Cancer Letters 300 (2011) 30–39

Contents lists available at ScienceDirect

Cancer Letters

journal homepage: www.elsevier.com/locate/canlet

A novel class of specific Hsp90 small molecule inhibitors demonstrating *in vitro* and *in vivo* anti-tumor activity in human melanoma cells

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ARTICLE INFO

Article history:
Received 21 June 2010
Received in revised form 30 August 2010
Accepted 1 September 2010

Keywords:
Hsp90
B-Raf
Melanoma
Cancer drug

ABSTRACT

Hsp90 is required for conformational maturation and stability of numerous key signaling proteins (clients) involved in cell proliferation and transformation. Here we describe two novel Hsp90 inhibitors, PF-04470296 and PF-03823863, demonstrate a differential sensitivity in B-Raf mutant (V600E) versus wild-type protein degradation. These two inhibitors inhibit proliferation and anchorage-dependent growth, and abolish *in vivo* xenograft tumor growth in melanoma cells regardless B-Raf mutation status. Mutant B-Raf protein and other Hsp90 clients such as cMet, ErbB2, C-Raf, and AKT, are degraded in cells and xenograft tumors. Our results indicate that Hsp90 inhibitors induce anti-tumor activity in melanoma cells and are expected to show therapeutic benefit in melanoma patients by collaboratively targeting multiple pathways.

1. Introduction

The heat shock protein 90 (Hsp90) is responsible for regulating the conformation, stability and activity of a variety of key signaling proteins, including protein kinases (e.g., Raf-1 AKT, ErbB family, Cdk4), steroid receptors (e.g., androgen receptor and estrogen receptor), and transcription factors (e.g., HIF-1α). These Hsp90 client proteins are involved in multiple pathways of cell transformation and tumor progression. Therefore, targeting Hsp90 offers an opportunity for the inhibition of multiple pathways in human cancers [1–3]. A number of mutant oncoproteins require Hsp90 to maintain protein stability and function such as v-Src, mutant epidermal growth factor receptor (EGFR), and mutant B-Raf, whereas their wild-type counterparts are either not dependent or only weakly dependent on Hsp90 activity [4–8]. Cancer cells have greater dependence on Hsp90 activity associated with the higher malignant over-stressed condition and oncogene addiction [3], such that Hsp90 inhibitors may have therapeutic advantages in cancer versus normal cells.

Several natural products, such as geldanamycin and radicicol, bind the amino-terminal ATP pocket of Hsp90, inhibit ATPase activity, and subsequently lead to client protein degradation mediated through the ubiquitin ligase machinery [9,10]. While geldanamycin toxicity precluded clinical development [11], geldanamycin derivatives, such as 17-allylamino-17-demethoxy-geldanamycin (17-AAG), exhibited a more favorable therapeutic window and anti-tumor activity has been observed in early-stage clinical trials [12,13]. However, 17-AAG is poorly soluble with resulting formulation issues as well as low oral bioavailability, metabolism issues, and hepatotoxicity [12,14–16]. 17-DMAG and the hydroquinone IPI-504 both have improved aqueous solubility, and are currently in Phase I and II clinical trials [17,18]. However, because of the potential toxicity of geldanamycin derivatives, specific small molecular Hsp90 inhibitors may be the more effective clinical agents.

The Ras/Raf/MEK/ERK(MAPK) pathway is mutationally activated and plays an important role in the phenotypic traits of melanomas [19]. N-Ras is mutated in approximately 25% of human melanomas, whereas mutations in the H-Ras

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doi:10.1016/j.canlet.2010.09.002

and K-Ras genes are rare [20]. B-Raf is mutated in nearly 70% of melanomas where the vast majority are V600E missense mutations, whereas C-Raf and A-Raf are rarely mutated in melanomas [19]. N-Ras and B-Raf mutations appear mutually exclusive in melanomas, suggesting that they make similar contributions to tumor progression of melanomas and that activation of this pathway plays a key role in the development of human melanomas [19,21]. V600E B-Raf mutant protein has been shown to be a client protein of Hsp90, but wild type B-Raf is resistant to 17-AAG induced protein degradation [7,8]. However, *in vitro* anti-proliferation activity induced by 17-AAG was similar in the melanoma cell lines harboring either mutant V600E B-Raf or wild type B-Raf [7,8]. While the phase II trial of 17-AAG in patients with metastatic melanoma did not achieve objective anti-melanoma responses [22], a more potent Hsp90 inhibitor with better pharmacokinetic properties may demonstrate greater therapeutic benefit in this patient population.

A series of resorcinol amide compounds have been identified as potent and specific Hsp90 inhibitors which demonstrate activity to induce Hsp90 client protein degradation in a variety of cell lines tested; detailed structure–activity relationship (SAR) has previously been described [23,24]. Here we show data from two representative compounds, PF-4470296 and PF-3823863 in the resorcinol amide series. PF-4470296 represents the pyrrolidine series that has greater potency towards Hsp90 client protein degradation but lacks sufficient bioavailability for *in vivo* studies. PF-3823863 is an example of the isoindoline series that has slightly less activity towards Hsp90 client protein degradation but better bioavailability for animal studies. In this study, we focused on these two representatives to investigate anti-tumor activity in six melanoma cell lines. We show that these two representatives exhibit anti-tumor activity in both *in vitro* and *in vivo* assays in melanoma cell lines with either mutant B-Raf or wild type B-Raf. In mutant B-Raf melanoma cells, these Hsp90 inhibitors induce mutant B-Raf proteasomal degradation and subsequently inhibit the phosphorylation of MEK and ERK which results in the inhibition of cell proliferation, anchorage-independent growth activity, and *in vivo* tumor growth in an A2058 (B-Raf V600E) xenograft model. In the wild type B-Raf melanoma cells, Hsp90 inhibitors induce cMet, ErbB2, C-Raf and A-Raf but not wild type B-Raf proteasomal degradation and subsequently induce inhibition of cell proliferation, anchorage-independent growth, and *in vivo* tumor growth in a MeWo (B-Raf WT) xenograft model. Results demonstrate that these two Hsp90 inhibitors exhibit anti-tumor activity in both wild type and mutant B-Raf melanoma cells by targeting multiple client proteins and suggest that PF-3823863 may show therapeutic benefit in melanoma patients.

2. Materials and methods

2.1. Hsp90 inhibitors

PF-3823863 and PF-4470296 were synthesized as previously described [23,24]. All compounds were dissolved in DMSO for *in vitro* cellular assays. PF-3823863 was formulated in 40%PEG-400/60% saline (v/v) for animal studies.

