by Western blot analysis (Fig. 1D, right) and CaspACE Assay System (\( P < 0.001 \); Fig. 1D, left). These results suggest that PF-04928473 induces apoptosis in a caspase-3–dependent manner.

**PF-04928473 downregulates HSP90 client proteins but upregulates several HSPs in prostate cancer cell lines**

Inhibition of HSP90 causes proteasomal degradation of a subset of cellular proteins. We investigated the effect of PF-04928473 on HSP90 protein client and others HSPs. Expression of HSP90, HSP70, HSP27, and clustatin as markers of heat shock factor (HSF)-1–dependent stress response (22, 23) were increased by PF-04928473 in a dose- and time-dependent manner in LNCaP cells (Fig. 2A) and all other prostate cancer cell lines tested (Supplementary Fig. S2). These results show a typical HSF-1 stress response following HSP90 inhibition and also demonstrate for the first time that clustatin can be considered a stress biomarker like HSP70 after treatment with an HSP90 inhibitor. P23, a protein required in the HSP90 complex, was degraded in a dose- and time-dependent manner in LNCaP and C4-2 cells (Fig. 2A and Supplementary Fig. S2), confirming inhibition of the HSP90 complex. PF-04928473 also decreased levels of various HSP90 client proteins including Akt, ERK, p-ERK, and Her2 (Figure 2A and Supplementary Figure S2).

**PF-04928473 inhibits AR pathway by its degradation in prostate cell lines**

We next evaluated the effects of PF-04928473 on AR levels and activity in LNCaP and C4-2 cells. PF-04928473 induced AR degradation as shown by Western blotting analysis and consequently decreased PSA protein levels (Fig. 2A). Similar results were seen in C4-2 cells (Supplementary Fig. S3). In addition, PF-04928473 significantly abrogated R1881-induced AR-mediated gene activation as shown by a decrease in PSA–luciferase activity at concentrations as low as 10 nmol/L (+1.7-fold, \( P < 0.001 \)) in LNCaP (Fig. 2B) and C4-2 (Supplementary Fig. S3) cells. Quantitative real-time PCR indicated that PF-04928473 does not alter AR mRNA levels but does significantly decrease mRNA levels of target genes of AR: PSA, NKX3.1, and FKBP5 (Fig. 2C). AR localization was also altered after treatment with PF-04928473. While abundant AR resides in the nucleus following R1881 treatment (Fig. 2D), nuclear staining of AR was lost and only weak cytoplasmic staining was identified after treatment with PF-04928473. Similar results were obtained in C4-2 cells (Supplementary Fig. S3).
Figure 2. PF-04928473 induces degradation of HSP90 client proteins, inhibits AR transactivation and nuclear translocation, and downregulates AR-regulated genes. A, LNCaP cells were cultured in presence of PF-04928473 at indicated concentration for 2 days and proteins regulated by HSP90 and expression levels of HSPs were determined by Western blotting. B, LNCaP cells were transiently transfected with 1 μg of PSA–luciferase for 24 hours, followed by 1 nmol/L R1881 treatment for 12 hours in media supplemented with CSS and luciferase activity was determined and expressed in arbitrary units. ***, P < 0.001. C, LNCaP cells were treated with increasing concentrations of PF-04928473 for 2 days and AR, PSA, NKX3.1, and FKBP5 mRNA levels were determined by quantitative real-time PCR. mRNA levels were normalized to level of GAPDH mRNA and expressed as mean ± SE. D, LNCaP cells were treated with 1 μmol/L PF-04928473 for indicated time and 0.1 nmol/L R1881 for 12 hours and AR localization was assessed by immunofluorescence staining. Nucleus was stained with DAPI (4',6-diamidino-2-phenylindole). All experiments were repeated at least three times.
Figure 3. PF-04929113 significantly delays castrate-resistant LNCaP tumor growth and prolongs cancer specific survival. Mice were orally treated with 50 mg/kg PF-04929113 3 times per week, starting when serum PSA values relapsed to precastration levels as described in the "Materials and Methods" section. The mean (A) or the individual (B) tumor volume of mice treated was compared to the control using a paired two-sample t test, with a significance level of 0.05. ***, P < 0.001. C, tumor progression was estimated as the relative tumor volumes (RTV) calculated from the formula: RTV = (V_{45}/V_{10}) where V_{45} is the mean tumor volume at day 45 and V_{10} is the mean tumor volume at day 10. ***, P < 0.001. D, in Kaplan-Meier curves, cancer-specific survival (left) and progression-free survival (right) were compared between mice treated with PF-04929113 and control over a 60-day period. P < 0.001. Progression-free survival was defined as time for the first tumor volume doubling.
treatment did not reduce circulating PSA level in parallel with changes in tumor volume. In controls, as previously reported (24), serum PSA levels increase proportionally with increases in tumor volume with a high degree of correlation ($r = 0.7233$; Fig. 5C), In contrast, in PF-04929113–treated mice, serum PSA correlated poorly with tumor volume ($r = 0.4694$; $P < 0.001$; Fig. 5C), being higher than expected on a per volume basis in this group. Figure 5D illustrates kinetics of serum PSA and corresponding tumor volume of mice treated after 75 days of PF-04929113. During treatment, mean PSA levels increased from 70 to 1,000 ng/mL, whereas mean tumor volume remained stable up to day 60. Interestingly, when PF-04929113 treatment was stopped, serum PSA levels decreased 5-fold over 25 days while tumor volume increased. These in vivo data indicate that serum PSA correlates poorly with CRPC progression and may be enhanced during PF-04929113 treatment. This in vivo finding was particularly surprising, considering that in vitro treatment with PF-04928473 caused AR degradation and reduced PSA expression levels (Fig. 2). Consequently, serum PSA may not a good biomarker for HSP90 inhibition.

