Blocking HSP90 addiction inhibits tumor cell proliferation, metastasis development and synergistically acts with zoledronic acid to delay osteosarcoma progression.

Benjamin Ory\textsuperscript{a,b,c,e*}, Marc Baud’huin\textsuperscript{a,b,c,d,e*}, Franck Verrecchia\textsuperscript{a,b,c,e}, Bénédicte Brounais-Le Royer\textsuperscript{a,b,c,e}, Thibaut Quillard\textsuperscript{a,b,c,e}, Jérôme Amiaud\textsuperscript{a,b,c,e}, Séverine Battaglia\textsuperscript{a,b,c,e}, Dominique Heymann\textsuperscript{a,b,c,d,e}, Francoise Redini\textsuperscript{a,b,c,e}, Francois Lamoureux\textsuperscript{a,b,c,e}

\textsuperscript{a} Université de Nantes, Nantes atlantique universités, Laboratoire de Physiopathologie de la Résorption Osseuse et Thérapie des Tumeurs Osseuses Primitives, Nantes F-44035, France

\textsuperscript{b} INSERM, UMR 957, Nantes F-44035, France

\textsuperscript{c} LUNAM Université

\textsuperscript{d} CHU de Nantes, Nantes F-44035, France

\textsuperscript{e} Equipe labellisée LIGUE 2012, Nantes, cedex

* B. Ory and M. Baud’huin contributed equally to this work

Short title: HSP90 delays osteosarcoma progression

Key words: HSP90 inhibitor, osteosarcoma, bone tumor

Corresponding author:

Dr. Francois Lamoureux

UMR-S 957

Faculté de Médecine de Nantes

1 rue Gaston Veil 44035 NANTES cedex 1, FRANCE
STATEMENT OF TRANSLATIONAL RELEVANCE

Osteosarcoma (OS) is a very aggressive primary malignant bone tumor mainly affecting children that is associated with high rate of pulmonary metastasis. HSP90 is a central “node” for several signaling pathways that are known to modulate OS progression and pulmonary metastasis development. In this study, we demonstrate the antitumor activity of the HSP90 inhibitor PF4942847, which inhibits OS cell growth and induces apoptosis by disrupting Akt, ERK and C-Met pathways. PF4942847 delays OS progression and prevents pulmonary metastasis development by blocking C-Met, FAK and MMP9 signaling in a OS xenograft model. Combined with zoledronic acid, PF4942847 synergistically inhibits tumor growth and prevents tumor-associated bone lesions and lung metastasis. Together, these data are the proof of principle to target HSP90 alone or in combination with zoledronic acid as an alternative therapeutic strategy to traditional chemotherapy for OS patients with poor prognosis.
Abstract

**Purpose:** Despite recent improvements in therapeutic management of osteosarcoma, ongoing challenges in improving the response to chemotherapy warrants the development of new strategies to improve overall patient survival. Among them, HSP90 is a molecular chaperone involved in the maturation and stability of various oncogenic proteins leading to tumor cells survival and disease progression. We assessed the antitumor properties of a synthetic HSP90 inhibitor, PF4942847, alone or in combination with zoledronic acid in osteosarcoma.

**Methods:** The effects of PF4942847 were evaluated on human osteosarcoma cells growth and apoptosis. Signaling pathways were analyzed by western blotting. The consequence of HSP90 therapy combined or not with zoledronic acid was evaluated in mice bearing HOS-MNNG xenografts on tumor growth, associated-bone-lesions and pulmonary metastasis. The effect of PF4942847 on osteoclastogenesis was assessed on human CD14+ monocytes.

**Results:** In osteosarcoma cell lines, PF4942847 inhibited cell growth in a dose-dependent manner (IC50 ±50nM) and induced apoptosis with an increase of sub-G1 fraction and cleaved-PARP. These biologic events were accompanied by decreased expression of Akt, p-ERK, C-Met and C-RAF1. When administered orally to mice bearing osteosarcoma tumors, PF4942847 significantly inhibited tumor growth by 80%, prolonged survival compared to controls, and inhibited pulmonary metastasis by blocking c-Met, FAK and MMP9 signaling. In contrast to 17-AAG, PF4942847 didn’t induce osteoclast differentiation, and synergistically acted with zoledronic acid to delay osteosarcoma progression and prevent bone lesions.

**Conclusions:** All these data provide a strong rationale for clinical evaluation of PF4942847 alone or in combination with zoledronic acid in osteosarcoma.
Introduction

Osteosarcoma (OS) is the most common primary malignant bone tumor in both children and young adults with a peak of incidence at 18 years (1, 2). The tumor generally develops on the average part of the long bones (femur and tibia). Current therapeutic protocols combine neoadjuvant poly-chemotherapy with conservative surgery. The long-term survival rate is 60% to 75% at 5 years for patients with localized tumor but is drastically lowered to 20% if pulmonary metastases are detected at diagnosis. To significantly improve survival in children with OS, new therapeutic strategies targeting the molecular basis of OS are required.

