KU675, a Concomitant Heat-Shock Protein Inhibitor of Hsp90 and Hsc70 that Manifests Isoform Selectivity for Hsp90 α in Prostate Cancer Cells^[S]

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ABSTRACT

The 90-kDa heat-shock protein (Hsp90) assists in the proper folding of numerous mutated or overexpressed signal transduction proteins that are involved in cancer. Inhibiting Hsp90 consequently is an attractive strategy for cancer therapy as the concomitant degradation of multiple oncoproteins may lead to effective antineoplastic agents. Here we report a novel C-terminal Hsp90 inhibitor, designated KU675, that exhibits potent antiproliferative and cytotoxic activity along with client protein degradation without induction of the heat-shock response in both androgen-dependent and -independent prostate cancer cell lines. In addition, KU675 demonstrates direct inhibition of Hsp90 complexes as measured by the inhibition of luciferase refolding in prostate cancer cells. In direct binding studies, the internal

fluorescence signal of KU675 was used to determine the binding affinity of KU675 to recombinant Hsp90 α , Hsp90 β , and Hsc70 proteins. The binding affinity (K_d) for Hsp90 α was determined to be 191 μ M, whereas the K_d for Hsp90 β was 726 μ M, demonstrating a preference for Hsp90 α . Western blot experiments with four different prostate cancer cell lines treated with KU675 supported this selectivity by inducing the degradation of Hsp90 α -dependent client proteins. KU675 also displayed binding to Hsc70 with a K_d value at 76.3 μ M, which was supported in cellular by lower levels of Hsc70-specific client proteins on Western blot analyses. Overall, these findings suggest that KU675 is an Hsp90 C-terminal inhibitor, as well as a dual inhibitor of Hsc70, and may have potential use for the treatment of cancer.

Introduction

Prostate cancer is the sixth leading cause of cancer-related death in men globally. In the United States, it is responsible for approximately 30,000 deaths annually and ranks as the second leading cancer killer among men (Siegel et al., 2011). Prostate cancer tends to be detected in men over the age of 50, and the treatments of patients with metastatic, locally recurrent, and androgen-independent prostate cancer are particularly problematic and often fatal. Huggins and Hodges reported the susceptibility of prostate tumors to undergo androgen withdrawal in 1952 (Huggins, 1952; Huggins and Hodges, 2002). Since then, patients with advanced prostate cancer have undergone treatment with therapies directed at inhibition of the androgen receptor (AR) (Lassi and Dawson, 2010); however, androgen withdrawal is not sufficient in the treatment of metastatic disease because androgen-deprivation therapies

usually fail for most patients. Although recent trials with docetaxel have demonstrated a reasonable survival advantage, the long-term effectiveness of such chemotherapy remains limited (Petrylak et al., 2004; Tannock et al., 2004; Chi et al., 2010; Kelly et al., 2012). Hence, there remains a critical need for the development of novel therapies to treat advanced prostate cancer.

Heat-shock protein 90 (Hsp90) represents one of the most promising biologic targets identified for the treatment of cancer. Hsp90 is commonly overexpressed in many cancer cells, including prostate cancer (Hanahan and Weinberg, 2000; Isaacs et al., 2003; Holzbeierlein et al., 2010). As a molecular chaperone, Hsp90 is responsible for the maturation of proteins directly associated with malignant progression; therefore, inhibition of this protein folding function results in a combinatorial attack on numerous pathways Schulte and Neckers, 1998; Sato et al., 2000; Basso et al., 2002; Xu et al., 2001). Currently, many clinical trials for prostate cancer involve the targeting of proteins within the AR pathway, including kinases, growth factor receptors, or antiapoptotic proteins. Notably, 75% of these drug targets are Hsp90-dependent client proteins (Blagosklonny et al., 1995; Schulte et al., 1995; An

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ABBREVIATIONS: 17-AAG, 17-allylamino-17-demethoxygeldanamycin; AR, androgen receptor; BN, Blue-native; DMSO, dimethylsulfoxide; HSP, heat-shock protein; HSR, heat-shock response; KU174, *N*-(7-((2S,3R,4S,5R)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yloxy)-8-methoxy-2-oxo-2H-chromen-3-yl)-3',6-dimethoxybiphenyl-3-carboxamide; MEM, minimum Eagle's medium; NB, novobiocin; shRNA, small hairpin RNA.

et al., 2000). Consequently, Hsp90 inhibition can target most of these pathways and provide a powerful new approach toward the treatment of prostate cancer.

Hsp90 exists as a dimer, and the binding of ATP to the N-terminal pocket of each monomer leads to formation of a “closed” formation that binds client proteins, facilitates folding, discourages protein aggregation, and mediates proteasomal degradation. A number of Hsp90 inhibitors that target the N-terminal ATP-binding pocket have demonstrated potent antiproliferative effects (Roe et al., 1999; Whitesell and Lindquist, 2005; Avila et al., 2006); however, a major drawback associated with their use is that they induce a prosurvival heat-shock response (HSR), which results in increased levels of Hsp27, Hsp40, Hsp70, and Hsp90 at the same concentration that leads to client protein degradation (Chiosis et al., 2003; Powers and Workman, 2007). Consequently, scheduling and dosing of these drugs are difficult and have limited their potential use. Compared with N-terminal inhibitors, the antibiotic novobiocin (NB) binds the C terminus of Hsp90 and, through Hsp90 C-terminal inhibitions, induces client protein degradation without induction of the prosurvival HSR (Burlison et al., 2008; Shelton et al., 2009; Matthews et al., 2010; Eskew et al., 2011). We have previously reported the synthesis and evaluation of NB analogs that exhibit improved potency over NB (Shelton et al., 2009; Matthews et al., 2010; Eskew et al., 2011; Kusuma et al., 2014). In this article, we report a new C-terminal inhibitor, KU675, that exhibits potent antiproliferative effects in prostate cancer cells and a preference for the inhibition of Hsp90 α .