**PF-04928473 inhibits osteoclastogenesis**

Because 17-AAG has been reported to induce osteoclastogenesis and consequently stimulate tumor progression in bone environments (17, 18), we next investigated the effect of PF-04928473 on osteoclastogenesis. Raw 264.7 cells were cultured with 100 ng/ml RANKL for 5 days, in
Figure 5. Serum PSA changes in PF-04929113-treated mice do not correlate to suppression of tumor volume progression. Serum samples were obtained from the tail vein of the mice once weekly to measure serum PSA by ELISA and showed as a mean PSA levels (A) or individual PSA level (B) ± SE from the first day of treatment. C, serum PSA progression from treated mice is not correlated to tumor volume progression ($r = 0.4694$) and significantly different of control serum PSA which increases proportionally to tumor volume with a high degree of correlation ($r = 0.7233$). D, the relationship between the serum PSA and tumor volume of mice treated with PF-04929113 from the castration time to the sacrifice of mice ± SE.
presence of 10 or 500 nmol/L of PF-04928473 or 17-AAG. PF-4928473 potently inhibited osteoclast formation from Raw 264.7 preosteoclast cells cultured in presence of RANKL, whereas 17-AAG significantly induced osteoclast formation alone (300 nmol/L) or in presence of RANKL at the indicated concentrations (Fig. 6A). Since activation of NF-κB is an essential step for osteoclast differentiation (25), we compared NF-κB–luciferase activity in presence of 17-AAG and PF-04928473 in Raw 264.7 cells to explain differences of osteoclastogenesis between these 2 HSP90 inhibitors. Compared with 17-AAG, PF-04928473 significantly decreased NF-κB–luciferase activity in osteoclast precursors (Fig. 6B). Because Src kinase is reported to be essential for osteoclast maturation (26, 27) and is activated shortly after 17-AAG treatment (18), we next compared Src activation by PF-04928473 or 17-AAG. Phospho-Src was rapidly and significantly increased within 1 hour after treatment with 17-AAG (P < 0.05; Fig. 6C), whereas PF-04928473 significantly decreased p-Src in Raw 264.7 cells cultured in presence of RANKL (P < 0.01; Fig. 6C). Total Src was not changed during the treatment.