Heat shock protein-90 (HSP90) is an ATPase-dependent molecular chaperone that is abundantly and ubiquitously expressed in normal cells (3). HSP90 plays a pivotal role in assisting correct folding and functionality of its client proteins in cells, protecting them from aggregation (4). Tumor cells are constantly stressed (aneuploidy, hypoxia, oncoproteins, treatment…) and consequently exhibit higher HSP90 activity and expression compared with normal cells (4, 5). Thus, HSP90 appears as a therapeutic target of relevance in OS since it acts as “hyper-node” to control multiple survival pathways involved in tumor progression, migration and invasion. Many HSP90 inhibitors targeting the ATPase pocket have been developed. Geldanamycin derivatives (e.g. 17-AAG) were the first proof-of-concept showing HSP90 as a druggable target for cancer therapy. However, 17-AAG contains a benzoquinone moiety that can cause liver toxicity (6), and is poorly soluble leading to formulation challenges and additional toxicities, that have limited its clinical development. Synthetic HSP90 inhibitors with improved pharmacological properties and safety profiles might overcome some of the constraints for clinical development of the first generation of HSP90 inhibitors. For example, other novel HSP90 inhibitors (PU-H71, NVP-AUY922…) were described to induce apoptosis in preclinical studies of various cancers (4, 7-9), including primary bone tumors (10, 11), and are currently in clinical trials in various cancers. However, little is known regarding the potential activity of HSP90 inhibitors in sarcomas. PF4942847, an orally nonquinone-based HSP90 inhibitor that competitively binds to the N-terminal adenosine trisphosphate-binding site of HSP90 (12), and described as extremely potent in triple-negative breast cancer (12), could be a promising therapeutic approach for patients with OS.
There are however, some limitations. Bone microenvironment plays a major role in OS tumor progression by the establishment of a vicious cycle between tumor proliferation and bone resorption (1). In this context, the role of HSP90 inhibition is controversial. Indeed, conflicting studies suggest that in one hand HSP90 inhibition may inhibit both primary tumor growth and bone tumor metastasis (13), while in the other hand several studies have reported that HSP90 inhibition using 17-AAG promotes tumor growth in bone by inducing osteoclast differentiation and activation (14, 15), raising concerns as for patients with tumor-associated bone lesions. 17-AAG was reported to potentiate tumor growth in bone by inducing osteoclastogenesis in bone metastasis models (14, 15). In this study, we show that 17-AAG, but not PF4942847, induces osteoclast formation. Thus, combining PF4942847 with anti-osteoclastic drugs could be an alternative therapeutic strategy. Among these drugs, zoledronic acid (ZOL) is the second generation of nitrogen-containing bisphosphonates (N-BPs), that are stable synthetic analogues of endogenous pyrophosphate (PPI) (16). ZOL is one of the most potent inhibitors of bone resorption in clinical use inducing osteoclast apoptosis by inhibiting enzymes of the mevalonate pathway (17, 18). Moreover, ZOL also directly inhibits proliferation and induces apoptosis of tumor cells, thus delaying tumor growth in OS models (19, 20). Consequently, in the vicious cycle context, combining ZOL with PF4942847 would represent an exciting therapeutic alternative to treat OS by targeting both tumor cells and bone microenvironment.

In the present study, we elucidated the mechanistic and anti-cancer activity of PF4942847, as a single agent or in combination with ZOL, both in vitro and in vivo, in a panel of human osteosarcoma cell lines and xenograft models. We performed our experiments on tumor growth, metastatic dissemination and tumor-associated bone lesions to determine its potential clinical utility.
Materials and Methods:

**Tumor cell lines and reagents:**
The human osteosarcoma cancer cell lines HOS-MNNG (CRL-1547), KHOS (CRL-1544) MG63, U2OS, SJSA1, G292, Cal72, 143B and SaOS2, were purchased from the American Type Culture Collection and maintained in DMEM (Invitrogen-Life Technologies, Inc.) supplemented with 5% fetal bovine serum (FBS) and 2mmol/L L-glutamine. All cell lines were cultured in a humidified 5% CO\textsubscript{2}/air atmosphere at 37°C. All cell lines were passaged for less than 3 months after resurrection and were authenticated by short tandem repeat (STR) profiling.

**Therapeutic agents**
HSP90 inhibitor, PF4942847, orally bioavailable was kindly provided from Pfizer (La Jolla, CA) and used for *in vitro* and *in vivo* studies. This compound is a synthetic small molecule inhibitor that bind the N-terminal adenosine triphosphate binding site of HSP90. For the *in vitro* studies, PF4942847 was dissolved in dimethyl sulfoxide (DMSO) at 10 mM stock solution and stored at -20°C. For the *in vivo* studies, PF4942847 was dissolved in 40% PEG + 60% PBS (at 15 mg/ml and stored at 4°C).

17-allylamino-17-demethoxygeldanamycin (17-AAG) was purchased from Sigma-Aldrich and dissolved in DMSO at 10mM stock solutions and stored at -20°C.

ZOL, 1-hydroxy-2-(1H-imidazole-1-yl) ethylidene-bisphosphonic acid supplied as the disodium salt by Novartis Pharma AG, was dissolved in PBS at 10mM stock solution and stored at -20°C.

**Cell proliferation assay, pulse exposure and apoptosis assays:**
Human OS cells lines were treated with PF4942847 at indicated concentration and time. After time course exposure, cell growth was measured using the crystal violet assay. Detection and quantitation of apoptotic cells were done by flow-cytometry and western blotting analysis. Each assay was repeated in triplicate.

For pulse-exposure experiments, HOS-MNNG and U2OS cells were exposed to 100nM PF4942847. After 2, 6, 16 and 24h of exposure, cells were washed extensively with PBS and returned to PF4942847-free culture. Cell growth was checked by crystal violet assay at 24, 48, 72 and 96 h.
The combination index (CI) was evaluated using CalcuSyn dose effect analysis software (Biosoft, Cambridge, UK). This method, based on the multiple drug effect equation of Chou-Talalay (21).

Caspase 3 activity was assessed two days after treatment using the Fluorometric CaspACE Assay System, (Promega, Madison, USA) as already described in Lamoureux et al. (22).

**Cell cycle analysis**

OS cells were treated with 100 or 200nM PF4942847 for 48 hrs, trypsinized, washed twice and incubated in PBS containing 0.12% Triton X-100, 0.12mM EDTA and 100μg/ml ribonuclease A. 50μg/ml propidium iodide was then added to each sample for 20min at 4°C. Cell cycle distribution was analyzed by flow cytometry (Beckman Coulter Epics Elite, Beckman, Miami, FL), based on 2N and 4N DNA content. Each assay was done in triplicate.

**Quantitative Reverse Transcription-PCR**

Total RNA was extracted using TRIzol reagent (Invitrogen Life Technologies, Inc.). Real-time monitoring of PCR amplification of cDNA was performed using DNA primers (supplemental table S1) on CFX96 Detector System (Bio-Rad) with SYBR PCR Master Mix (Bio-Rad). Target gene expression was normalized to GAPDH level in respective samples as an internal standard, and the comparative cycle threshold (Ct) method was used to calculated relative quantification of target mRNAs. Each assay was performed in triplicate.