In mammalian cells, there are two Hsp90 isomers in the cytosol (Hsp90 α and Hsp90 β in humans). Additional Hsp90 homologs include Grp94, which is found in the endoplasmic reticulum, and Hsp75/TRAP1, found in the mitochondrial matrix (Chen et al., 2006). There are two or more genes encoding cytosolic Hsp90 homologs, with the human Hsp90 α showing 85% identity to Hsp90 β (Chen et al., 2005). Certain areas within the amino acid sequence differ between Hsp90 α and Hsp90 β , raising the potential to exhibit isoform-specific functions, such as differential binding to client proteins (Sreedhar et al., 2004). These differences in the function and expression of Hsp90 isoforms provide the potential to develop isoform specific inhibitors of Hsp90 for antitumor therapies, which may have clinical importance (Csermely et al., 1998). Selective inhibition of individual Hsp90 isoforms may decrease toxicity or enhance potency, depending on the pharmacology of the cancer. Unfortunately, most Hsp90 inhibitors undergoing clinical evaluation are pan-inhibitors and bind all isoforms with similar affinity (Martin et al., 2008; Stuhmer et al., 2009; Ohba et al., 2010; Suda et al., 2014).

The aim of this study was to characterize the effects of the C-terminal Hsp90 inhibitor, KU675, a second-generation analog of NB, in different prostate cancer cell lines. The results indicate that KU675 binds directly to Hsp90 and exhibits a robust antiproliferative and cytotoxic activity, along with client protein degradation and disruption of Hsp90 native complexes without induction of the HSR. KU675 also manifested some isoform selectivity for Hsp90 α , with a binding affinity 3 times greater than Hsp90 β . In addition, KU675 also binds to Hsc70 and reduces the expression of Hsc70-specific client proteins. These findings reveal a novel direction for the design and synthesis of future C-terminal Hsp90 inhibitors.

Materials and Methods

KU675 was synthesized as previously described (Burlison et al., 2008) (Fig. 1A). KU675, KU174 [N-(7-((2S,3R,4S,5R)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl)-3',6-dimethoxybiphenyl-3-carboxamide), NB, and 17-allylamino-17-demethoxygeldanamycin (17-AAG) were dissolved in dimethylsulfoxide (DMSO) and stored at -80°C until use. Hsc70 recombinant protein was purchased from Stress Marq Biosciences Inc. (Victoria, BC, Canada). The antibodies used for Western blot analysis include rabbit anti-Her2/ErB2, rabbit anti-Akt, mouse anti-survivin, rabbit anti-cdc25C, mouse anti-Hsp27, rabbit anti-Hsf-1, rabbit anti- β -actin, rabbit anti-cyclin D1 (Cell Signaling Technologies, Danvers, MA); rabbit anti-B-Raf (Upstate Cell Signaling Solutions, Lake Placid, NY); rabbit anti-Hsc70, rat anti-Hsp90 α (Assay Designs, Ann Arbor, MI), goat anti-Hsp90 β , mouse anti-AR, mouse anti-Sp1, mouse anti-c-Src (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); rabbit anti-CXCR4, rabbit anti-pAkt (S473), rabbit anti-pAkt (T308), and rabbit anti-ERK5 (Abcam Inc., Cambridge, MA); mouse anti-Nestin and mouse anti-Myb (Millipore Corporation, Temecula, CA).

Hsp90 α and Hsp90 β Recombinant Proteins. Overexpression and purification of Hsp90 α and Hsp90 β were carried out in the vector pTBSG1 (Qin et al., 2008) by the Center of Biomedical Research Excellence in Protein Structure and Function, University of Kansas (Lawrence, KS). Hsp90 α and β recombinant proteins were further purified by AKTA Xpress purification system (GE Healthcare, Little Chalfont, UK), aliquoted, and stored at -80°C before use.

Cell Culture. PC3MM2 (androgen independent) and LNCaP-LN3 (androgen dependent) prostate cancer cell lines (Pettaway et al., 1996) were obtained from M.D. Anderson Cancer Center (Houston, TX) and cultured in minimum Eagle's medium (MEM; Sigma-Aldrich, St. Louis, MO) with 10% fetal bovine serum, penicillin/streptomycin (100 IU/ml penicillin, 100 mg/ml streptomycin), MEM vitamins, and MEM nonessential amino acid. LAPC-4 (androgen-dependent) and C4-2 (androgen-dependent) prostate cancer cell lines were provided by Dr. Benyi Li (Department of Urology, University of Kansas Cancer Center). LAPC-4 and C4-2 cells were cultured in Iscove's modified Dulbecco's medium (Sigma-Aldrich), fetal bovine serum, and RPMI 1640 Medium (Invitrogen, Carlsbad, CA), respectively, supplemented with 10% and penicillin/streptomycin (100 IU/ml penicillin, 100 mg/ml streptomycin). All cells were maintained at 37°C with 5% CO_2 . The stably transduced Hsp90 α and Hsp90 β knockdown PC3MM2 cells were cultured as described but with the addition of 2.5 $\mu\text{g}/\text{ml}$ of puromycin. The small hairpin RNA (shRNA) expression of Hsp90 α was induced with the addition of 12 or 24 $\mu\text{g}/\text{ml}$ of doxycycline. Induction of Hsp90 α shRNA expression with tetracycline was monitored by the TurboRFP fluorescence. The Hsp90 β shRNA was constitutively expressed and monitored by TurboGFP fluorescent cells. Freeze-down stocks of the original characterized cell line were stored under liquid nitrogen. All experiments were performed using cells with a passage number less than 20 and less than 3 months in continuous culture.

Antiproliferative Assay. Cellular viability was assessed using the Cell Titer-Glo luminescent cell viability assay (Promega, Madison, WI) according to the manufacturer's instructions. This approach is a homogeneous method to determine the number of viable cells in culture based on quantitation of the ATP present, which signals the presence of metabolically active cells. Briefly, 5×10^3 cells/well were cultured in 96-well white plates in medium for 24 hours and then incubated with KU675 for 24 and 48 hours. Luminescent signals were measured on the BioTek Synergy 4 plate reader (BioTek Instruments, Winooski, VT). Data were analyzed from three independent experiments performed in triplicate, and nonlinear regression and sigmoidal dose-response curves (GraphPad Prism 5.0, La Jolla, CA) were used to calculate IC_{50} and R^2 values.