2.2. Cell lines

All cell lines were purchased from the American Type Culture Collection. A2058, WM266-4, A375, MeWo, and CHL-1 were grown in DMEM with 10% FBS. SK-MEL2 was grown in MEM with 10% FBS. The gene mutation status of the melanoma cell lines used in this study is based on the information from the COSMIC database (http://www.sanger.ac.uk/perl/genetics/CGP/cosmic).

2.3. Immunoblotting

Cells and tumors were lysed in lysis buffer (50 mM Tris–HCl, 1% NP-40, 0.25% TX-100, 150 mM NaCl, 1 mM Na_3VO_4, 1 mM NaF and complete mini protease inhibitor cocktail [Roche]). Protein concentration was determined using BCA kit [Pierce Chemical] per the manufacturer's instructions. Protein (50 μg) was resolved by SDS–PAGE and transferred onto nitrocellulose membrane. All primary and secondary antibodies were purchased from Cell Signaling Technology, Inc. with the exceptions of ErbB2 (Santa Cruz) and actin (AbCam). After incubation with secondary antibodies, membranes were visualized by chemiluminescence. The intensity of protein bands was quantitatively determined using the phosphor-imager and normalized with the intensity of Actin band in each gel.

2.4. Luminex, MesoScale, and ELISA assays

Cells (10,000 cells/well) were seeded in a 96-well microtiter plate and cultured overnight. The next day, Hsp90 compounds were added to each well starting at a high concentration of 10,000 nM with a threefold serial dilution and ending at a low concentration of 0.0169 nM. Twenty-four hours post-compound addition, cells were washed with PBS twice and cell lysates were prepared and analyzed following the manufacturer's instructions. The Luminex 100 system (Upstate) was used for AKT protein measurement, ELISA was performed to measure the phosphorylation levels of ERK and MEK (Cell Signaling), and MesoScale technology was used for Hsp70 protein quantitation.

2.5. Proliferation, anchorage-independent growth and caspase 3/7 assays

For cellular proliferation, cells (3000/well) were cultured in a 96-well microtiter plate and compounds were added to each well with a threefold serial dilution as described above. Seventy-two hours post-compound addition, resazurin (250 μg/ml final, Sigma) was added to each well and incubated at 37 °C for 6 h. Plates were read by a florescence reader using emission and excitation wavelengths of 530 nm and 590 nm, respectively. To measure anchorage-independent growth in soft agar, growth media containing 0.6% agarose were plated in each well of a 96-well plate as bottom agar and allowed to solidify. Cells (10,000 cells/well) were mixed with growth media

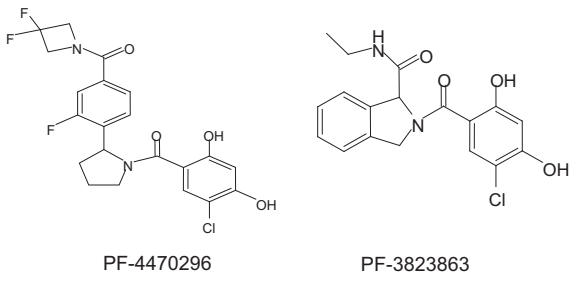

Fig. 1. Structures of PF-4470296 and PF-3823863.

containing 0.3% agarose and layered over the bottom agar in each well. A threefold serial dilution concentration of compounds was prepared with growth media and added to each well. On day 3, medium was replaced in all wells with freshly prepared compounds, and at day 6, resazurin was added to each well and a florescent reading was collected after 6 h. All experiments were run at least twice in duplicate; IC50 values were calculated by utilizing SigmaPlot. For caspase 3/7 assay, A2058 and MeWo cells were plated in a 96-well plate and treated with compounds for 48hrs, then analyzed by using the Promega Caspase-Glo® 3/7 assay as described in the manufacturer's instructions.