**Western-blotting analysis**:

Samples containing equal amounts of protein from lysates of cultured OS cells underwent electrophoresis on SDS-polyacrylamide gel and were transferred to nitrocellulose filters blocked in PBS-BSA 3%-Tween 0.05%. And then, blots were probed overnight at 4°C with primary antibodies (supplementary Table S2). After incubation, the filters were washed in PBS containing 0.1% Tween. Filters were then probed with the secondary antibody coupled to horseradish peroxidase. Antibody binding was visualized with the enhanced chemiluminescence system (Roche Molecular Biomedicals).
**Transwell motility and invasion**

Cell migration and invasion assays were performed in Transwell chamber (8-µm pore size; Falcon). For cell invasion experiments, inserts were coated with 100 µl basement membrane Matrigel (3 µg ml⁻¹; Becton Dickinson) for 90 min at 37°C. HOS-MNNG cells (1×10⁵ cells per ml) were re-suspended in DMEM containing 0.1% FBS, and 300 µl of this cell suspension was loaded into each insert (upper chamber). The chemo-attractant (10% FBS) was placed in the lower chamber (750 µl per well). The plates were incubated for 8h at 37°C. Then, the inserts were carefully collected, the non-migrating cells were removed, and the migrating cells on the under-surface of the inserts were fixed and stained with crystal violet and, then counted by bright-field microscopy.

**Zymography**

The presence of MMP-2 and MMP-9 in serum-free conditioned media was analyzed by zymography in 10% sodium dodecyl sulfate–polyacrylamide gels containing 1 mg/mL gelatin (Sigma-Aldrich). After removal of sodium dodecyl sulfate with Triton X-100, neutral protease activity was revealed by incubation of the gels for 48 hours at 37°C in 50mM Tris buffer (pH 7.4) containing 2mM CaCl₂, followed by staining in 0.05% Coomassie blue in an aqueous solution containing 20% isopropanol and 10% acetic acid. Pictures were taken using G-Box (Syngene, Cambridge, UK).

**Animal Treatment**

Female athymic nude mice (Harlan Sprague-Dawley, Inc.) were injected intra-muscularly close to the tibia with 2x10⁶ HOS-MNNG cells. Once their tumors reached 50mm³, mice were randomly assigned to vehicle, PF4942847 (50 mg/kg for the monotherapy experiment or 25mg/kg for the combined therapy experiment), ZOL (50µg/kg) and treated orally three times per week for PF4942847 and subcutaneously twice a week for ZOL. Each experimental group consisted of 8 mice. Tumor volume was measured 3 times per week. Tumor volume was calculated by the formula: length x width x depth x 0.5432. When the tumor was over 10% of body weight, the mice were sacrificed. Data points were expressed as average tumor volume ±SEM. Tumors and tibia were harvested for further analyses (microscanner, western-blot, histology...). Animal care and experimental protocols were approved by the French Ministry of Research and were done in accordance with the institutional guidelines of the
French Ethical Committee protocol agreement number 1280.01 and under the supervision of authorized investigators.

**MicroCT analysis.**

Tibias were scanned using high-resolution microcomputed tomography (Skyscan 1076, Kontich, Belgium) at 50kV and 200mA using a 0.5mm aluminium filter and a detection pixel size of 9mm. Images were captured every 0.6° through 180° rotation, and analysed using Skyscan software. Trabecular structures positioned 0.2mm below the growth plate were quantified over a length of 1mm. BV/TV and Tb.N of trabecular bone as well as BS and BV of cortical bone were determined.

**Immunohistochemistry**

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded 3µm sections of tumor samples. Immunostaining for Ki67, cleaved-caspase-3, CD146 were conducted using a primary antibody anti-human Ki67 (1:100; Dako), anti-human cleaved-caspase-3 (1/400; Cell Signaling) or anti-human CD146 (1/200; Abcam) on tumor section.

Tibias were decalcified with 4.13% EDTA and 0.2% paraformaldehyde in PBS using the KOS microwave histostation (Milestone, Kalamazoo, MI) before embedding in paraffin. Sections (Leica Microsystems) were analysed by TRAP staining. Quantification of relative OC surface (TRAP+ cells) was evaluated by Qwin (Leica) and ImageJ (NIH, Bethesda, MD) softwares. All analyses were assessed by light microscopy using a DMRXA microscope (Leica). All comparisons were made at x200 magnifications.

**Osteoclast differentiation assays**

Osteoclasts were generated from human CD14+ monocytes of different donors. Purified CD14+ cells were cultured in α-MEM (Invitrogen Life Technologies) with 10% FBS and 25ng/mL human macrophage colony stimulating factor (hM-CSF; R&D Systems). After 4 days, 100ng/mL hRANKL +/-100nM 17-AAG or PF4942847 were added. Multinucleated cells with more than three nuclei were counted after Tartrate-Resistant Acid Phosphatase (TRAP) staining (Sigma, France). All experiments were performed at least three times.
**Statistical analysis**

All *in vitro* data were assessed using the Student t test and Mann-Whitney test. Tumor volumes of mice were compared using Kruskal-Wallis test. Overall survival was analyzed using Kaplan-Meier curves and statistical significance between the groups was assessed with the log-rank test (Graphpad Prism). Levels of statistical significance were set at $P<0.05$. 
Results:

**PF4942847 inhibits osteosarcoma cell proliferation**

Before planning any therapeutic strategy targeting HSP90, we evaluated the mRNA expression levels of HSP90 in human OS patient samples. Compared to normal osteoblast cells, all patients overexpressed HSP90, making all those OS patients good candidates for HSP90 inhibition as an alternative therapeutic strategy in OS treatment (Supp. Figure 1).

We first assessed the effects of inhibitors of HSP90, namely PF4942847 and the well-known 17-AAG, on cell growth in nine genetically defined human OS cell lines. In all cell lines, PF4942847 inhibited cell growth in a dose-dependent manner, with IC\textsubscript{50} values ranging from 13.48 to 97.31nM (Fig 1A). Moreover, PF4942847 was 2.5 to 27 fold more potent than 17-AAG (Fig 1A). Then, we chose the most PF4942847-sensitive HOS-MNNG, and the low/moderate sensitive U2OS cell lines for further in vitro investigations. Pulse exposure of these cell lines showed that 16h of drug exposition were sufficient to induce growth inhibition at 48h, 72h and 96h of culture, while 2 and 6 hours of drug exposition had no significant effect on cell proliferation (Fig. 1B, left panel). Moreover, colony formation assays after 48h of PF4942847 treatment showed that inhibition of HSP90 significantly reduced the ability of tumor cells to form colony (Fig 1B, right panel).