Trypan Blue Cytotoxicity Experiments. Cell viability was conducted as previously described (Matthews et al., 2010). Prostate cancer cells were treated with KU675 for the indicated time points,

Fig. 1. KU675, a novel analog of NB, causes antiproliferative effects in prostate cancer cells. (A) The structures of NB and KU675. PC3MM2 (B), LNCap-LN3 (C), LAPC-4 (D), and C4-2 (E) cells were cultured in a 96-well white plate with corresponding medium and treated with KU675 for 24 hours (●) and 48 hours (■). Cell Titer-Glo luminescent reagent was used to measure cellular proliferation. Data were analyzed from three independent experiments performed in triplicate; each data point represents the mean $\pm$ S.E.M.

and at the end of the incubation time for each cell treatment group, nonadherent cells were collected and combined with cells detached by trypsinization using TrypLE Express (Invitrogen) followed by centrifugation at 200g at 4°C. Cell pellet was then resuspended and washed twice with cold Dulbecco's phosphate-buffered saline (Invitrogen). Total cell counts and viability were conducted on an automated system Vi-Cell cell viability analyzer (Beckman Coulter Inc., Brea, CA). Data were statistically analyzed using a two-tailed *t* test (GraphPad Prism 5.0). All data displayed represent the mean $\pm$ S.E.M. from three independent experiments (*n* = 3); asterisks (*, **, and ***) indicate significant *P* value < 0.05, < 0.01, and < 0.001 respectively, compared with vehicle-treated (i.e., DMSO) control.

Western Blot Analysis. PC3MM2, LNCaP-LN3, LAPC-4, and C4-2 cells were seeded at a density of 1.0×10^6 in T75 flasks. After 24 hours, the *t* = 0 flask was harvested and cell number counted by Vi-Cell as described. Remaining flasks were dosed with drugs by serial dilution from DMSO stocks. Total cells after 24 hours of KU675 treatment were pelleted and suspended into phosphate-buffered saline. Suspended cells were aliquoted for Vi-Cell viability measurements, total protein SDS-PAGE analysis and Blue-native (BN) electrophoresis. SDS-PAGE lysates were prepared in RIPA buffer: 50 mM Tris-HCl, pH 7.5; 150 mM NaCl containing 0.1% SDS; 1% Igepal (Sigma-Aldrich); 1% sodium deoxycholate; protease; and phosphatase inhibitor cocktail (Sigma-Aldrich) and lysed by three

freeze-thaw cycles using liquid nitrogen and a 37°C water bath. Protein concentration was determined using a DC Protein Assay (Bio-Rad Laboratories, Hercules, CA). Equal amounts of protein (20 µg) were loaded on a Novex E-PAGE 8% protein gel (Life Technologies, Carlsbad, CA), transferred to a nitrocellulose membrane by Novex iBlot gel transfer system (Invitrogen), blocked in Tris-buffered saline-T containing 5% milk, and probed with primary antibodies (1:1000 dilution). Membranes were incubated with IRDye fluorescent secondary antibody (1:10,000 dilution) and visualized with the Odyssey Infrared Imager system (LI-COR, Lincoln, NE). All Western blots were probed for the loading control β -actin. The data were representative of at least three independent experiments ($n = 3$).

Blue Native Gel Electrophoresis. After 24 hours of KU675 treatment, BN lysates were prepared from PC3MM2 cells in 20 mM Bis-Tris (pH7.4), 125 mM caproic acid, 20 mM KCl, 2mM EDTA, 5 mM $MgCl_2$, 10% glycerol, and 2% *n*-dodecyl β -D-maltoside followed by three freezing and thawing cycles and centrifugation at 14,000g for 30 minutes at 4°C. Protein concentration was determined as described already, and equal amounts of protein were loaded on a native PAGE Novex 3–12% Bis-Tris gel (Invitrogen) and electrophoresed according to manufacturer's instructions. Membranes were incubated with a horseradish peroxidase-conjugated secondary antibody (1:40,000 dilution), developed using the UVP AutoChemi system (UVP, LLC, Upland, CA). A loading-control β -actin was included in each experiment.

Cancer Cell-Based Hsp90-Dependent Luciferase Refolding Assay. Use of a reporter enzyme, such as luciferase, for the study of heat shock and related stress has been well established in our laboratory (Sadikot et al., 2013). Luciferase can be reversibly denatured, and its recovery is an active process mediated by the heat-shock proteins (HSPs). The luciferase refolding assay was performed in PC3MM2 and LNCap-LN3 cells that had been previously stably transfected with lentivirus carrying Luc2/m cherry genes. Cell pellets were collected from 80–90% confluent flasks and resuspended in prewarmed media (50°C) for approximately 5 minutes. Previous experiments have demonstrated this to be sufficient to denature the endogenous luciferase to less than 2% of the basal activity but insufficient to decrease the viability of the cells. Cells were then plated at a density of 50,000 cells/well in a 96-well white plate in the presence of inhibitors. After 1 hour, the extent of refolded luciferase was measured by the addition of an equal volume of luciferase substrate solution and read on a Synergy 4 multi-detection microplate reader (BioTek Instruments Inc.) set for 0.1 second/well integration. Direct inhibition of luciferase was analyzed for each compound as previously described (Sadikot et al., 2013). EC_{50} values were calculated from raw data plotted or normalized to a percentage control using a nonlinear regression and sigmoidal dose response curves (GraphPad Prism 5.0).

Intrinsic Fluorescence Spectroscopy. Intrinsic fluorescence measurements were performed with a SpectraMax M5 spectrophotometer (Molecular Devices Corporation, Sunnyvale, CA). KU675 was diluted to 20 µM in assay buffer containing 20 mM Hepes, pH 7.4, and 50 mM NaCl. Recombinant Hsp90 α , Hsp90 β , and Hsc70 proteins were added to the KU675 solution, adjusted to designed concentration (0–100 µg/ml for Hsp90 α , 0–140 µg/ml for Hsp90 β , and 0–30 µg/ml for Hsc70 recombinant proteins), and the mixture was allowed to incubate for 30 minutes before measurement. All measurements were made at 25°C and done in triplicate. The excitation wavelength was 345 nm, and the emission was monitored from 350 to 600 nm. The concentration-dependent binding curves were analyzed using nonlinear fitting by GraphPad Prism 5 software, and the affinity of binding (K_d) was determined accordingly.