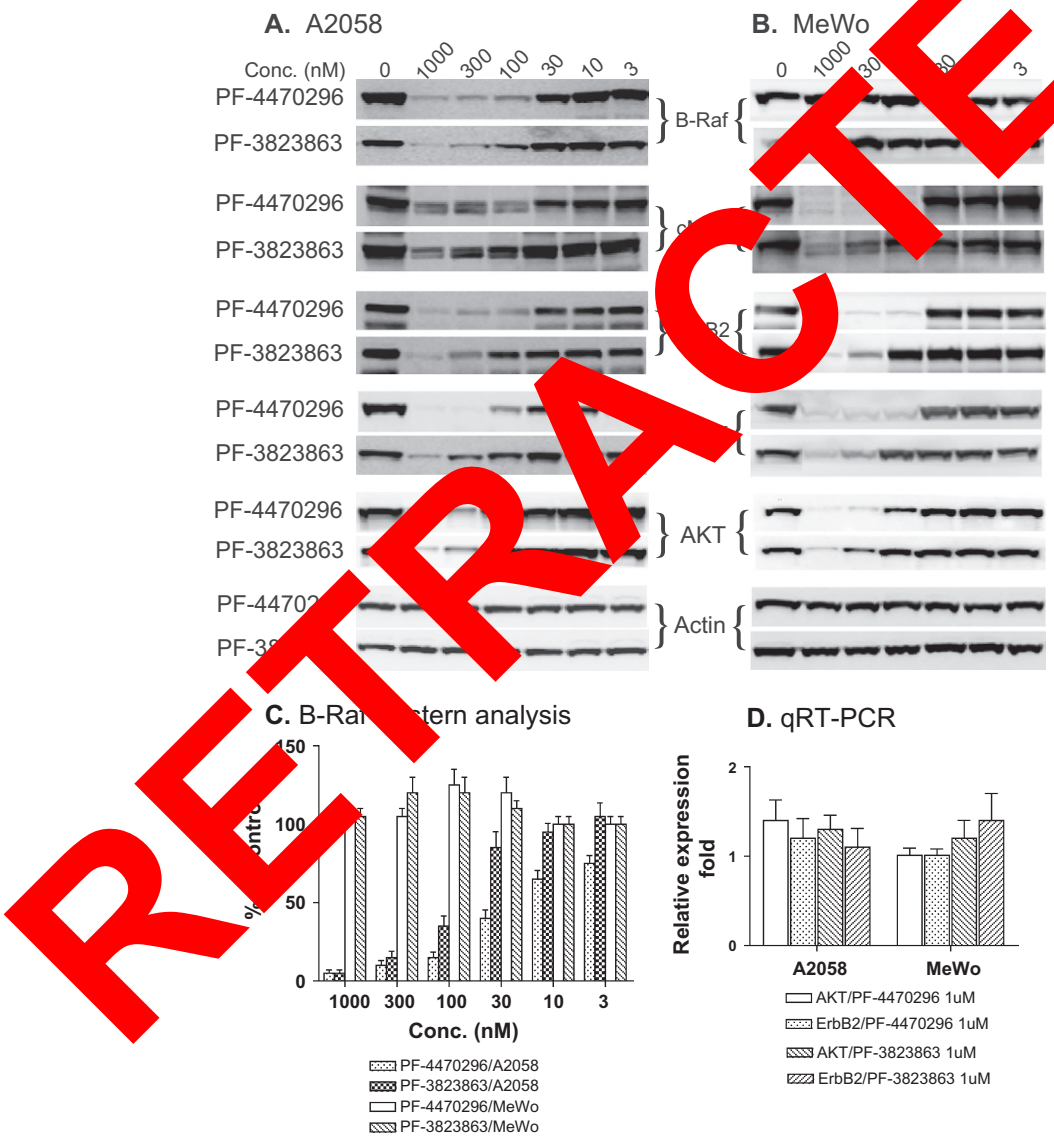

Fig. 2. PF-4470296 and PF-3823863 induce protein degradation but do not decrease mRNA expression of Hsp90 clients. Western blot analysis to determine the protein levels of Hsp90 client proteins in A2058 (A) and MeWo (B) cells. Hsp90 inhibitors, PF-4470296 and PF-3823863, were added to cells at approximately 80–90% confluence. After 24 h of treatment, cell pellets were collected and processed to be used for western analysis or quantitative PCR. Cell lysates (50 μg/lane) were subject to SDS–PAGE. Western blot analysis was performed to detect protein degradation of B-Raf, cMet, ErbB2, C-Raf, AKT, and actin. The intensity of B-Raf protein bands was determined and normalized with actin's intensity by using the phosphor-imager and plotted in (C). Quantitative real-time PCR was performed to determine the mRNA expression levels of AKT and ErbB2 as shown in (D).

2.6. Quantitative real-time PCR

Total RNA from A2058 and MeWo cells was extracted using the RNeasy Plus Mini Kit (Qiagen) following the manufacturer's instructions. cDNA was generated using the iScript Select cDNA Synthesis Kit (Bio-Rad) and analyzed by quantitative real-time PCR using SyberGreen qPCR primer assays (Qiagen) and the iCylceriQ multicolor real-time PCR detection system (Bio-Rad). Relative expression levels were normalized against GAPDH expression that was run concurrently as a reference control.

2.7. Animal studies

Six- to eight week-old nu/nu athymic female mice were obtained from Jackson Laboratory and maintained in pressurized ventilated caging at the Pfizer La Jolla animal facility. All studies were done in compliance with Institutional Animal Care and Use Committees guidelines. Tumors were established by injecting 2×10^6 cells suspended 1:1 (v/v) with reconstituted basement membrane (Matrigel, BD Biosciences). For tumor growth inhibition studies, mice with established tumors of ~150 mm^3 were selected and randomized to treatment groups approximately 10 days after implantation; mice were treated daily with PF-3823863 by intraperitoneal (ip) route using the doses of 10 mg/kg, 25 mg/kg, or 50 mg/kg. Tumor dimensions were measured with vernier calipers and tumor volumes were calcula[illegible] using the following formula: [$\pi/6 \times$ larger diame[illegible] (smaller diameter)^2]. Tumor growth inhibition percent[illegible] (TGI%) was calculated as $100 \times (1 - \Delta T/\Delta C)$.

2.8. Determination of plasma concentratio[illegible] PF-[illegible]3863

Plasma concentrations of PF-3823[illegible]ere[illegible] by liquid chromatography tande[illegible]lass s[illegible]ometry (LC–MS/MS) following protein pre[illegible]tation of pl[illegible]a samples. Chromatographic separatio[illegible] analytes w[illegible]hieved using an Agilent Zorbax, [illegible]B-C1[illegible]m, 2.1 × 50 mm column. Mass spectrom[illegible]ic detectio[illegible] the analytes was accomplished usi[illegible]e Turbo Ionspray[illegible]erface operated in the positive i[illegible]ation [illegible]de. Analyst® software (version 1.4.1) was use[illegible] da[illegible]cquisition and chromatographic peak integration. [illegible]titation [illegible]s performed by linear regressi[illegible]h a 1/2[illegible]eighi[illegible]g. Pharmacokinetic parameters [illegible]F-3[illegible]863 we[illegible]ulated using Watson TM Bioa[illegible]dical LI[illegible] software ([illegible]ersion. 7.2.0.03).

3. Resu[illegible]

3.1. PF-4470[illegible]d PF-3823863 are potent Hsp90 inhibitors capable of inducing protein degradation of mutant B-Raf and the other Hsp90 client proteins

The resorcinol amide series of compounds have shown potent activity against Hsp90 function with the biochemical Ki against Hsp90 activity and cellular activity against AKT degradation ranging from 0.002 to 2 μM and 0.02 to 20 μM, respectively, as described [23,24]. In this study, we examined the activity of representatives of the resorcinol amide series of compounds, PF-4470296 and PF-3823863 (as shown in Fig. 1), against a variety of melanoma cell lines. Both compounds induced mutant B-Raf (V600E) protein degradation in the melanoma cell line A2058 which harbors the V600E B-Raf mutation (Fig. 2A). However, neither compound induced significant degradation of wild type B-Raf protein in MeWo cells which maintain wild type B-Raf (Fig. 2B). Consistent with the biochemical Ki data, PF-4470296 exhibited better potency than PF-3823863 in the degradation of V600E B-Raf protein as determined by western blotting and band intensity analysis using the phosphor-imager quantitation method (Fig. 2C). Both compounds showed activity in the degradation of other Hsp90 client proteins including C-Raf, ErbB2, cMet, and AKT in both A2058 and MeWo cell lines (Fig. 2). Quantitative real-time PCR (qRT-PCR) was also performed to determine the mRNA levels of C-Raf, ErbB2, cMet, B-Raf, and AKT in cells treated with Hsp90 compounds, a representative qRT-PCR graph of the AKT and ErbB2 genes is shown in Fig. 2D. Results indicate that the mRNA levels of [illegible] client proteins were not changed by the treatment of PF-4[illegible]-3823863.