**PF4942847 induces apoptosis and down-regulates HSP90 client proteins in osteosarcoma tumor cell lines**

We next assessed the effects of HSP90 inhibition on OS cancer cell apoptosis using flow-cytometric, western blot analyses and enzymatic caspase-3/7 activity. The fraction of cells undergoing apoptosis (subG1 fraction) significantly increased in a dose-dependent manner with PF4942847 compared to control, in both HOS-MNNG and U2OS cells (respectively 88% and 34% vs 3% and 6.1% in control; Fig 1C, upper panel). Furthermore, PF4942847 induced apoptosis as shown by increasing caspase-3 activation in a dose-dependent manner in both cell lines (Fig 1C, bottom panel). These results were confirmed by western-blot analysis showing an increase of both cleaved-caspase-3 and cleaved-PARP expression in tumor cells (Fig 1D).
Because inhibition of HSP90 causes proteasomal degradation of a subset of cellular proteins, we next investigated the effect of PF4942847 on HSP90-client proteins and HSP70. Expression of HSP70, a typical marker of HSP90 inhibition (23, 24), was increased by PF4942847 in a dose-dependent manner in OS cells at both mRNA and protein levels (Fig 1D and Supp. Fig.2A). PF4942847 also decreased protein levels of client proteins (Fig 1D), while mRNA levels did not change (Supp. Fig.2A), suggesting a regulation at the protein level. We then checked whether PF4942847 induced proteasomal degradation of client proteins by using the proteasome inhibitor MG132. While treatment with PF4942847 decreased expression of HSP90 client proteins, adding MG132 inhibited these degradations (Supp. Fig.2B).

**PF4942847 delays tumor growth on HOS-MNNG xenograft model.**

We next evaluated the therapeutic potential of PF4942847 in OS xenograft model. Mice bearing OS tumors were orally treated three times per week with 50 mg/kg PF4942847 or with vehicle starting at the day 7. All animals treated with PF4942847 (n=7) exhibited a significant decrease in tumor volume compared with control mice starting at the day 12 (82.6mm$^3$ and 187.7mm$^3$, respectively) and after 34 days (654.3mm$^3$ and 3148.3mm$^3$, respectively; Fig. 2A). When each animal is considered individually, the incidence of mice progressing with a tumor volume ≥1000mm$^3$ was significantly diminished by day 23 in PF4942847-treated animals (0/7) compared with controls (7/7, Fig 2B). Consequently, overall survival was significantly prolonged in the PF4942847-treated group compared with control group (Fig 2C). Indeed, by day 45, all mice were died or euthanized due to a high tumor burden in the vehicle group, while all mice were still alive in the PF4942847-treated group. These data demonstrate that targeting HSP90 significantly delays OS tumor growth and prolongs the overall survival in the human HOS-MNNG xenograft model.

Immunohistochemical analysis indicated a decreased Ki67 expression after treatment with PF4942847 (26.8% in PF4942847-groups vs 48.75% in control-group; Fig 2D). Moreover, significant increase of cleaved-caspase-3 expression as compared to control suggests a PF4942847-induced apoptosis (Fig. 2D). Overall, inhibition of tumor progression by PF4942847 may result from a combination of decreased proliferative (reduced Ki67 expression) and increased apoptosis (increased cleaved-caspase-3 expression) rates (Fig 2).
Importantly, no apparent side effects were observed except a non-significant loss of bodyweight, probably due to a small toxicity.

**PF4942847 reduced development of pulmonary metastasis by inhibiting invasion, migration and angiogenesis**

OS progression is associated with the development of pulmonary metastasis. In our HOS-MMNG model, PF4942847 significantly reduced the incidence of pulmonary metastases in mice (37.5% versus 100% in control) and the number of pulmonary metastases per mouse (0.87 versus 7.1 metastasis in control; Fig 3A). Moreover, the HE staining showed vascular emboli in 100% of mice in control group as compared to only 14.3% in treated mice (Fig 3A). We also assessed CD146 staining in order to evaluate angiogenesis in tumor tissue. PF4942847 significantly reduced the number and the development of vessels in tumor (Fig 3B). We then examined the effect of PF4942847 on several aspects of tumor cell behavior in vitro. As shown in Fig. 3C, PF4942847 significantly reduced (by 4-fold at 50nM) cells migration and invasion in a dose-dependent manner in HOS-MNNG cells (Fig. 3C), in accordance with the in vivo results (Fig. 3B). In addition, zymography assay demonstrated that PF4942847 strongly reduced the activation of matrix metalloproteinase MMP9 in a dose-dependent manner without affecting the MMP2 activity in OS cells (Fig. 3D). Western blot analysis indicated a reduction of C-MET, MMP9 and Focal adhesion kinase (FAK) expressions at protein level in PF4942847-treated cells (Fig. 3D) without affecting mRNA expressions (Supp Fig.2A). These decreases of C-MET, FAK and MMP9 expressions at protein level were confirmed in vivo by immunohistochemistry in tumor treated with PF4942847 (Supp. Fig.3). All these in vitro and in vivo data demonstrate that targeting HSP90 blocks the formation of lung metastasis.

**Effect of PF4942847 on tumor-associated bone lesions**

17-AAG has been reported to induce osteoclastogenesis and consequently stimulate tumor progression in bone environment (17, 18). We thus investigated the effect of PF4942847 on the bone micro-architecture of tumor-bearing tibia using micro-computed tomography (microCT) on bone parameters in vivo and on osteoclastogenesis in vitro. While the tumor
volume was reduced after treatment (Fig. 2A), PF4942847 failed to significantly prevent the tumor-associated osteolysis as shown by an extensive analysis of bone morphometric parameters of cortical and trabecular bone (Fig. 4A). However, the increased bone degradation reported in literature with 17-AAG was not reproduced with PF4942847. Indeed, the trabecular bone volume (BV/TV; from 7.3% to 10.5%) and the trabecular number (Tb.N (/mm); from 0.93 to 1.1) were slightly improved after treatment (Fig. 4A). The same tendency was observed for the bone volume (BV; 4.3mm$^3$ in control vs 4.9mm$^3$ in treated group) and the bone surface (BS; 77.5mm$^2$ in control vs 85mm$^2$ in treated group) of the cortical bone (Fig. 4A). Histological analysis of the tumor-bearing bone with TRAP staining confirmed that PF4942847 treatment slightly decreased the number of osteoclasts (Fig. 4B).