Discussion

Most treatments for CRPC have been approved for symptomatic benefit such as mitoxantrone chemotherapy, the bisphosphonate zoledronic acid, and radioactive isotopes. Only docetaxel, and more recently, cabazitaxel and abiraterone, have been shown to improve overall survival. New treatments, preferably based on selective targeting of mechanistically relevant cancer proteins, are urgently needed for patients with advanced disease. HSP90 is an attractive therapeutic target because as a molecular chaperone, it guides the normal folding, stability, maturation, and activity of many client proteins critical for cell survival. Cancer cells are particularly sensitive to HSP90 inhibition because many client proteins play critical roles in oncogenesis. Several HSP90, such as 17-AAG, and other novel ansamycins, including the orally bioavailable geldanamycin derivative 17-(dimethylaminoethylamino)-17 demethoxygeldanamycin, and the soluble prodrug of 17-DMA are already in clinic (28, 29). But these drugs necessitate complex formulations associated with limited bioavailability and hepatotoxicity (30).

Owing to these limitations of the ansamycin-derived HSP90 inhibitors, we examined the effects of a novel HSP90 inhibitor, PF-04928473 and its prodrug, PF-04929113, in prostate tumor cell lines and castrate-resistant LNCaP xenograft model. PF-04928473 binds the ATP pocket and thereby inhibits many HSP90 client proteins and downstream target genes. In vitro, PF-04928473 demonstrated superior potency in a panel of prostate cell lines compared with 17-AAG, inducing apoptosis and degradation of client proteins, such as Her2, AR, Akt, and ERK, in a dose-dependent manner in prostate tumor cell lines. PF-04928473 has a high oral bioavailability with good solubility delivered via its prodrug PF-04929113 and is active in various preclinical cancer cell lines and models (19, 20). We found that PF-04929113 significantly delayed tumor growth of castrate-resistant LNCaP xenografts and prolonged cancer-specific free survival. After 60 days of treatment, all 8 mice in the treatment group were still alive, whereas all 8 control mice died or required sacrifice. No side effects or toxicity were observed after treatment with PF-04929113, in contrast to reports of hepatotoxicity with 17-AAG (30). PF-04929113 decreased Ki67 expression, increased apoptotic rates, decreased levels of HSP90 client proteins such as Akt (15, 31), and increased levels of HSP70 in tumors in vivo as previously documented (15, 31).

Bone metastasis is associated with significant morbidity and mortality in advanced prostate cancer, affecting more than 80% of CRPC patients (32). Quinn and colleagues identified that HSP90 expression correlates with bone metastasis in an in vivo mouse model (33). Also, c-Src and other HSP90 client proteins like matrix metalloproteinase-2 (MMP2) or epidermal growth factor receptor (EGFR) lead to bone metastasis by differentiation and activation of osteoclasts (34, 35). Conflicting reports suggest that HSP90 inhibition may inhibit both primary tumor growth and bone tumor metastasis (36, 37), whereas several more recent studies have reported that HSP90 inhibition using 17-AAG promotes prostate and breast cancer growth in bone by mechanisms involving osteoclast differentiation and activation (17, 18). These results provide a potential risk for the use of 17-AAG therapies in patients with bone metastasis. In this report, we observed induction of osteoclastogenesis with 17-AAG, whereas the opposite effect was observed with PF-04928473, which abrogates RANKL-induced osteoclast differentiation and is associated with downregulation of NF-κB activity. The classical NF-κB pathway is known to play a pivotal role in osteoclast formation, function, and survival (38, 39). Indeed, the double knockdown of NF-κB causes an osteopetrotic phenotype with growth retardation and craniofacial abnormalities in mice (40). Our results show that, in contrast to 17-AAG, PF-04929113 inhibits key proteins involved in osteoclastogenesis such as p-Src and Akt.