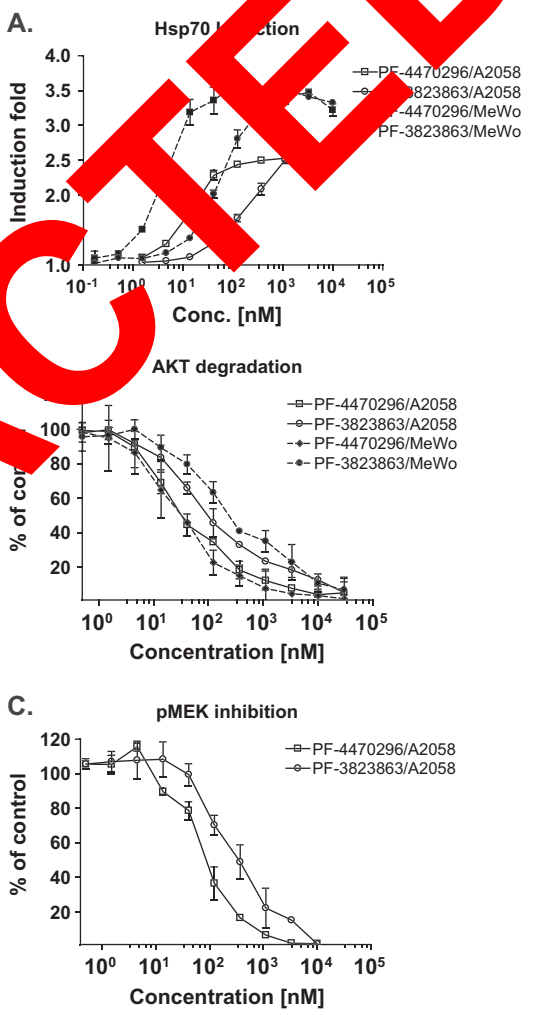

Fig. 3. PF-4470296 and PF-3823863 induce Hsp70 elevation, AKT protein degradation, and decreases in MEK phosphorylation and ERK phosphorylation in melanoma cells. EC50 curves for Hsp70 induction (A), and IC50 curves for AKT degradation (B), pMEK inhibition (C), and pERK inhibition (D) were graphed in A2058 and MeWo melanoma cell lines. Each cell line was plated in microtiter 96-well plates at 10,000 cells/well; after 24 h, cell lysates were prepared and analyzed. Endogenous phosphorylation levels of MEK and ERK in melanoma cell lines were shown in (E) and (F). A2058, WM266-4, MeWo, and CHL-1 cells were plated at 3000 cells/well or 5000 cells/well for 24 h. Cell lysates were prepared and subject to pMEK (E) or pERK (F) ELISA assay to determine the endogenous levels of pMEK and pERK in each cell line.

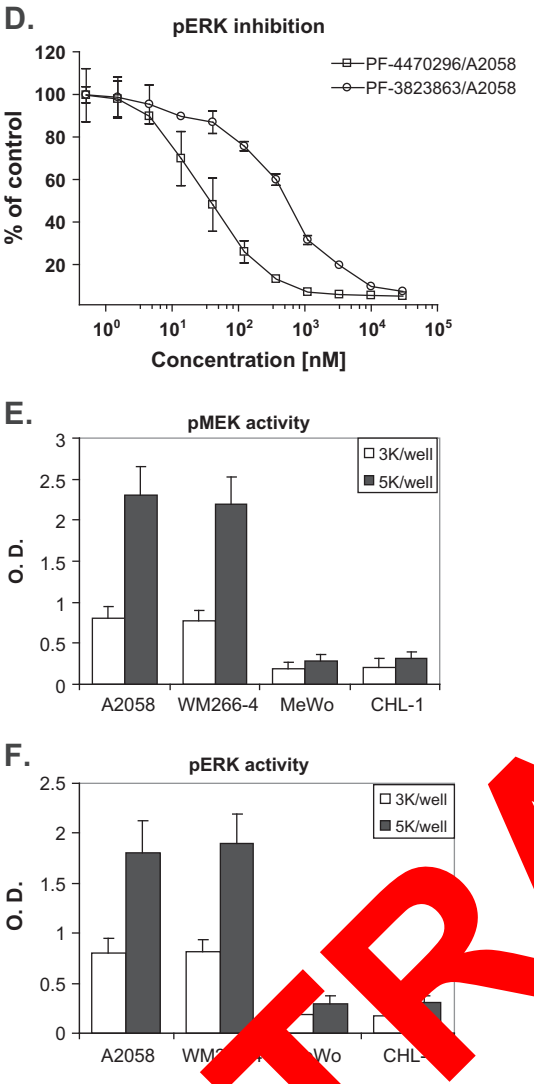

Fig. 3 (*continue*

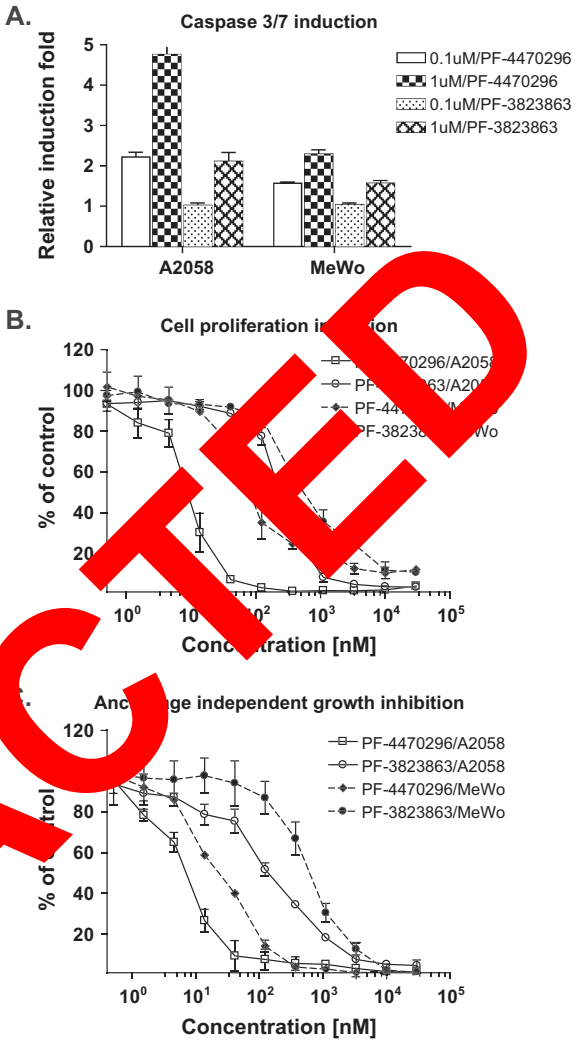

Fig. 4. PF-4470296 and PF-3823863 induce cell apoptosis and inhibit cell proliferation and anchorage-independent growth in melanoma cells. Cells were plated in 96-well plate and treated with compounds as described. Caspase 3/7 activity was measured to investigate cell apoptosis (A). Resazurin was added to measure the cell viability and colony formation ability; representative graphs from A2058 and MeWo cells are shown for cell proliferation (B) and anchorage-independent growth (C).