We then evaluated the effects of PF4942847 vs. 17-AAG on osteoclast differentiation. 17-AAG increased osteoclast formation as compared to control, while PF4942847 treatment did not (Fig. 4C). To identify the underlying mechanism of HSP90 inhibitors on osteoclast differentiation, we examined the activation of NF-kB (p-P105) and Src pathways, essential steps for osteoclast differentiation and maturation, in presence of 17-AAG or PF4942847. While 17-AAG increased p-NF-KB (p-P105) and p-Src expressions, PF4942847 did not change their expressions as compared to control (Fig. 4D). Consequently, the mRNA expression of NF-kB target gene NFATC1 was increased after 17-AAG but not after PF4942847 treatments, corroborating the osteoclast differentiation results (Fig. 4C).

All these data demonstrate that PF4942847 does not have any significant effect on osteoclast differentiation and tumor-associated bone lesions, whereas 17-AAG induced osteoclasts formation.

**HF4942847 synergistically acts with ZOL to reduce cell proliferation and induce apoptosis in OS cell lines.**

Since PF4942847 strongly inhibits tumor progression (Fig. 2), but does not prevent tumor-associated bone lesions (Fig. 4), we next evaluated whether the combination of PF4942847 with ZOL prevented tumor-associated osteolysis with a good therapeutic effect on OS tumor growth. The constant ratio design and the combination index (CI) values were performed and calculated according to the Chou and Talalay median effect principal (21). The dose-response curve (combination treatment, PF4942847 and ZOL monotherapy) and the combination index
plots indicate that PF4942847 synergistically acted with ZOL to inhibit tumor cell growth (Fig. 5A). Then we performed a colony formation assay to evaluate capabilities to recover after 2 days of PF4942847 combined with ZOL. While ZOL did not change the number of colonies, PF4942847 decreased the colony formation compared with control (Fig. 5B). However, the combination of PF4942847 with ZOL induced the highest decrease of colony formation compared with each single drug alone (Fig. 5B).

Flow cytometric analysis shows that apoptotic rates (subG1 fraction) increased significantly when PF4942847 is combined with ZOL (64.8%), compared to control (5.7%), ZOL (8.3%), PF4942847 (34.5%; Fig. 5C). Moreover, PF4942847+ZOL increased caspase-dependent apoptosis compared to PF4942847 or ZOL monotherapy, as shown by a significant increase of caspase-3 activity, and cleaved-PARP and cleaved-caspase-3 expression (Fig. 5D). Reduced cell viability from combined ZOL and HSP90 inhibition may result, in part, from decreased Akt, p-ERK and C-RAF1 expression. Moreover C-MET expression is also reduced in combination therapy (Fig. 5D).

**Combined treatment with PF4942847 and ZOL synergistically delays OS tumor growth, and prevents bone lesions and pulmonary metastasis.**

We next assessed the effect in HOS-MNNG model. In order to reduce toxicity and to visualize a synergistic effect of the combined therapy, we used 25mg/kg PF4942847 in this experiment, which corresponds to half-dose of PF4942847 of what was used for the first in vivo experiment (Fig. 2). Mice treated with PF4942847+ZOL had significant delays in tumor growth compared with all other groups (Fig. 6A). Indeed, by day 35, all control mice died or had to be euthanized (tumor volume in the PF4942847+ZOL group was 1090.5mm³ compared to 1930.2mm³ for PF4942847 alone and 2444mm³ for ZOL alone; Fig. 6A). At individual level, the incidence of mice progressing with a tumor volume ≥1000mm³ was significantly diminished by day 30 in PF4942847+ZOL-treated animals (0/8) compared with PF4942847 alone (7/8), ZOL alone (8/8) or control (8/8, Fig. 6A). Overall survival was significantly prolonged in mice treated with combined therapy compared to the other groups (Fig 6B, left panel). In addition, PF4942847+ZOL significantly reduced the incidence of pulmonary metastasis in mice (12.5% versus 100% in control, 87.5% in ZOL group and 37.5% in PF4942847 group; Fig 6B). These data demonstrate that HSP90 inhibition using
PF4942847 synergistically acts with ZOL to significantly inhibit tumor growth and reduce the development of pulmonary metastasis in HOS-MNNG model.

Consistent with the \textit{in vitro} findings, immunohistochemical analysis suggest that delay in tumor progression in PF4942847+ZOL treated mice results from both reduced proliferation rate (significant decrease of Ki67 expression) as well as increased apoptosis rate (increased cleaved-caspase-3 expression; Fig. 6D).

Because PF4942847 monotherapy failed to protect bone from tumor-associated lesions (Fig 4), we next evaluated the combined therapy PF4942847+ZOL on bone parameters of tibia bearing tumors. The combined therapy significantly prevented the cortical bone lesions as shown by increased bone volume (BV=5.07mm$^3$) and bone surface (BS= 79.01mm$^2$) compared with PF4942847 alone (BV=4.52mm$^3$ and BS=72.19mm$^2$) or control (BV=4.01mm$^3$ and BS=67.4mm$^2$). However, the combination was not better compared than ZOL alone (BV=4.96mm$^3$ and BS=5.07mm$^2$; Fig 6D). The same results were observed for the trabecular bone volume (BV/TV; from 7% in control to 15.5% after combined therapy, versus 9.9% after PF4942847 treatment and 16% after ZOL treatment) and the trabecular number (Tb.N (/mm); from 0.91mm in control to 1.63mm after combined therapy, versus 1.06mm after PF4942847 treatment and 2.01mm after ZOL treatment; Fig. 6D). Altogether these data suggest that combined therapy had synergistic effect to delay tumor progression but the prevention of bone lesions is mainly due to the effect of ZOL.
Discussion

While great advances have been made in treating OS in the past few decades, still more research is needed to learn how to better manage hard-to-treat or advanced OS (resistant or metastatic OS) (1). Many clinical trials are focusing on treating OS using a variety of targeted therapies (1, 25). Among them, HSP90 inhibition is a promising therapeutic strategy showing clinical activity in various tumor types, including OS (10, 26-28). Indeed, HSP90 client proteins are proteins playing crucial roles in establishing cancer cell hallmarks (29). Consequently, the malignancy is maintained with the aid of HSP90 acting on mutated proteins, that become particularly dependent to its activity leading to “HSP90 addiction” for cancer progression and making the cancer cells more susceptible to HSP90 inhibitors than the normal cells (30).