Results

Antiproliferative Effects of KU675 in Prostate Cancer Cells. The antiproliferative effects of KU675 were examined over a 48-hour time course against PC3MM2, LNCap-LN3, LAPC-4, and C4-2 prostate cancer cells (Fig. 1,

B–E). Prostate cancer cells were incubated with an increasing concentration of KU675 for 24 and 48 hours, after which cell viability was determined by Cell Titer-Glo luminescent cell viability assay. As illustrated in Fig. 1, KU675 inhibited cell proliferation against PC3MM2 cells (androgen-independent) in both a concentration- and time-dependent manner. For PC3MM2 cells, the IC_{50} values for KU675 were determined to be 7.5 and 4.4 µM for 24- and 48-hour time points, respectively. Consistent with previous data, KU675 required longer treatment to achieve superior efficacy against the androgen-independent PC3MM2 cell line, as one might expect; however, for LNCap-LN3, LAPC-4, and C4-2 cells, the IC_{50} values for KU675 were determined to be 2.3, 1.7, and 1.6 µM for 24-hour treatment and 2.0, 1.6, and 1.1 µM for 48-hour treatment, respectively. Thus, the maximal potency of KU675 was essentially achieved at 24 hours for those three androgen-dependent prostate cancer cells. In particular, compared with previous studies (Matthews et al., 2010), at our 24-hour time point, KU675 was 290-fold and 77-fold more potent (IC_{50}) compared with the parent compound, NB, against the LNCap-LN3 and PC3MM2 cell lines, respectively. These findings indicate that, compared with NB, KU675 possesses potent antiproliferative activity against both androgen-dependent and -independent prostate cancer cell lines. As the AR is a client protein of Hsp90 and LNCap-LN3, LAPC-4, and C4-2 cells all contain a functional AR, it is not surprising that there is greater potency against those three cancer cell lines than the AR-deficient PC3MM2 cell line.

Cytotoxicity of KU675 in Prostate Cancer Cells. Prostate cancer cells were further examined to discern the relationship between antiproliferative effects and cytotoxicity. LNCap-LN3 (Fig. 2A), LAPC-4 (Fig. 2B), and C4-2 (Fig. 2C) cells were treated with KU675 for 24 hours, and PC3MM2 cells were treated for both 24 hours (Fig. 2D) and 48 hours (Fig. 2E). A dose-dependent increase in cell death was observed at concentrations of KU675 ranging from 0.5 to 10 µM in LNCap-LN3 cells, 1.0–5.0 µM in LAPC-4 cells, 0.5–5.0 µM in C4-2 cells, and 5.0–25.0 µM in PC3MM2 cells over 24 hours of treatment. Comparing the total cell number on KU675 treatment at each dose to time zero (Fig. 2, right panels) revealed that 10 µM KU675 is as cytostatic as 250 µM NB for LNCap-LN3 cells, 5 µM for LAPC4 cells, 2.5 µM for C4-2 cells, and 5 µM for PC3MM2. With respect to cytotoxicity, KU675 appears more potent in androgen-dependent prostate cancer cells than in androgen-independent cancer cells; for example, 10 µM KU675 is almost completely cytotoxic to LNCap-LN3 cells (Fig. 2A, left panel), and 25 µM KU675 was necessary to kill most of the PC3MM2 cells (Fig. 2D, left panel). Additionally, PC3MM2 cells dosed with KU675 were further investigated at a 48-hour time point (Fig. 2E) since the antiproliferative results suggested that KU675 might require longer exposure to elicit the maximal response for the PC3MM2 cell line (Fig. 1). At the 48-hour time point, KU675 demonstrated appreciable cytotoxicity at doses as low as 5 µM (Fig. 2E) in PC3MM3 cells, which not only correlated well with previous antiproliferative data but also suggested that androgen-independent cancer cells such as PC3MM2 might require a higher dose and longer treatment to achieve the optimal result.

Cancer Cell-Based Hsp90-Dependent Luciferase Refolding Assay. Evidence suggests that Hsp90 is present in cancer cells as part of a large macromolecular complex; therefore, drugs that target Hsp90 activity should be engineered

Fig. 2. Cytotoxicity of KU675 in prostate cancer cells. LNCap-LN3 (A), LAPC-4 (B), and C4-2 cells (C) were treated with KU675 for 24 hours, and PC3MM2 cells were treated for both 24 hours (D) and 48 hours (E). Samples were mixed with equal parts trypan blue and assessed for viability by Vi-Cell cell viability analyzer. Percent viability was calculated as described in *Materials and Methods*. Columns depict the mean $\pm$ S.E.M. from three independent experiments ($n = 3$). * $P < 0.05$, ** $P < 0.01$, and *** $P < 0.001$, by two-tailed t test compared with vehicle-treated (DMSO) control.

toward binding Hsp90 within physiologically relevant cancer cellular environment. Direct inhibition of the Hsp90 protein folding activity was assessed using a cancer-based luciferase refolding assay developed in our laboratory (Sadikot et al., 2013). The extent of luciferase refolding in the presence of the N-terminal inhibitor 17-AAG and the C-terminal inhibitor KU675 was evaluated in both PC3MM2 (Fig. 3A) and LNCap-LN3 (Fig. 3B) cells. The N-terminal inhibitor 17-AAG was potent, with EC_{50} values in the low micromolar range against both PC3MM2 and LNCap-LN3 cells (Fig. 3), in agreement with our prior studies. KU675, the C-terminal inhibitor, also showed significant inhibition, with similar EC_{50} values of $\sim 50 \mu M$ for both PC3MM2 and LNCap-LN3 cells. Although KU675 exhibited a higher EC_{50} value than 17-AAG, after we normalized the data to percent of control for comparison, we found the maximum inhibition 17-AAG can achieve is $\sim 50\%$, whereas KU675 can inhibit $>80\%$ (Fig. 3, A and B). Overall, these data demonstrate that the luciferase refolding assay is a reliable method to determine on-target Hsp90 inhibition in intact cancer cells and that KU675 shows significant inhibition of Hsp90 activity in both androgen-dependent and -independent prostate cancer cells with greater than 80% inhibition.