These results indi[illegible]hat Hsp[illegible]ient protein degradation by the treatment of PF-447[illegible]nd PF[illegible]3863 is due to the inhibition of protein conformation but i[illegible]ecrease of [illegible] transcription.

Next, [illegible]al plat[illegible]d cellul[illegible]ssays were performed to determine t[illegible]lues of [illegible]n degradation by the treatment of PF-[illegible]296 a[illegible]F-382386[illegible]ltiple melanoma cell lines. Melanoma c[illegible]nes were[illegible]osen based [illegible] the mutation status of B-Raf, including V[illegible]B-Ra[illegible](A2058, WM266-4, A375), a wild type B-Raf and N-Ras mutant cell line (SK-MEL-2), and wild type B-Raf cell lines (MeWo and CHL-1). It has previously been shown that 17-AAG induced

Table 1
Summary [illegible] values for AKT degradation and pMEK/pERK inhibition, and EC50 value for Hsp70 induction in a variety of human melanoma cell lines treated with PF-447[illegible] and PF-3823863.

Cell lines	AKT IC50 (nM)		Hsp70 EC50 (nM)		pMEK IC50 (nM)		pERK IC50 (nM)	
	PF-4470296	PF-3823863	PF-4470296	PF-3823863	PF-4470296	PF-3823863	PF-4470296	PF-3823863
A2058 (V600E)	19.8 ± 3.6	114.2 ± 21.4	12.9 ± 0.6	154.2 ± 23.6	42.3 ± 9.2	266.4 ± 22.2	21.4 ± 4.2	531.4 ± 43.2
A375 (V600E)	15.7 ± 2.3	341.3 ± 36.5	14.7 ± 2.7	127.2 ± 20.9	88.6 ± 1 9.2	838.4 ± 62.2	12.5 ± 1.2	435.4 ± 39.6
WM266-4 (V600E)	40.6 ± 2.4	648.3 ± 51.8	10.8 ± 2.2	161.6 ± 22.2	27.5 ± 0.5	381.4 ± 4.2	42.1 ± 4.6	614.4 ± 33.2
SK-MEL-2 (WT/N-Ras)	56.6 ± 5.4	437.7 ± 31.5	10.1 ± 3.5	152.8 ± 20.3	99.8 ± 20.5	282.1 ± 14.2	22.3 ± 1.6	168.4 ± 9.6
MeWo(WT/WT)	21.3 ± 1.9	209.4 ± 25.6	3.6 ± 1.1	148.6 ± 10.2	ND	ND	ND	ND
CHL-1(WT/WT)	25.6 ± 2.8	259.4 ± 14.3	3.2 ± 1.6	92.6 ± 26.3	ND	ND	ND	ND

ND: not determined.

Table 2
Summary of IC50 values of cellular proliferation and anchorage-independent growth assays in human melanoma cells treated with PF-4470296 and PF-3823863.

Cell lines	Cell proliferation IC50 (nM)		Anchorage-independent growth IC50 (nM)	
	PF-4470296	PF-3823863	PF-4470296	PF-3823863
A-2058 (V600E)	8.3 ± 0.6	228.2 ± 21.9	3.6 ± 0.6	121.6 ± 12.6
A375 (V600E)	28.3 ± 3.6	200.2 ± 15.2	23.2 ± 3.2	98.8 ± 46.9
WM266-4 (V600E)	13.6 ± 1.4	348.3 ± 21.8	7.8 ± 2.6	191.3 ± 12.9
SK-MEL-2 (WT/N-Ras)	25.9 ± 3.4	311.9 ± 28.5	29.6 ± 4.6	661.2 ± 21.5
MeWo(WT/WT)	107.3 ± 13.4	648.3 ± 51.8	10.7 ± 1.5	214.3 ± 12.2
CHL-1(WT/WT)	26.6 ± 9.8	480.4 ± 54.3	45.7 ± 11.5	

Hsp70 protein levels in treated cells due to a feedback mechanism in the heat shock protein machinery [25]. To determine if a specific Hsp90 inhibitor would have the same effect, an Hsp70 induction assay was performed using MesoScale technology. Representative results from A2058 and MeWo are shown in Fig. 3A and data for all melanoma lines are summarized in Table 1. PF-4470296 induced significant elevation of Hsp70 in all the melanoma cells tested with EC50s ranging from 3 nM to 15 nM, PF-3823863 similarly induced high levels of Hsp70 increases in all melanoma cells tested with EC50s ranging from 90 nM to 160 nM. Both compounds are potent inhibitors capable of inducing Hsp70 elevation with approximate similar potency among six melanoma cell lines tested in our study. AKT is one of the Hsp90 client proteins and controls cell proliferation and survival through multiple downstream signaling pathways [26,27]. Therefore, measuring AKT protein levels in melanoma cells treated by Hsp90 inhibitors is an indicator for not only Hsp90 inhibition but also downstream inhibitory effects. The AKT protein degradation assay was performed using Luminex technology. AKT degradation results were graphed for A2058 and MeWo and shown in Fig. 3B, data for all the melanoma cell lines are summarized in Table 1. PF-4470296 effectively induced AKT protein degradation in six melanoma cells tested with IC50s ranging from 15 nM to 60 nM while PF-3823863 induced AKT protein degradation with IC50s ranging from 115 nM to 650 nM. We did not observe differential AKT degradation in the melanoma cells based on B-Raf mutation status. MEK and ERK are downstream molecules of B-Raf or C-Raf signaling pathway. Therefore, we measured phospho-ERK and phospho-MEK levels by ELISA in melanoma cells to determine inhibition of the B-Raf/C-Raf pathway by specific Hsp90 small molecule inhibitors. The endogenous levels of phosphorylated MEK are much lower in the B-Raf wild type lines, MeWo and CHL-1 compared to those in mutant B-Raf lines, A2058 and WM266 as shown in Fig. 3E and F. The phosphorylation window between untreated and treated MeWo and CHL-1 was minimal due to the low endogenous phospho-ERK and phospho-MEK levels in MeWo and CHL1 cells such that IC50 values for pERK and pMEK in MeWo and CHL-1 cells were not determined. ELISA analysis of pMEK and pERK in A2058 are shown in Fig. 3C and D, all data are summarized in Table 1. PF-4470296 inhibited the phosphorylation level of MEK and ERK with IC50s ranging from 12 nM to 100 nM, PF-3823863 inhibited the pMEK and pERK signal in melanoma cells with IC50s ranging from 170 nM to 850 nM. Similar to the biochemical potency, PF-4470296 was more potent than PF3823863 in the cell assays. Data from western analysis and plate-based assays clearly indicate that both PF-4470296 and PF-3823863 are potent Hsp90 inhibitors capable of Hsp90 client protein degradation in mutant B-Raf cell lines (Fig. 2), and AKT as well as inhibition in pMEK and pERK (Fig. 3).