In this study, we examined the effects of the orally bioavailable HSP90 inhibitor, PF4942847, in human OS xenograft model. PF4942847 demonstrated superior potency in vitro in a panel of OS cell lines compared to 17-AAG on inhibition of cell survival and induction of apoptosis and, delayed tumor growth in OS model in vivo. All the data obtained in this study clearly identify significant anti-cancer effects of HSP90 inhibition in OS model in accordance with the literature. Indeed, decreased cell survival, decreased cell proliferation, induced apoptosis, and depletion of HSP90 client proteins have also been observed in osteosarcoma using HSP90 inhibitors (17-AAG, SNX-2112…) alone or in combination with chemotherapy or targeted therapies (telomerase inhibitor, CDK inhibitor…) (10, 26-28). However, the authors only focused on primary tumor often using subcutaneous xenograft models without studying the bone microenvironment and/or pulmonary metastases.

Indeed, at the time of diagnosis, approximately 80% of OS patients have metastatic disease usually involving the lungs (31). In this case the survival rates drastically drop down to 20% at 5 years (32), consequently there is an urgent need to block or prevent lung metastasis in these patients. In our study, we evidently demonstrated that targeting HSP90 affects C-Met signaling leading to delayed tumor growth and reduced angiogenesis and pulmonary metastases development in OS model. The C-Met oncogene encodes the tyrosine kinase receptor for Hepatocyte Growth Factor and is overexpressed in OS (33, 34) playing an important role in development of OS (35). Indeed, Patane et al. demonstrated the importance of C-Met signaling in OS by the ability of either wild-type or constitutively activated C-Met to drive osteoblast transformation to OS tumor cells (36), while introduction of C-Met
dominant-negative inhibits the in vivo tumorigenicity (36). C-Met elicits a physiological program leading to invasive tumor growth in OS (37), and inhibition of C-Met signaling as an anticancer strategy has been validated in preclinical model of human cancer (38).

Added to this, we also found that PF4942847 inhibited MMP9 activity and expression and FAK expression. MMP9 is overexpressed in malignant tumors compared with benign or non-invasive tumors and plays a role in tumor invasion and metastasis through the degradation of the extracellular membrane leading to cancer cell spread to distal organs in OS (39, 40). Wang et al. reported a strong correlation between HSP90 and MMP9 in gastric cancer and suggested an important role of HSP90 in tumor invasion and metastasis (41). We confirmed that inhibition of HSP90 affects MMP9 expression and activity, and reduces the apparition of metastases in OS model. FAK is also overexpressed in OS and plays an important role in metastasis by affecting the migration ability of OS cells by suppressing lamellipodia formation (42) and making FAK as a therapeutic target of interest in pediatric tumors (43). Our data demonstrate for the first time that targeting HSP90 using PF4942847 inhibits migration, invasion and metastasis development by disrupting at once C-Met, FAK and MMP9 signaling.

Although we demonstrated that HSP90 is a good anti-cancer target in OS, HSP90 inhibitors present major limitations: the necessity of complex formulations, poor solubility, limited bioavailability and hepatotoxicity (44), and several inhibitors failed in clinical trial because of limited efficacy (45). In this study, no side effects or toxicity were observed after treatment with PF4942847, except a non-significant loss of body weight, in contrast to reports of hepatotoxicity with 17-AAG (44). Moreover, the use of HSP90 inhibitors in tumor associated with bone lesions is actually controversial. Contradictory reports suggested that blocking HSP90 inhibits both primary tumor growth and bone metastasis (13, 24), while other recent studies have reported that HSP90 inhibition using 17-AAG promotes prostate and breast cancer growth in bone by inducing both osteoclast differentiation and activation via induction of Src signaling or HSF1 dependent mechanism (14, 15, 46). These results highlight a potential risk to use HSP90 inhibitors therapies in patients with tumor-associated bone lesions. In this report, we effectively confirmed induction of osteoclastogenesis with 17-AAG, while PF4942847 did not increase RANKL-induced osteoclast differentiation. However, PF4942847 failed to inhibit osteoclastogenesis and to prevent bone lesions associated with the tumor in OS model, even if the bone phenotype was slightly better than in control group.
In order to prevent tumor-associated bone lesions, we evaluated the combined therapy of PF4942847 with ZOL. The main aims of drug combination are to achieve synergistic therapeutic effect, dose and toxicity reduction and to minimize or delay the induction of drug resistance (47). Combination studies of HSP90 inhibitors and chemotherapeutic agents or targeted therapies have been previously explored, generally showing either an additive or synergistic effect (23, 48). We here clearly demonstrated that combination of PF4942847 and ZOL synergistically inhibits OS cell proliferation to a greater extent than PF4942847 alone via induction of apoptosis. In vivo, we used a dose of PF4942847 (25mg/kg), which did not have any significant effect on tumor growth in order to reduce a potential toxicity. PF4942847+ZOL synergistically delayed tumor growth, prolonged the survival and reduced the incidence of metastasis in OS model by reducing proliferation rate and inducing apoptosis. Moreover, the combined therapy significantly prevented tumor-associated bone lesions just as well as ZOL alone, compared to PF4942847 alone or control group. We also confirmed the antitumor activity of ZOL as described (47, 49, 50).

In conclusion, there are many types of targeted therapies, but all share the same characteristic of attempting to capitalize on a biologic characteristic of the cancer cell to eradicate the tumor. This report provides evidence that blocking “HSP90 addiction” in cancer cells may represent a novel therapeutic approach to treat patients with advanced-osteosarcoma, by hitting important cancer hallmarks such as cell viability, angiogenesis and metastatic development, with only one drug. Moreover, these data are a proof of principle for the combination of an HSP90 inhibitor with anti-osteoclastic drugs, making this type of treatment as a new potential therapeutic alternative to traditional chemotherapy, by preventing tumor-associated bone lesions and potentially reducing many side effects.