KU675-Mediated Degradation of Client Proteins. The level of expression of Hsp90 client proteins has been shown to correlate with prostate cancer cell survival (Isaacs et al., 2003; Ayala et al., 2004; Zhang and Burrows, 2004; Kleeberger et al., 2007; Mohler, 2008); so the potential of KU675 to trigger degradation of client proteins, effect on HSP modulators, and effect on HSP induction were analyzed in PC3MM2 (Fig. 4A), LNCap-LN3 (Fig. 4B), LAPC4 (Fig. 4C), and C4-2 (Fig. 4D) cells after 24 hours of treatment. In the PC3MM2 cell line, KU675 demonstrated a dose-dependent reduction in many Hsp90 client proteins such as survivin, Akt, B-Raf, cdc25C, Her2/ErB2, and Nestin, whereas no obvious changes were observed in the expression level of CXCR4. KU675 also significantly decreased the expression of HSP modulators (Hsf-1) in PC3MM2 cells. In the LNCap-LN3, LAPC-4, and C4-2 cell lines, we also observed a dramatic degradation of several client proteins such as survivin, Akt, cdc25C, Her2/ErB2, AR, and B-Raf without induction of the HSR. Unlike the N-terminal inhibitor 17-AAG, which causes a robust HSR and induction of Hsp27 and Hsc70 in all four prostate cancer cell lines tested (Fig. 4, A–D), KU675 did not cause a HSR in PC3MM2. Moreover, KU675 demonstrated a modest dose-dependent

Fig. 3. Cancer cell-based Hsp90-dependent luciferase refolding assay. Inhibition of luciferase refolding in PC3MM2 (A) and LNCap-LN3 (B) cancer cells in dose response with N-terminal (17AAG) and C-terminal Hsp90 (KU675) inhibitors was measured at 60-minute time points. Luciferase activity is reported as a percentage of its control activity for each drug concentration. These results are the mean $\pm$ S.E.M. from three independent experiments performed in duplicate ($n = 3$).

reduction in HSPs (Hsc70 and Hsp27) in the LNCap-LN3, LAPC-4, and C4-2 cells (Fig. 4, B–D), demonstrating again that androgen-dependent prostate cancer cells are more sensitive to KU675 treatment. Interestingly, in all these prostate cancer cell lines, the expression level of CXCR4 remains unaffected. Additional studies with the Hsp90 α and Hsp90 β knockdown cell lines (Peterson et al., 2012) revealed that, among many Hsp90 client proteins, survivin and B-raf are Hsp90 α -specific client proteins, whereas CXCR4 is a client protein specifically dependent on Hsp90 β (Fig. 4E). Our client protein degradation data suggest that KU675 may possess Hsp90 isoform selectivity for Hsp90 α since in four prostate cancer cell lines tested, we observed significant reduction of Hsp90 α client proteins, but the Hsp90 β -specific protein, CXCR4, remained unchanged.

Specific Binding of KU675 to Hsp90 α/β Recombinant Proteins. The intensity of KU675's intrinsic fluorescence spectra, as well as its maximum wavelength, are sensitive to the environments of the fluorescent side chain; thus, to determine the specific binding of KU675 to Hsp90, the interaction between KU675 and Hsp90 α/β recombinant proteins was investigated by intrinsic fluorescent spectroscopy. When excited at 345 nm, KU675 exhibits a fluorescence emission peak located at 450 nm, and the binding of KU675 to Hsp90 protein results in a small red shift of its fluorescence peak together with increased peak intensity. As can be seen in

Fig. 5, A and B (upper panels), in which the fluorescence emission peaks of KU675 are shown in the absence and presence of varying concentrations of recombinant Hsp90 α and β proteins, Hsp90 causes around 5-nm red shift of KU675's maximum wavelength and also enhances KU675's fluorescence peak intensity on binding. As illustrated in the concentration-dependent binding curves (Fig. 5, A and B, bottom panels), the binding of KU675 to Hsp90 was saturable with a calculated K_d of 191 μ M for Hsp90 α (Fig. 5A) and 726 μ M for Hsp90 β (Fig. 5B). One negative control was also used in this study, phosphorylase kinase, which does not bind KU675; as expected, only a slight fluorescence fluctuation was observed (Fig. 5C). Taken together, these results suggest that KU675 exhibits selective binding to Hsp90 and that the binding affinity for Hsp90 α is 3.8 times greater than Hsp90 β , which corresponds well with the Western blot data that show client protein degradations were Hsp90 α specific.

Analysis of Native Hsp90 Chaperone Complexes by BN-PAGE. To further analyze KU675's binding selectivity for Hsp90 α , BN-PAGE Western blot was performed to assess disruption of the Hsp90 complex on binding by KU675. Since Hsp90 functions as part of a large multiprotein complex, inhibition of Hsp90 leads to disruption of these complexes. These complexes resolve at a molecular mass of 400 kDa for Hsp90 α and Hsp90 β and a lower molecular mass of 150 kDa

Binding of KU675 to Hsc70 and the Degradation of Hsc70's Client Proteins. Further binding studies showed that KU675 also binds to Hsc70 protein and that KU675 interacts with Hsc70 in a way that differs from its interaction with Hsp90 proteins. As shown in Fig. 6A, when Hsp90 was added to KU675, Hsp90 α and β enhanced KU675's fluorescence peak intensity with a small red shift for the peak position; however, the addition of Hsc70 led to a blue shift of KU675's fluorescence peak position along with an increase in peak intensity. Shifting of the peak toward

a shorter wavelength indicates that the fluorescent side chains of KU675 are buried in a less polar environment on binding Hsc70, which is different from Hsp90. To further support that Hsc70 specifically binds to KU675, a protein titration was performed; the concentration-dependent curve for the binding of KU675 to Hsc70 is shown in Fig. 6B. As illustrated in Fig. 6B, the binding of KU675 to Hsc70 was saturable with a calculated K_d of 76.3 μ M and a stoichiometry of 1 mole per mole of Hsc70. By comparison, Hsp90 exhibits a higher binding affinity for KU675 than Hsp90. Next, we tested the effect of KU675 binding to Hsc70 and its subsequent effect on Hsc70-dependent client proteins. Two Hsc70 client proteins, Myb and Sp1, were chosen, and their expression and subsequent degradation were examined in the PC3MM2 and LNCap-LN3 cell lines exposed to KU675 for 24-hour degradation of the Hsc70 client proteins, Myb and Sp1, was observed in a dose-dependent manner in both cell lines (Fig. 6C), which again strongly suggest that KU675 is a dual inhibitor of both Hsp90 and Hsc70 in prostate cancer cells.

