KU675, a Concomitant Heat-Shock Protein Inhibitor of Hsp90 and Hsc70 that Manifests Isoform Selectivity for Hsp90α in Prostate Cancer Cells

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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 Hsp90α, Hsp90β, and Hsc70 proteins. The binding affinity (Kd) for Hsp90α was determined to be 191 μM, whereas the Kd for Hsp90β was 726 μM, demonstrating a preference for Hsp90α. Western blot experiments with four different prostate cancer cell lines treated with KU675 supported this selectivity by inducing the degradation of Hsp90α-dependent client proteins. KU675 also displayed binding to Hsc70 with a Kd value at 76.3 μM, 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 prostate cancer 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,SR)-3,4-dihydroxy-5-methoxy-6,6-dimethyltetrahydro-2H-pyran-2-yl oxy)-8-methoxy-2-oxo-2H-chromen-3-yl)-3’6-dimethoxybiphenyl-3-carboxamide; MEM, minimum Eagle’s medium; NB, novobio- cin; 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, 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 Hsp90α.

In mammalian cells, there are two Hsp90 isomers in the cytosol (Hsp90α and Hsp90β 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 Hsp90α showing 85% identity to Hsp90β (Chen et al., 2005). Certain areas within the amino acid sequence differ between Hsp90α and Hsp90β, 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 Hsp90α, with a binding affinity 3 times greater than Hsp90β. 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-6,8-dimethyltetrahydro-2H-pyran-2-yloxy)-8-methoxy-2-oxo-2H-chromen-3-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/ErbB2, rabbit anti-Akt, mouse anti-survivin, rabbit anti-cdc25C, mouse anti-Hsp27, rabbit anti-Hsf1, 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-Hsp90α (Assay Designs, Ann Arbor, MI), goat anti-Hsp90β, 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).

Hsp90α and Hsp90β Recombinant Proteins. Overexpression and purification of Hsp90α and Hsp90β were performed in the baculovirus expression system. To obtain a homogeneous method to determine the number of viable cells in continuous culture.

Cell Culture. PC3MM2 (androgen dependent) 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% CO2. The stably transduced Hsp90α and Hsp90β knockdown PC3MM2 cells were cultured as described but with the addition of 2.5 µg/ml of puromycin. The small hairpin RNA (shRNA) expression of Hsp90α was induced with the addition of 12 or 24 µg/ml of doxycycline. Induction of Hsp90α shRNA expression with tetracycline was monitored by the TarboRFp fluorescence. The Hsp90β shRNA was constitutively expressed and monitored by TarboRFp 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 × 105 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 IC50 and R2 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,
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 200 g 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 ± 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 × 10⁶ 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

**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 ± S.E.M.
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 X-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 μM capric acid, 20 mM KCl, 2 mM EDTA, 5 mM MgCl2, 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 horse-radish peroxidase-conjugated secondary antibody (1:40,000 dilution), developed using the UVP AutoChem 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 is 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 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). EC50 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 Hsp90α, Hsp90β, and Hsc70 proteins were added to the KU675 solution, adjusted to desired concentration (0–100 μg/ml for Hsp90α, 0–140 μg/ml for Hsp90β, 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 non-linear fitting by GraphPad Prism 5 software, and the affinity of binding (Kd) 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 IC50 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 IC50 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 (IC50) 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
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<sub>50</sub> 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<sub>50</sub> values of ~50 μM for both PC3MM2 and LNCap-LN3 cells. Although KU675 exhibited a higher EC<sub>50</sub> 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 ~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, B-Raf, 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
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 Hsp90α and Hsp90β knockdown cell lines (Peterson et al., 2012) revealed that, among many Hsp90 client proteins, survivin and B-raf are Hsp90α-specific client proteins, whereas CXCR4 is a client protein specifically dependent on Hsp90β (Fig. 4E). Our client protein degradation data suggest that KU675 may possess Hsp90 isoform selectivity for Hsp90α since in four prostate cancer cell lines tested, we observed significant reduction of Hsp90α client proteins, but the Hsp90β-specific protein, CXCR4, remained unchanged.

Specific Binding of KU675 to Hsp90α/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 Hsp90α/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 Hsp90α 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 μM for Hsp90α (Fig. 5A) and 726 μM for Hsp90β (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 Hsp90α is 3.8 times greater than Hsp90β, which corresponds well with the Western blot data that show client protein degradations were Hsp90α specific.

Analysis of Native Hsp90 Chaperone Complexes by BN-PAGE. To further analyze KU675’s binding selectivity for Hsp90α, 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 Hsp90α and Hsp90β and a lower molecular mass of 150 kDa.
for Hsp90α/β monomer/dimer (Fig. 5D). In BN studies, differential disruption of Hsp90α and Hsp90β complexes was observed on binding to KU675. A robust disruption of Hsp90α complex was observed with KU675’s concentration as low as 1 μM for both Hsp90α large complex and Hsp90α monomer/dimer, whereas the Hsp90β complex was only moderately disrupted by KU675 at a concentration of 10 μM. These observations, together with the previous Western blot and binding affinity studies, further support KU675’s ability to preferentially disrupt Hsp90α.

**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, Hsp90α 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 μM and a stoichiometry of 1 mole per mole of Hsc70. By comparison, Hsc70 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. 4.** KU675-mediated client protein degradation in the absence of HSP induction. PC3MM2 (A), LNCap-LN3 (B), LAPC-4 (C), and C4-2 cells (D) were examined for client protein degradation and the HSP induction. 17-AAG demonstrates HSP induction in all four cancer cell lines, and KU675 triggers a dose-dependent decrease in several client proteins known to play a role in the development of prostate cancer. (E) Western blots were run with the wild-type (WT), Hsp90α, and Hsp90β knockdown PC3MM2 cell lines and blotted against Hsp90α, Akt, pAkt (S473), pAkt (T308), cyclin D1, survivin, c-Src, Raf, Erk5, Hsp90β, and CXCR4. Actin was used as loading control. Dox, doxycycline; KD, knockdown.
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 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
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 prosurvival 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 Hsp90α’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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 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 the protein folding machinery >80% (Fig. 3).
inhibits Hsp70. 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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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.

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