Acknowledgments

This study was supported by l’Association pour la Recherche sur le Cancer (France), and Pfizer Global Research & Development (United States).
Bibliography


Legends

Figure 1. PF4942847 inhibits cell growth and induces apoptosis and degradation of HSP90 client proteins in OS cell lines. A, tumor cell lines (U2OS, HOS-MNNG, MG63, KHOS, SJSA1, Cal72, G292, SaOS2 and 143B) were cultured for 72 hours in the presence of PF4942847 or 17-AAG at the indicated concentration and cell growth was determined by crystal violet assay and compared with control. The table compares the IC50 of each tumor cell lines between PF4942847 and 17-AAG. B, left, time dependent anti-proliferative activity of PF4942847 was evaluated by pulse exposure assay. Tumor cells were exposed to 100nM PF4942847 for 2, 6, 16 or 24 hours, washed extensively, and cultured in PF4942847-free media. Cell growth at 24-96 hours was determined by crystal violet. Data represent mean % cell growth ± SD. B, right, In order to perform clonogenic assay, HOS-MNNG tumor cells were treated with 100nM PF4942847 or with DMSO in control condition for 2 days, and then plated at clonal density and cultured in PF4942847-free media followed by colony counts. C, upper, tumor cells were treated with 100nM or 200nM of PF4942847 for 2 days, and the proportion of cells in subG1, G0-G1, S, G2-M was determined by propidium iodide staining. C, bottom, HOS-MNNG and U2OS cells were treated with PF492847 at indicated concentration for 2 days, and Caspase-3/7 activity was determined on the cell lysates and the results are expressed in arbitrary units and corrected for protein content. *, p<0.05; ***, p<0.001. D, tumor cells were cultured in presence of 100nM or 200nM PF492847 for 2 days, and proteins regulated by HSP90, PARP, Caspase-3 and HSP70 expression levels were determined by Western blotting. All experiments were repeated at least thrice.

Figure 2. PF4942847 significantly delays osteosarcoma HOS-MNNG tumor growth and prolongs the overall survival. Mice were orally treated with 50mg/kg PF4942847 three times per week, starting when the tumor is palpable as described in Materials and Methods. The mean (A) or the individual (B) tumor volume of mice treated was compared with control group ± SEM (n=7). C, in Kaplan-Meier curve, overall survival was compared between mice treated with PF4942847 and control over a 42-d period. p<0.001. D, tumors were collected after 42 days and Ki67 and cleaved-Caspase-3 were evaluated by immunohistochemical analysis. Specimens were scored and estimated in percentage of positive cells or in arbitrary unit (UA). ***, p<0.001; **, p<0.01.
Figure 3. PF4942847 inhibits invasion, migration and pulmonary metastasis development in osteosarcoma HOS-MNNG xenograft model. A, After sacrifice lungs were harvested, embedded in paraffin and stained by H&E. Black Arrows indicate metastatic tumor cells, whereas red arrows indicate emboli. B, tumors were collected after 42 days, embedded in paraffin, and CD146 was evaluated by immunohistochemical analysis. Representative photomicrographs per group for HOS-MNNG osteosarcoma mice are shown. Histograms represent the percentage of positive area for CD146, the number of vessels per total area (in pixel) and the average size of vessels in each group of mice (Mean ±SEM). C, Cell migration and invasion were performed using boyden chambers transwell inserts (8 µm pores) as described in M&M. Cells were treated 24 hours with PF4942847 at indicated concentration and plated in the transwell inserts. After 8h, cells which invaded through the membrane were fixed, stained with crystal violet and counted. D, left, Zymography analysis of serum-free conditioned media from HOS-MNNG cells treated with PF4942847 at indicated concentration for 24 hours. Coomassie blue stained gel representative of three independent experiments is shown. D, right, tumor cells were cultured in presence of 100nM PF4942847 for 2 days, and C-Met, MMP9, FAK and GAPDH expression levels were determined by Western blotting. **, p<0.01; ***, p<0.001.

Figure 4. Effect of PF4942847 on tumor-associated bone lesions. A, MicroCT quantification of the specific trabecular bone volume (BV/TV (%)) and trabecular number (Tb.N (/mm)) as well as the cortical bone volume (BV (mm³)) and the cortical bone surface (BS (mm²)) were calculated on the tibia of tumor-bearing mice of the different groups. B, Multinucleated TRAP+ cells (osteoclasts) were evaluated on the tibia of tumor-bearing mice by immunohistochemical analysis (Black arrows; original magnification, x200). Specimens were scored and estimated in osteoclast per bone surface and osteoclast number per mm of bone using ImageJ software. C, Purified human CD14+ monocytes were cultured for 13 days in the presence of M-CSF, RANKL and treated or not with 100nM PF4942847 or 17-AAG. TRAP coloration was performed at the end of culture period and multinucleated TRAP-positive cells were counted under light microscope. D left, human CD14+ monocytes were cultured in the presence of M-CSF and 100nM PF4942847 or 17-AAG for 24 hours and stimulated with 100 ng/mL RANKL for 20 minutes. Proteins were extracted and western blotting were performed to evaluate expressions of p105, phospho-p105, Src and p-Src. D
right, human CD14+ monocytes were cultured in the presence of M-CSF, RANKL and 100nM PF4942847 or 17-AAG for 3 days, and qRT-PCR was performed to evaluate NFATC1 RNA levels. Results are expressed as fold increase compared with the control condition without RANKL and treatment (PF4942847 or 17-AAG). *, p<0.05.

**Figure 5.** PF4942847 synergistically acts with ZOL to inhibit cell proliferation and induce apoptosis in OS cell lines. A, Dose-dependent effects and Combination Index (CI) values calculated by CalcuSyn software were assessed in HOS-MNNG cells treated for 48 hours with PF4942847 alone, ZOL alone, or combined treatment at indicated concentration with constant ratio design between both drugs. Cell growth was determined by crystal violet. The CI for ED$_{50}$ and ED$_{75}$ was respectively 0.67 and 0.69 indicating a synergistic effect (lower than 1) of HSP90 inhibition combined with ZOL. B, osteosarcoma cells were treated with 100nM PF4942847 +/- 1μM ZOL for 2 days, and then plated at clonal density for colony counts after crystal violet staining. C, HOS-MNNG cells were treated with 100nM PF4942847 +/- 1μM ZOL for 2 days and the proportion of cells in subG1, G1, S and G2/M phase was determined by propidium iodide staining. D, left, Cells were harvested, and Caspase-3/7 activity was determined on the cell lysates and the results are expressed in arbitrary units and corrected for protein content. D, right, tumor cells were cultured in presence of 100nM PF4942847 and +/- 1μM ZOL for 2 days, and expression levels of proteins of interest were determined by Western blotting. All experiments were repeated at least three times. ***, p<0.001; **, p<0.01; *, p<0.05.