Fig. 5. Analysis of the binding of KU675 to Hsp90 exhibits isoform selectivity for Hsp90α. Intrinsic fluorescence spectra were used to test the specific binding of KU675 to Hsp90. KU675 (20 μM) was incubated with Hsp90α (A), Hsp90β (B), and a negative control protein phosphorylase kinase (PhK) (C) at different concentrations: 0–100 μg/ml for Hsp90α, 0–140 μg/ml for Hsp90β, and 0–100 μg/ml for PhK. After 30 minutes, KU675 (20 μM) was excited at 345 nm, and the emission peak was monitored from 350 to 600 nm. Concentration-dependent binding curves for the interaction of KU675 with Hsp90α and β were generated using the nonlinear regression of the data with Scatchard plot inserted. Dose-dependent effects of KU675 on Hsp90α and Hsp90β native complexes were determined by BN Western blot after treatment of PC3MM2 cells by KU675 along with 17-AAG and NB (D); protein concentration was determined using a DC Protein Assay to ensure that equal amounts of protein were loaded and a loading control β-actin was included in each experiment. Hsp90α complex was significantly disrupted by KU675, whereas Hsp90β complex only showed moderate change.

Discussion

Hsp90 is a molecular chaperone required for the folding of nascent and denatured proteins. Hsp90 is often present at elevated levels in cancer cells, and it functions to stabilize oncogenic proteins involved in signal transduction, growth, and apoptosis regulation. There are approximately 200 reported cytosolic and nuclear client proteins of Hsp90, including protein kinases (e.g., Akt and Her2), transcription factors (e.g., mutant p53 and HIF-1α), chimeric signaling proteins, steroid receptors, and several proteins involved in apoptosis. Whereas many of the aforementioned Hsp90 client proteins are pursued individually as targets for anticancer

drug development, inhibition of Hsp90 would prevent the maturation and stabilization of numerous Hsp90 client proteins simultaneously, leading to their degradation via the ubiquitin-proteasome pathway. Since 1995, when the first Hsp90 inhibitor demonstrated antitumor efficacy in a mouse xenograft tumor model, considerable efforts have focused on the development of Hsp90 inhibitors for the treatment of cancer. Although several N-terminal Hsp90 inhibitors, such as 17-AAG and its parent compound geldanamycin, have demonstrated client protein degradation, many of these agents have been hampered in clinical trials by high toxicity or poor solubility. A hallmark of N-terminal inhibition is induction of

Fig. 6. KU675 binds to Hsc70 and decreases the expression levels of Hsc70 client proteins (A) Intrinsic fluorescence spectra of 20 μM KU675 in the presence of Hsp90 α , β , and Hsc70. (B) Replot of relative fluorescence intensity versus concentration of Hsc70 (0 to 30 $\mu\text{g/ml}$) with a Scatchard plot inserted. (C) Decreased expression of Hsc70-specific client proteins induced by KU675.

HSPs, which is mediated through HSF-1 transcriptional activation of the HSR element, and is a significant concern because clinical resistance has been attributed to the induction of these pro-survival HSPs. Efforts to increase the doses of the N-terminal inhibitors to overcome this resistance have been prevented by toxicity, which may limit the clinical potential for these compounds.

A new approach to target Hsp90 began with the observation that the antibiotic NB binds with low affinity to a C-terminal ATP-binding pocket (Marcu et al., 2000). Since then, more potent analogs of NB have been developed, and we report here one such candidate, KU675. This compound binds directly to Hsp90 and suppresses cell proliferation against androgen-dependent and -independent prostate cancer lines. In comparison with NB, KU675's antiproliferative effects were found to be 290-fold more potent against the LNCap-LN3 cells and 77-fold more against PC3MM2 cells (Figs. 1 and 2). In a cancer cell-based Hsp90-dependent luciferase refolding assay, KU675 also exhibited greater inhibition of Hsp90's protein folding function than the N-terminal inhibitor, 17-AAG. The maximum inhibition achieved by 17-AAG was ~50%, whereas KU675 suppressed the protein folding machinery >80% (Fig. 3). More importantly, KU675 in prostate cancer cells not only induced the degradation of client proteins, but it also caused concomitant reduction of Hsp27, Hsc70, and Hsf-1 (Supplemental Fig. 1). The induction of a HSR, which was initially believed to be a hallmark of N-terminal Hsp90 inhibition, was not observed in androgen-dependent LNCap-LN3, LAPC4, and C4-2 cells or androgen-independent PC3MM2 cells after KU675 treatment, which may have important clinical implications. The most interesting finding in this study is that some Hsp90 α -specific client proteins, such as B-Raf and survivin, were affected across prostate cancer cell lines; however, the Hsp90 β -specific client protein, CXCR4, was minimally affected. The fact that not all client proteins would be equally degraded on KU675 treatment suggests that KU675 may be selective for Hsp90 α inhibition.

In this study, for the first time, we revealed that the C-terminal Hsp90 inhibitor, KU675, exhibited preferential

inhibition of Hsp90 α . KU675 appeared to have distinct effects on Hsp90 α specific client proteins, such as B-Raf and survivin, in all four prostate cancer cell lines we tested (Fig. 4); however, the level of the Hsp90 β -dependent client protein, CXCR4, was minimally affected. Also, analysis of the binding of KU675 to Hsp90 by intrinsic fluorescent spectroscopy indicated that KU675 binds to Hsp90 preferentially with a K_d of 191 μM for Hsp90 α , and a K_d of 726 μM for Hsp90 β . The binding affinity of KU675 for Hsp90 α is 3 times greater than its affinity for Hsp90 β (Fig. 5). Finally, BN Western blots also showed that KU675 disrupts the Hsp90 α complex, including both the Hsp90 α monomer and dimer at 1 μM , but affecting only the Hsp90 β complex moderately at the same concentration. Combined, these data support the isoform selectivity of KU675 for Hsp90 α .

Furthermore, KU675 also displayed binding to Hsc70 with a K_d value of 76.3 μM and, as a promising Hsc70 inhibitor, KU675, also decreased the levels of Hsc70 specific client proteins. The fact that KU675 is a dual inhibitor for both Hsp90 and Hsc70 was also supported by our luciferase refolding assay, in which the N-terminal inhibitor 17-AAG, a geldanamycin analog, inhibited luciferase folding by only around 50%, but KU675 resulted in inhibition of more than 80%. Research by Thulasiraman and Matts (1996) demonstrated that geldanamycin did not inhibit luciferase folding by 100% and that there was an alternate slower pathway through which luciferase could refold. This alternate pathway may represent an Hsp70-dependent pathway since the study showed that direct Hsp70-inhibitors also block luciferase refolding (Thulasiraman and Matts, 1998). KU675 being a dual inhibitor would block both pathways, which accounts for the 80% inhibition of the luciferase refolding activity.