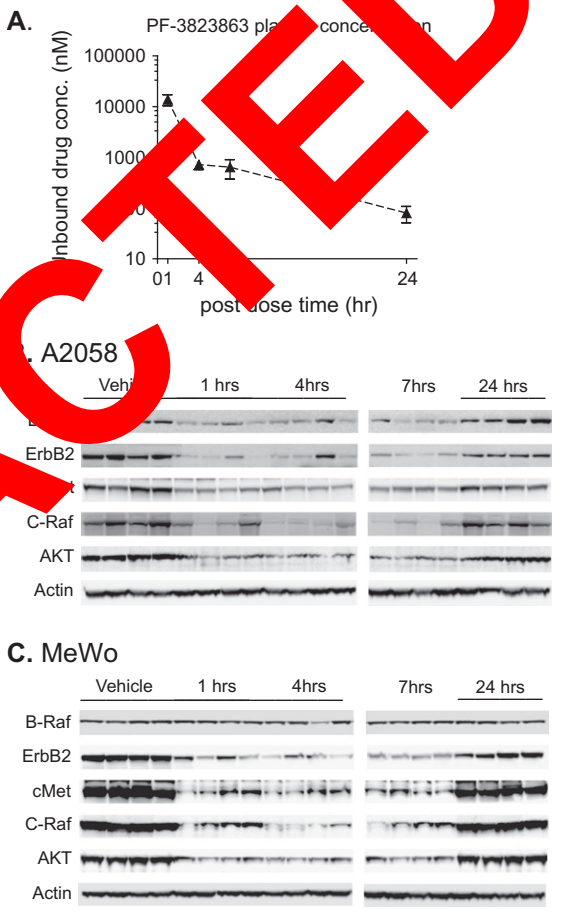

Fig. 5. PF-3823863 induces target modulation of the Hsp90 clients in the murine xenograft models. Human melanoma cells A2058 (B) and MeWo (C) were implanted in nude mice to establish xenograft tumors. PF-3823863 (50 mg/kg) was administrated to the tumor bearing animals bv intraperitoneal injection. Xenograft tumors were harvested at the indicated time points. Unbound drug concentration in plasma was measured and plotted in (A). Tumor lysates were prepared and subject to SDS–PAGE to determine Hsp90 client protein degradation in (B) and (C).

3.2. PF-4470296 and PF-3823863 induce apoptosis and inhibit cell proliferation and transformation in melanoma cells lines

To investigate whether mutant B-Raf (V600E) and other client protein degradation leads to anti-tumor activity in melanoma cells by treatment with an Hsp90 inhibitor, the cell apoptosis assay, the resazurin assay to measure cell viability, and the soft agar assay for anchorage-independent growth were performed in melanoma cell lines with or without B-Raf mutation. Results are shown in Fig. 4 and IC50 values are summarized in Table 2. Both PF-4470296 and PF-3823863 demonstrated induction of caspase 3/7 activity in A2058 and MeWo cells in a dose dependent manner. PF-4470296 inhibited cell proliferation with IC50 values ranging from 8 nM to 30 nM for all lines except for the wild type B-Raf MeWo cell line (IC50 is approximately 100 nM). PF-3823863 showed an IC50 ranging from 200 to 650 nM in the cell proliferation inhibition in the melanoma lines. There is no significant difference associated with B-Raf mutation status in the inhibition of cell proliferation among the melanoma cells. Both PF-4470296 and PF-3823863 exhibited potent activity to inhibit

colony formation of melanoma cells in soft agar assays. PF-4470296 inhibited cell transformation with IC50 values ranging from 4 nM to 50 nM while PF-3823863 inhibited cell transformation with IC50 values between 100 and 650 nM in the melanoma cell lines. Similarly, inhibition of colony formation in melanoma cells by these two Hsp90 inhibitors did not show distinct differentiation based on the mutation status of B-Raf. Results from *in vitro* anti-tumor assays clearly indicate that both PF-4470296 and PF-3823863 are potent Hsp90 inhibitors that induce Hsp90 client protein degradation and cell apoptosis and lead to the inhibition of cell proliferation and transformation in melanoma cell lines and that this inhibition is independent of the mutation status of B-Raf in melanoma cell lines.

3.3. PF-3823863 induces Hsp90 protein client protein degradation in the xenograft tumors of human melanoma models and subsequently leads to tumor growth inhibition