**Figure 6.** PF4942847 combined with ZOL synergistically delay tumor growth and prevents tumor-associated bone lesions in HOS-MNNG osteosarcoma xenograft model. Mice were treated twice a week with 50μg/kg ZOL and 3 times per week with 25mg/kg PF4942847 starting when tumors were palpable as described in M&M. A, The mean tumor volume (left panel) and individual tumor volume (right panel) were compared between the 4 groups ± SEM (n=8). ***, p<0.001; **, p<0.01. B, in Kaplan-Meier curve, overall survival was compared between the 4 groups over a 55-d period (left panel), and presence of pulmonary metastasis were evaluated after autopsy (right panel). C, tumors were collected and Ki67, and cleaved-caspase-3 expression were evaluated by immunohistochemical analysis. Specimens were scored and estimated in percentage of positive cells or in arbitrary unit (UA). The control group corresponds to the mice bearing tumor that did not receive any
treatment. This group only received vehicle. **, p<0.01; ***, p<0.001. D, MicroCT quantification of the specific trabecular bone volume (BV/TV (%)) and trabecular number (Tb.N (/mm)) as well as the cortical bone volume (BV (mm³)) and the cortical bone surface (BS (mm²)) were calculated on the tibia of tumor-bearing mice of the different groups. ***, p<0.001; **, p<0.01; *, p<0.05.
<table>
<thead>
<tr>
<th>Cell line</th>
<th>PF-4924847 GI&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
<th>17-AAG GI&lt;sub&gt;50&lt;/sub&gt; (nM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>U2OS</td>
<td>72.18</td>
<td>177.5</td>
</tr>
<tr>
<td>MMNG/HOS</td>
<td>15.37</td>
<td>104.6</td>
</tr>
<tr>
<td>MG63</td>
<td>44.62</td>
<td>361.8</td>
</tr>
<tr>
<td>KHOS</td>
<td>18.97</td>
<td>185.9</td>
</tr>
<tr>
<td>SJSA1</td>
<td>13.48</td>
<td>231.2</td>
</tr>
<tr>
<td>143B</td>
<td>31.86</td>
<td>285.6</td>
</tr>
<tr>
<td>Cal72</td>
<td>49.97</td>
<td>856.7</td>
</tr>
<tr>
<td>G292</td>
<td>21.53</td>
<td>985.2</td>
</tr>
<tr>
<td>SaOS2</td>
<td>97.31</td>
<td>1262</td>
</tr>
</tbody>
</table>

**Figure 1**

(A) Cell viability (%) vs. time (hours) for different cell lines with varying concentrations of PF-4924847 and 17-AAG.

(B) Cell viability (%) vs. time (hours) for HOS-MNNG and U2OS cell lines with different treatments.

(C) Percentage of cell-cycle repartition for HOS-MNNG and U2OS cell lines with different treatments.

(D) Caspase 3/7 activity (%) for HOS-MNNG and U2OS cell lines with different treatments.
Figure 2

A

Tumor cells injection  Treatment initiation  Analysis

5 weeks  1 week  4-5 weeks

Mean tumor volume (mm$^3$)

Control  PF-4942847

***

B

Tumor volume (mm$^3$)

Control  PF4942847

C

Percent survival

Control  PF4942847

$P<0.001$

D

Ki67

% positive cells

Control  PF4942847

**

Cleaved-Casp3

Expression (UA)

Control  PF4942847

***
Figure 4

A

Cortical bone

[Graphs showing BV (mm²) and BS (mm²) for CT, RANKL, and PF4942847]

Trabecular bone

[Graphs showing BV/TV (%) and Tb.N. (mm) for CT, RANKL, and PF4942847]

B

Control

PF4942847

[Images and graphs showing TRAP, OcS/BS, and OC number/mm]

C

[Images showing RANKL, RANKL + 17-AAG, and RANKL + PF4942847]

D

[Images showing Western blots for Phospho-p105, Total-p105, Phospho-Src, Total-Src, and NFATC1 with relative mRNA expression]

Research.

on July 15, 2017. © 2015 American Association for Cancer Research.

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

Author Manuscript Published OnlineFirst on December 28, 2015; DOI: 10.1158/1078-0432.CCR-15-1925
Figure 5

A. Dose-effect curve

B. Colonies/well

C. % cell-cycle repartition

D. Caspase 3/7 activity (U/µg of protein)

- HSP90: 90kDa
- HSP70: 70kDa
- PARP: 116kDa, 89kDa
- Casp-3: 35kDa, 19kDa
- AKT: 60kDa
- p-ERK: 44kDa, 42kDa
- ERK: 44kDa, 42kDa
- C-RAF1: 80kDa
- C-MET: 170kDa, 140kDa
- GAPDH: 37kDa
Figure 6

A

B

C

D

Mean tumor volume (mm$^3$)

Days

Percent survival

Days

% positive cells

Expression (UA)

% of mice with metastasis

Days

Tumor volume (mm$^3$)

Days

Ki67

Cleaved-Casp3

Trabecular bone

Cortical bone

BS (mm$^2$)

BV (mm$^3$)

BV/TV (%)

Tb.N. (/mm)

Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.
Blocking HSP90 addiction inhibits tumor cell proliferation, metastasis development and synergistically acts with zoledronic acid to delay osteosarcoma progression.

Benjamin Ory, Marc Baud'huin, Franck Verrecchia, et al.

Clin Cancer Res  Published OnlineFirst December 28, 2015.

Updated version  Access the most recent version of this article at: doi:10.1158/1078-0432.CCR-15-1925

Supplementary Material  Access the most recent supplemental material at: http://clincancerres.aacrjournals.org/content/suppl/2015/12/23/1078-0432.CCR-15-1925.DC1

Author Manuscript  Author manuscripts have been peer reviewed and accepted for publication but have not yet been edited.

E-mail alerts  Sign up to receive free email-alerts related to this article or journal.

Reprints and Subscriptions  To order reprints of this article or to subscribe to the journal, contact the AACR Publications Department at pubs@aacr.org.

Permissions  To request permission to re-use all or part of this article, contact the AACR Publications Department at permissions@aacr.org.