In summary, KU675, a novel C-terminal Hsp90 inhibitor, exhibits potent antiproliferative and cytotoxic activity along with client protein degradation, without induction of the HSR in both androgen-dependent and -independent prostate cancer cell lines. KU675 displays preferential inhibition of the Hsp90 α isoform, and at the same time, KU675 also

inhibits Hsc70. The presented data suggest the potential to design isoform-selective inhibitors of Hsp90 as well as dual inhibitors of other HSPs for the treatment of cancer.

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Authorship Contributions

Participated in research design: Liu, Vielhauer, Holzbeierlein, Blagg.

Conducted experiments: Liu, Brown, Ghosh.

Contributed new reagents or analytic tools: Zhao.

Performed data analysis: Liu, Brown, Ghosh.

Wrote or contributed to the writing of the manuscript: Liu, Holzbeierlein, Lee, Blagg.

References

- An WG, Schulte TW, and Neckers LM (2000) The heat shock protein 90 antagonist geldanamycin alters chaperone association with p210bcr-abl and v-src proteins before their degradation by the proteasome. *Cell Growth Differ* 11:355–360.
- Avila C, Hadden MK, Ma Z, Kornilayev BA, Ye QZ, and Blagg BS (2006) High-throughput screening for Hsp90 ATPase inhibitors. *Bioorg Med Chem Lett* 16: 3005–3008.
- Ayala G, Thompson T, Yang G, Frolov A, Li R, Scardino P, Otori M, Wheeler T, and Harper W (2004) High levels of phosphorylated form of Akt-1 in prostate cancer and non-neoplastic prostate tissues are strong predictors of biochemical recurrence. *Clin Cancer Res* 10:6572–6578.
- Basso AD, Solit DB, Munster PN, and Rosen N (2002) Ansamycin antibiotics inhibit Akt activation and cyclin D expression in breast cancer cells that overexpress HER2. *Oncogene* 21:1159–1166.
- Blagosklonny MV, Toretzky J, and Neckers L (1995) Geldanamycin selectively destabilizes and conformationally alters mutated p53. *Oncogene* 11:933–939.
- Burlison JA, Avila C, Vielhauer G, Lubbers DJ, Holzbeierlein J, and Blagg BS (2008) Development of novobiocin analogues that manifest anti-proliferative activity against several cancer cell lines. *J Org Chem* 73:2130–2137.
- Chen B, Piel WH, Gui L, Bruford E, and Monteiro A (2005) The HSP90 family of genes in the human genome: insights into their divergence and evolution. *Genomics* 86: 627–637.
- Chen B, Zhong D, and Monteiro A (2006) Comparative genomics and evolution of the HSP90 family of genes across all kingdoms of organisms. *BMC Genomics* 7:156–175.
- Chi KN, Hotte SJ, Yu EY, Tu D, Eigl BJ, Tannock I, Saad F, North S, Powers J, and Gleave ME et al. (2010) Randomized phase II study of docetaxel and prednisone with or without OGX-011 in patients with metastatic castration-resistant prostate cancer. *J Clin Oncol* 28:4247–4254.
- Chiosis G, Huezio H, Rosen N, Mimnaugh E, Whitesell L, and Neckers L (2003) 17AAG: low target binding affinity and potent cell activity: finding an explanation. *Mol Cancer Ther* 2:123–129.
- Csermely P, Schnaider T, Soti C, Prohászka Z, and Nardai G (1998) The 90-kDa molecular chaperone family: structure, function, and clinical applications: a comprehensive review. *Pharmacol Ther* 79:129–168.
- Eskew JD, Sadikot T, Morales P, Duren A, Dunwiddie I, Swink M, Zhang X, Hembruff S, Donnelly A, and Rajewski RA et al. (2011) Development and characterization of a novel C-terminal inhibitor of Hsp90 in androgen dependent and independent prostate cancer cells. *BMC Cancer* 11:468–484.
- Hanahan D and Weinberg RA (2000) The hallmarks of cancer. *Cell* 100:57–70.
- Holzbeierlein JM, Windsperger A, and Vielhauer G (2010) Hsp90: a drug target? *Curr Oncol Rep* 12:95–101.
- Huggins C (1952) Endocrine factors in cancer. *J Urol* 68:875–884.
- Huggins C and Hodges CV (2002) Studies on prostatic cancer: I. The effect of castration, of estrogen and of androgen injection on serum phosphatases in metastatic carcinoma of the prostate. 1941. *J Urol* 168:9–12.
- Isaacs JS, Xu W, and Neckers L (2003) Heat shock protein 90 as a molecular target for cancer therapeutics. *Cancer Cell* 3:213–217.
- Kelly WK, Halabi S, Carducci M, George D, Mahoney JF, Stadler WM, Morris M, Kantoff P, Monk JP, and Kaplan E et al. (2012) Randomized, double-blind, placebo-controlled phase III trial comparing docetaxel and prednisone with or without bevacizumab in men with metastatic castration-resistant prostate cancer: CALGB 90401. *J Clin Oncol* 30:1534–1540.
- Kleeberger W, Bova GS, Nielsen ME, Herawi M, Chuang AY, Epstein JI, and Berman DM (2007) Roles for the stem cell associated intermediate filament Nestin in prostate cancer migration and metastasis. *Cancer Res* 67:9199–9206.
- Kusuma BR, Khandelwal A, Gu W, Brown D, Liu W, Vielhauer G, Holzbeierlein J, and Blagg BS (2014) Synthesis and biological evaluation of coumarin replacements of novobiocin as Hsp90 inhibitors. *Bioorg Med Chem* 22:1441–1449.
- Lassi K and Dawson NA (2010) Update on castrate-resistant prostate cancer: 2010. *Curr Opin Oncol* 22:263–267.
- Marcu MG, Chadli A, Bouhouche I, Catelli M, and Neckers LM (2000) The heat shock protein 90 antagonist novobiocin interacts with a previously unrecognized ATP-binding domain in the carboxyl terminus of the chaperone. *J Biol Chem* 275:37181–37186.