LNCaP tumors secrete PSA, express AR, and relapse after androgen ablation therapy, mimicking many aspects of CRPC including intratumoral androgen synthesis (41), increased expression of stress-activated molecular chaperones (CLJ, HSP27), reactivation of the AR (42), and increased AR-driven serum PSA levels (24). The LNCaP model shows an accurate correlation between tumor volume and serum PSA levels and undergoes an 80% decrease of PSA after castration (24). PSA is considered the most useful biomarker in prostate cancer (43) and used as a clinically useful tool in the follow-up of patients posttreatment. Several clinical investigations indicate that the serum PSA levels are roughly proportional to tumor volume and stage (44, 45). In reported in vitro studies, inhibition of HSP90 induced degradation of the AR associated with a decrease of PSA expression (15, 46, 47). No in vivo data were reported. In the present study, we found that serum PSA levels continued to climb with PF-04929113 despite significant delays in tumor growth of
Figure 6. PF-04928473 inhibits osteoclastogenesis. A, osteoclastogenesis was determined by MGG staining. Raw 264.7 osteoclast precursors were cultured for 5 days in the presence of 100 ng/mL of RANKL with or without 10 nmol/L of PF-04928473 or 17-AAG. Multinucleated cells were counted and compared between all conditions ± SD. ***, \( P < 0.001 \). B, PF-04928473 inhibits NF-κB transactivation. Raw 264.7 cells were transiently transfected with NF-κB-Luc and Renilla plasmids. After 48 hours and stimulation with 20 ng/mL TNF-α, cells were harvested and luciferase activity was determined. C, Raw 264.7 cells were treated with RANKL for 5 days and then treated with 1 μmol/L PF-04928473 or 17-AAG for indicated time. Total lysates were analyzed by Western blotting using total (T)-Src and p-Src antibodies. Western blots were quantitated using Odyssey infrared imaging system application software version 3.0. All experiments were repeated at least three times. *, \( P < 0.05 \); **, \( P < 0.01 \).
castrate-resistant LNCaP xenografts, highlighting that serum PSA level does not always correspond with clinical response as reported in human patients (48, 49). PF-04929113 induces a significant decrease of AKT and a slight reduction of AR expression in tumor tissues, whereas the drug completely reduces AR and PSA expressions in vitro. One explanation for the discrepancy between in vitro and in vivo data is that, compared with AR, AKT is inhibited at lower concentrations of PF-04929113, and the concentration of PF-04929113 used in vivo is not enough to suppress AR-driven PSA levels. There were conflicting data in the literature on the cross-talk between PI3K/AKT and AR signaling pathways. AKT pathway was recently reported to be dominant over AR signaling in prostate cancer cells (50). Indeed, the authors suggest that the inhibition of PI3K/AKT signaling activates AR transactivation potential (50). This hypothesis could explain the in vivo results without inhibition of PSA level, in spite of the opposite in vitro data. Consequently, serum PSA does not reflect the anticancer activity to monitor response to HSP90 inhibition in vivo.

In summary, these data identify significant anticancer effects of the HSP90 inhibitor PF-04929113 in a model of CRPC and that unlike 17-AAG analogues is not associated with src activation and osteoclastogenesis. However, PSA should not be considered as a pharmacodynamic marker of on-target inhibition or anticancer activity for this drug.

Disclosure of Potential Conflicts of Interest

M.-J. Yin is an employee of Pfizer.

Acknowledgments

The authors thank Gerri Los for his support and discussion and Eilàna Beraldi, Virginia Yago, and Estelle Li for technical assistance.

Grant Support

This study was supported by l’Association pour la Recherche sur le Cancer (France), the Canadian Institutes of Health Research, and Pfizer Global Research & Development (United States). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked this fact.

Received November 24, 2010; revised January 20, 2011; accepted January 21, 2011; published OnlineFirst February 24, 2011.

References

15. Solit DB, Zheng FP, Dobrinjak M, Munster PN, Higgins B, Verbel D, et al. 17-Allylamino-17-demethoxygeldanamycin induces the degra-
HSP90 Delays Progression to CRPC

Correction: A Novel HSP90 Inhibitor Delays Castrate-Resistant Prostate Cancer without Altering Serum PSA Levels and Inhibits Osteoclastogenesis

In this article (Clin Cancer Res 2011;17:2301–13), which was published in the April 15, 2011 issue of Clinical Cancer Research (1), the following funding support is not included: Pacific Northwest Prostate SPORE NCI CA097186.

Reference


Published OnlineFirst June 14, 2011.
©2011 American Association for Cancer Research.
doi: 10.1158/1078-0432.CCR-11-1314
Clinical Cancer Research

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Clin Cancer Res 2011;17:2301-2313. Published OnlineFirst February 24, 2011.

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