PF-4470296 and related pyrrolidine resorcinol amide compounds are highly metabolized to glucuronides [45]. PF-3823863 was designed to improve metabolic stability to evaluate anti-tumor activity in mouse xenograft models [24]. An initial study was run to determine the pharmacokinetic and pharmacodynamic profile of PF-3823863. When administered to tumor-bearing mice by intraperitoneal (ip) injection (50 mg/kg), PF-3823863 maintained a high plasma exposure until seven hours post-dose with a free drug concentration of 638 nM (mean value from 4 animals/group). The pERK IC50 of PF-3823863 in A2058 cells was determined to be 531 nM, while the IC50 for AKT degradation of PF-3823863 was 114 nM in A2058 and 209 nM in MeWo; therefore, the free drug concentration of PF-3823863 in plasma can be maintained at a concentration above the cellular IC50s of pERK and AKT for more than 7 h in both the A2058 and MeWo models (Fig. 5A). At the end of the study, detection of target modulation in PF-3823863 treated xenograft tumors was performed in both A2058 and MeWo model[illegible] western blotting. Cells were implanted in female nude mice su[illegible] on the right flank. After tumors reached a volume of 30[illegible]0 mm^3, P[illegible]3863 was administered at 50 mg/kg and tumors [illegible] dissected at [illegible]us time points post-last dosing as indicated in Fi[illegible] and C. Deg[illegible]tion of Hsp90 client proteins including B-R[illegible]AKT, c[illegible]-Raf, and [illegible]2 were analyzed in tumor lysates as pha[illegible]odynamic e[illegible]nts t[illegible]nfirm target modulation by PF-3823[illegible] PF-3823863 in[illegible]utant B-Raf (V600E) protein degradati[illegible]the A20[illegible]odel, but d[illegible]not induce wild type B-Raf protein degradat[illegible]th[illegible]Wo xenog[illegible]aft model, confirming the results from cell[illegible]ed as[illegible]-3823863[illegible]uced expected client protein degradati[illegible]c-Met, Er[illegible]-Raf,[illegible]AKT in both A2058 and MeWo xenogra[illegible]ors for up to [illegible]nt protein levels returned to baseline l[illegible]e[illegible]ime point wh[illegible]e plasma drug exposure was lower (Fig. [illegible] and C)[illegible] we investigated whether target modulation by PF-3823863 treatmen[illegible] sufficient to induce tumor growth inhibition[illegible]3 and MeWo x[illegible]aft models. A2058 and MeWo cells were s[illegible]taneously implanted in[illegible]ude mice. After tumor volume reached [illegible]–150 mm^3, tumor bearing animals were randomized and treated with [illegible]3823863 at v[illegible]us doses IP using a BID regimen for three weeks. As [illegible]vn in Fig. 6, [illegible]823863 was a potent inhibitor that induced tumor g[illegible] inhibitio[illegible]GI) in both melanoma xenograft models, with a maximal[illegible]d 85% at the high dose (50 mg/kg) in the A2058 and MeWo [illegible], respectively. The inhibition of tumor growth in both the [illegible]2058 and MeWo models was dose dependent. At the 10 mg/kg and [illegible]g doses, the anti-tumor activity of PF-3823863 was greater in the [illegible]058 than the MeWo model; however, at the maximal tolerated doses of 50 mg/kg, PF-3823863 induced similar tumor growth inhibition in both xenograft models. PF-3823863 was well tolerated by animals in both studies such that maximal body weight loss was less than 5% and no adverse events were observed during the study period. Therefore, PF-3823863 is a potent Hsp90 inhibitor capable of inhibiting Hsp90 client protein stability and subsequently resulting in tumor growth inhibition in melanoma xenograft models.

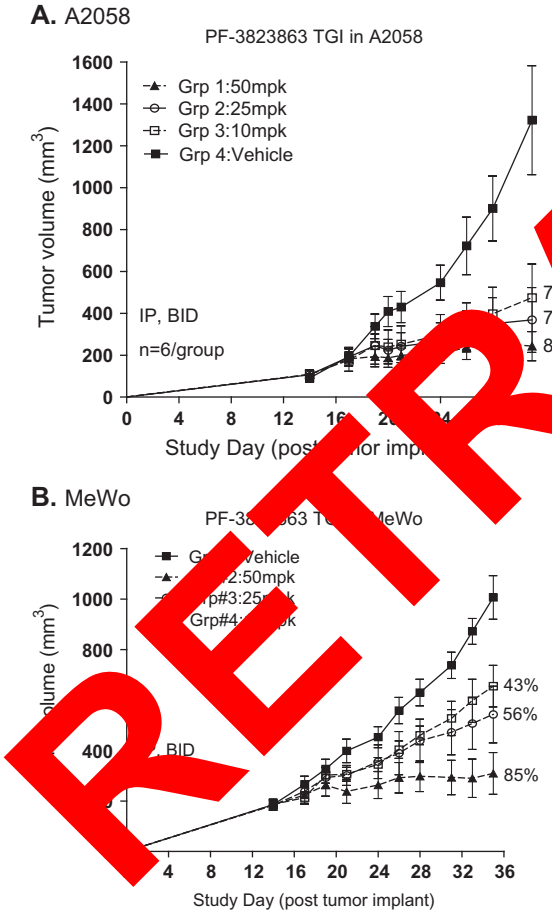

Fig. 6. Hsp90 inhibitor PF-3823863 demonstrates anti-tumor activity in A2058 and MeWo xenograft models. Human melanoma cells A2058 and MeWo were implanted into nude mice; when the tumor sizes reached ~150 mm^3, the tumor-bearing mice were randomized to various groups and treated with vehicle or various doses of PF-3823863 as indicated for 17 days in A2058 model (A) or 20 days in MeWo model (B). Tumors were measured three times a week, and tumor sizes were recorded and plotted against the study days. Tumor growth inhibition by the treatment of PF-3823863 was calculated and labeled accordingly in A and B.

4. Discussion

The activation of N-Ras or B-Raf plays a key role in the development of human melanomas yet N-Ras and B-Raf mutations are mutually exclusive in melanoma [19,21]. V600E B-Raf mutant protein is sensitive to 17-AAG induced protein degradation while wild type B-Raf is resistant to 17-AAG [7,8]. 17-AAG has previously been shown to block xenograft tumor growth in a B-Raf (V600E) SK-MEL28 model [7]; however, 17-AAG had no effect on or only moderately delayed tumor growth in a panel of human melanoma xenografts [28]. Therefore, we were interested in testing Pfizer novel Hsp90 inhibitors in melanoma models to examine whether there is differential sensitivity against these Hsp90 inhibitors in regards to the stability of mutant B-Raf protein over wild type B-Raf protein. Moreover, it was important to determine whether the differential sensitivity of mutant versus wild type B-Raf protein leads to differential *in vitro* and *in vivo* anti-tumor activity in melanoma. Our results show that two resorcinol amide compounds PF-4470296 and PF-3823863 induce multiple Hsp90 client protein degradation in melanoma cell lines.

These client proteins include cMet, ErbB2, AKT, C-Raf and mutant B-Raf but exclude wild type B-Raf. Similar to the activity of 17-AAG, both PF-4470296 and PF-3823863 demonstrate great potency in the degradation of mutant B-Raf protein but had no activity against wild type B-Raf protein in the human melanoma cells tested in this study. Both PF-4470296 and PF-3823863 inhibit *in vitro* cell proliferation and transformation activity in melanoma cells regardless of the mutation status of B-Raf. Similarly, PF-3823863 inhibits tumor growth in the A2058 (mutant B-Raf) as well as MeWo (wild type B-Raf) xenograft models. Therefore, the specific Hsp90 inhibitors, PF-4470296 and PF-3823863, demonstrate differential activity in the degradation of mutant B-Raf protein versus wild type B-Raf protein. However, differential B-Raf degradation activity does not correlate differential anti-tumor activity in *in vitro* cellular assays or *in vivo* animal studies. In addition to the degradation of mutant B-Raf by PF-4470296 and PF-3823863, these two compounds also induce protein degradation of other Hsp90 client proteins including ErbB2, C-Raf, AKT, and cMet in both A2058 and MeWo cells. These client proteins are known to drive tumor progression through their various corresponding signaling pathways. Therefore, reduced protein stability of other client proteins likely contributes to enhance the anti-tumor activity of Hsp90 inhibitors.