- Martin CJ, Gaisser S, Challis IR, Carletti I, Wilkinson B, Gregory M, Prodromou C, Roe SM, Pearl LH, and Boyd SM et al. (2008) Molecular characterization of macbecin as an Hsp90 inhibitor. *J Med Chem* 51:2853–2857.
- Matthews SB, Vielhauer GA, Manthe CA, Chaguturu VK, Szabla K, Matts RL, Donnelly AC, Blagg BS, and Holzbeierlein JM (2010) Characterization of a novel novobiocin analogue as a putative C-terminal inhibitor of heat shock protein 90 in prostate cancer cells. *Prostate* 70:27–36.
- Mohler JL (2008) A role for the androgen-receptor in clinically localized and advanced prostate cancer. *Best Pract Res Clin Endocrinol Metab* 22:357–372.
- Ohba S, Hirose Y, Yoshida K, Yazaki T, and Kawase T (2010) Inhibition of 90-kD heat shock protein potentiates the cytotoxicity of chemotherapeutic agents in human glioma cells. *J Neurosurg* 112:33–42.
- Peterson LB, Eskew JD, Vielhauer GA, and Blagg BS (2012) The hERG channel is dependent upon the Hsp90α isoform for maturation and trafficking. *Mol Pharm* 9: 1841–1846.
- Petrylak DP, Tangen CM, Hussain MH, Lara PN, Jr, Jones JA, Taplin ME, Burch PA, Berry D, Moynour C, and Kohli M et al. (2004) Docetaxel and estramustine compared with mitoxantrone and prednisone for advanced refractory prostate cancer. *N Engl J Med* 351:1513–1520.
- Pettaway CA, Pathak S, Greene G, Ramirez E, Wilson MR, Killion JJ, and Fidler IJ (1996) Selection of highly metastatic variants of different human prostatic carcinomas using orthotopic implantation in nude mice. *Clin Cancer Res* 2:1627–1636.
- Powers MV and Workman P (2007) Inhibitors of the heat shock response: biology and pharmacology. *FEBS Lett* 581:3758–3769.
- Qin H, Hu J, Hua Y, Challa SV, Cross TA, and Gao FP (2008) Construction of a series of vectors for high throughput cloning and expression screening of membrane proteins from Mycobacterium tuberculosis. *BMC Biotechnol* 8:51–59.
- Roe SM, Prodromou C, O'Brien R, Ladbury JE, Piper PW, and Pearl LH (1999) Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. *J Med Chem* 42:260–266.
- Sadikot T, Swink M, Eskew JD, Brown D, Zhao H, Kusuma BR, Rajewski RA, Blagg BS, Matts RL, and Holzbeierlein JM et al. (2013) Development of a high-throughput screening cancer cell-based luciferase refolding assay for identifying Hsp90 inhibitors. *Assay Drug Dev Technol* 11:478–488.
- Sato S, Fujita N, and Tsuruo T (2000) Modulation of Akt kinase activity by binding to Hsp90. *Proc Natl Acad Sci USA* 97:10832–10837.
- Schulte TW, Blagosklonny MV, Ingui C, and Neckers L (1995) Disruption of the Raf-1-Hsp90 molecular complex results in destabilization of Raf-1 and loss of Raf-1-Ras association. *J Biol Chem* 270:24585–24588.
- Schulte TW and Neckers LM (1998) The benzoquinone ansamycin 17-allylamino-17-demethoxygeldanamycin binds to HSP90 and shares important biologic activities with geldanamycin. *Cancer Chemother Pharmacol* 42:273–279.
- Shelton SN, Shawgo ME, Matthews SB, Lu Y, Donnelly AC, Szabla K, Tanol M, Vielhauer GA, Rajewski RA, and Matts RL et al. (2009) KU135, a novel novobiocin-derived C-terminal inhibitor of the 90-kDa heat shock protein, exerts potent antiproliferative effects in human leukemic cells. *Mol Pharmacol* 76:1314–1322.
- Siegel R, Ward E, Brawley O, and Jemal A (2011) Cancer statistics, 2011: the impact of eliminating socioeconomic and racial disparities on premature cancer deaths. *CA Cancer J Clin* 61:212–236.
- Sreedhar AS, Kalmár E, Csermely P, and Shen YF (2004) Hsp90 isoforms: functions, expression and clinical importance. *FEBS Lett* 562:11–15.
- Stühmer T, Chatterjee M, Grella E, Seggewiss R, Langer C, Müller S, Schoepfer J, Garcia-Echeverria C, Quadt C, and Jensen MR et al. (2009) Anti-myeloma activity of the novel 2-aminothienopyrimidine Hsp90 inhibitor NVP-BEP800. *Br J Haematol* 147:319–327.
- Suda A, Kawasaki K, Komiyama S, Isshiki Y, Yoon DO, Kim SJ, Na YJ, Hasegawa K, Fukami TA, and Sato S et al. (2014) Design and synthesis of 2-amino-6-(1H,3H-benzo[de]isochromen-6-yl)-1,3,5-triazines as novel Hsp90 inhibitors. *Bioorg Med Chem* 22:892–905.
- Tannock IF, de Wit R, Berry WR, Horti J, Pluzanska A, Chi KN, Oudard S, Théodore C, James ND, and Turesson I et al.; TAX 327 Investigators (2004) Docetaxel plus prednisone or mitoxantrone plus prednisone for advanced prostate cancer. *N Engl J Med* 351:1502–1512.
- Thulasiraman V and Matts RL (1996) Effect of geldanamycin on the kinetics of chaperone-mediated renaturation of firefly luciferase in rabbit reticulocyte lysate. *Biochemistry* 35:13443–13450.
- Thulasiraman V and Matts RL (1998) Luciferase renaturation assays of chaperones and chaperone antagonists. *Methods Mol Biol* 102:129–141.
- Whitesell L and Lindquist SL (2005) HSP90 and the chaperoning of cancer. *Nat Rev Cancer* 5:761–772.
- Xu W, Mimnaugh E, Rosser MF, Nicchitta C, Marcu M, Yarden Y, and Neckers L (2001) Sensitivity of mature ErbB2 to geldanamycin is conferred by its kinase domain and is mediated by the chaperone protein Hsp90. *J Biol Chem* 276:3702–3708.
- Zhang H and Burrows F (2004) Targeting multiple signal transduction pathways through inhibition of Hsp90. *J Mol Med (Berl)* 82:488–499.

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