Melanoma is an aggressive form of skin cancer that arises from melanocytes. Mutated N-Ras or B-Raf play a key role in the development of human melanoma [19,21]. B-Raf is mutated in nearly 70% of human melanomas; a substitution of glutamic acid for valine at position 600 (V600E) is the most common B-Raf mutation in melanoma [19]. B-Raf (V600E) stimulates constitutive cell signaling, growth factor independent cell proliferation and transformation of immortalized melanocytes, allowing these cells to grow as tumors in nude mice [31]. Treatment by B-Raf inhibitors or siRNA knockdown by siRNAs blocks melanoma cell proliferation, induces apoptosis *in vitro*, and slows the growth of melanoma xenografts *in vivo* [32]. These data demonstrate that mutant B-Raf (V600E) is necessary for the maintenance and progression of melanoma in humans. However, Ras/Raf/MEK/ERK activation is a very complex signaling axis and feedback regulation is a critical component of the cellular signal transduction network. Additionally, such feedback regulation may be involved in determining the specificity of cellular responses to upstream receptor activation. Within the MAPK signaling network, two positive and negative regulatory feedback loops have been described that affect differential cellular responses [33–35]. It is possible that selective kinase inhibition of B-Raf or MEK may not effectively block tumor progression because the induction of the MAPK pathway through the activation of feedback regulation loops in a tumor-type specific manner [36–38]. In contrast, the inhibition of Hsp90 function would induce protein degradation in mutant B-Raf and C-Raf such that the feedback regulation of MAPK would not have an impact on the phosphorylation of B-Raf or C-Raf [38–40]. Furthermore, Hsp90 inhibitors also induce RTK degradation in melanoma cells to enhance the blockade of tumor progression; therefore, a Hsp90 inhibitor may provide better efficacy in the inhibition of melanoma progression than the specific B-Raf or MEK kinase inhibitors. However, the phase II clinical trial of 17-AAG did not achieve objective responses in melanoma patients, and western blot data did not show significant degradation in mutant B-Raf and other client proteins [22]. This is highly likely due to the toxicity of geldanamycin derivatives preventing sufficient drug exposure in patients to achieve efficacy. PF-3823863 demonstrates a superior preclinical anti-tumor activity in melanoma xenograft models compared to previous melanoma studies [28]. PF-3823863 also exhibits superior drug properties, bioavailability, and plasma exposure compared to geldanamycin derivatives, as previously described [24]. Therefore, our data suggest that PF-3823863 is likely to show clinical benefit in melanoma patients through inhibition of multiple signal transduction pathways.

In the mutant B-Raf xenograft model (A2058), degradation of mutant B-Raf may be a major contributor to anti-tumor activity since the B-Raf activation plays a major role in melanoma proliferation as previously described [19,21]. The degradation of other client proteins by Hsp90 inhibitors is likely to enhance anti-tumor activity in melanoma harboring mutant B-Raf while tumorigenesis is driven by the B-Raf mutation. PF-3823863 indeed had better efficacy at lower doses in A2058 compared to that in MeWo xenografts. Our data also indicate that protein degradation of Hsp90 clients including cMet, ErbB2, C-Raf and AKT results in anti-activity in melanoma harboring wild type B-Raf, likely through the inhibition of multiple signal pathways. Cancer cells often develop resistance against single targeted therapy by creating secondary mutations in key kinases or activating new pathways to compensate for defects in proliferation and transformation by the primary agents. Examples include gastrointestinal stromal tumor (GIST) patients with acquired resistance to imatinib by gain of secondary mutations in the *c-kit* gene [41], and the evasion of trastuzumab therapy by over-expression and activation of HER3, Notch-1, or cMet in breast cancer patients [42–44]. Hsp90 inhibitors may prevent cancer cells from developing resistance by targeting multiple signal transduction pathways. In melanoma cells carrying the B-Raf mutation, activation through B-Raf and subsequent downstream signaling is the major driving force for tumor progression. Hsp90 inhibitors can inhibit tumor progression in these cells by limiting signaling mediated by the B-Raf pathway. In addition, deregulation of other signaling pathways may enhance anti-tumor activity by also preventing the development of resistance to inhibition in the B-Raf pathway. In melanoma cells harboring wild type B-Raf, Hsp90 inhibitors induce protein degradation of RTKs (cMet, ErbB2, and others), C-Raf, and AKT to inhibit cell proliferation and transformation by targeting several key molecules in multiple pathways. This may provide optimal effects in anti-tumor activity and prevent further development of resistance. Our data also suggest that a single Hsp90 inhibitor is sufficient to shut down multiple pathways in tumors, similar to combination therapy of anti-tumor agents. Therefore, using an Hsp90 inhibitor in the clinic may lead to better benefits over single target therapy.

In conclusion, we have developed a novel series of Hsp90 specific compounds and have evaluated PF-4470296 and

PF-3823863 as two representatives in this new resorcinol amide series. We used these two compounds to examine both *in vitro* and *in vivo* anti-tumor activity in melanoma cells. Our results indicate that both compounds are potent Hsp90 compounds capable of inhibiting Hsp90 client protein activity in melanoma cells, and that PF-3823863 can induce tumor growth inhibition in human melanoma xenograft models. These two Hsp90 inhibitors demonstrate strong activity towards melanoma cells, and the inhibitory effect of these two compounds is independent of the mutation status of B-Raf. These data suggest that Hsp90 inhibitors impede melanoma cell growth and transformation through B-Raf and other signal transduction pathways, and that Hsp90 inhibitors can induce collaborative inhibition against multiple pathways. Also, these data support the clinical development of Hsp90 inhibitors for melanoma patients including both B-Raf mutant and wild type populations.

Conflicts of interest

None declared.

Disclosure

All authors are Pfizer employees.

Acknowledgments

We thank Samantha Lee for her assistance in cell ture and western blot analysis and Sangit M. Baxi her help in manuscript preparation.

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