Hsp90 is a chaperone required for the conformational maturation of certain signaling proteins including Raf, cdk4, and steroid receptors. Natural products and synthetic small molecules that bind to the ATP-binding pocket in the amino-terminal domain of Hsp90 inhibit its function and cause the degradation of these client proteins. Inhibition of Hsp90 function in cells causes down-regulation of an Akt kinase-dependent pathway required for D-cyclin expression and retinoblastoma protein-dependent G1 arrest. Intracellular Akt is associated with Hsp90 and Cdc37 in a complex in which Akt kinase is active and regulated by phosphatidylinositol 3-kinase. Functional Hsp90 is required for the stability of Akt in the complex. Occupancy of the ATP-binding pocket by inhibitors is associated with the ubiquitination of Akt and its targeting to the proteasome, where it is degraded. This results in a shortening of the half-life of Akt from 36 to 12 h and an 80% reduction in its expression. Akt and its activating kinase, PDK1, are the only members of the protein kinase A/protein kinase B/protein kinase C-like kinase family that are affected by Hsp90 inhibitors. Thus, transduction of growth factor signaling via the Akt and Raf pathways requires functional Hsp90 and can be coordinately blocked by its inhibition.

The members of the heat shock protein 90 (Hsp90)^† family are ubiquitous and abundant protein chaperones that have several physiologic roles. Hsp90α and Hsp90β are found in the cytosol, where they are required for the stability and functional maturation of certain signaling proteins such as steroid receptors, the Raf serine kinases, cyclin-dependent kinase 4 (cdk4), and some receptor tyrosine kinases (1–8). The Hsp90-containing chaperone complex is also required for sustaining the function of mutated proteins and for preventing protein aggregation (9, 10). These complexes also play a role in refolding denatured proteins in cells exposed to environmental stress (11). Grp94 and TRAP-1 are members of the Hsp90 family expressed in the endoplasmic reticulum and mitochondria, respectively (12, 13). The Hsp90 family members contain an ATP-binding pocket that is required for its function. ATP binding and hydrolysis are required for the last steps of refolding and release of the native protein from the chaperone complex (14).

Several natural products, including radicicol and the ansamycin antibiotics geldanamycin and herbimycin-A, bind tightly to the Hsp90 ATP/ADP pocket (15–17). Occupancy of the pocket by these drugs prevents ATP binding and the completion of client protein refolding. As a result, drug treatment leads to proteasome-dependent degradation of proteins that require Hsp90 for conformational maturation (18). Exposure of cells to ansamycins or radicicol leads to a decline in expression of Hsp90 client proteins such as Raf, HER2, and mutant p53 (2, 8, 9, 17, 19). These drugs have thus been used to probe Hsp90 function. Pharmacological inhibition of Hsp90 function and degradation of its client proteins do not lead to nonspecific cell death. Instead, in cancer cells, Hsp90 inhibitors cause growth arrest followed by differentiation and then apoptosis (20–22).

Treatment of cancer cells with these inhibitors causes retinoblastoma protein (RB)-dependent G1 cell cycle arrest associated with a down-regulation of D-cyclins, loss of D-cyclin-associated kinase activity, and hypophosphorylation of the RB protein (20). The RB dependence of the G1 arrest suggests that the effects of ansamycins on G1 progression are mediated by inhibition of pathways that selectively affect RB. RB is the only known target of the cyclin D-cdk4 complex. Ansamycins cause a rapid down-regulation of D-cyclin-dependent protein kinase by inhibiting the expression of both D-cyclins and cdk4. Cdk4 associates with Hsp90 and is a direct target of these drugs (5). In contrast, D-cyclins are not direct targets. Ansamycins cause their down-regulation by inhibiting a phosphatidylinositol 3-kinase (PI3 kinase)/Akt-dependent pathway required for their expression (23).

The coordinate down-regulation by ansamycins of D-cyclin and cdk4 expression and the RB dependence of the G1 block suggest that Hsp90 selectively regulates this pathway. The mechanism whereby these drugs down-regulate the PI3 kinase/Akt pathway is complex. In a subset of tumor cells in which Akt is activated by upstream pathways dependent on the Hsp90 client protein, HER2, drug treatment leads to degradation of...
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HER2 and a rapid loss of Akt activity (24). In addition, Hsp90 inhibitors reduce Akt expression as well in many cellular systems, albeit more slowly. In this article, we describe the mechanism underlying this effect. Endogenous cellular Akt is associated in a complex with Hsp90 and Cdc37. The Akt in this complex is active and stimulatable by extracellular growth factors. Association with functional Hsp90 is required for Akt stability but not for activity. Occupancy of the Hsp90 pocket by inhibitors does not alter the association of Akt with Hsp90 but does result in its destabilization. In cells exposed to drug, Akt is ubiquitinated and targeted to the proteasome, where it is degraded. The only members of the AGC kinase family that are affected in this manner by Akt inhibitors are Akt and its activating phosphoinositide-dependent kinase 1 (PDK1). These findings suggest that these inhibitors selectively down-regulate Akt signaling by coordinate decreasing the expression of both of these proteins.

EXPERIMENTAL PROCEDURES

MATERIALS—Hsp90 inhibitors were incubated with MCF-7, MDA-MB-468 (MDA-HER2), SKBr-3, and BT-474 (American Type Culture Collection, Manassas, VA) were maintained in a 1:1 mixture of Dulbecco's modified Eagle's medium:F12 supplemented with 2 mM glutamine, 50 units/ml penicillin, and 10% heat-inactivated fetal bovine serum (H9262). MCF-7 cells were transfected with hemagglutinin (HA)-tagged Akt and the PI3 kinase inhibitor, LY294002, or the ATP/ADP inhibitor I (Calbiochem), LY294002 (Biomol, Plymouth Meeting, PA), and EGF (Sigma) were dissolved in 100% Me2SO. 17-AAG (NSC 330507, National Cancer Institute, Frederick, MD), radicil and PU24F-CI (kindly provided, respectively, by Samuel Danishefsky and Gabriela Chiosis, Memorial Sloan-Kettering Cancer Center), proteasome inhibitor I, MG-132, calpeptin, and caspase inhibitor I (Calbiochem), L9294002 (Bioul, Plymouth Meeting, PA), and EGF (Sigma) were dissolved in 100% Me2SO.

Cell Culture—The human cancer lines MCF-7, MDA-MB-468 (MDA-HER2), SKBr-3, and BT-474 (American Type Culture Collection, Manassas, VA) were maintained in a 1:1 mixture of Dulbecco’s modified Eagle’s medium:F12 supplemented with 2 mM glutamine, 50 units/ml penicillin, 50 units/ml streptomycin, and 10% heat-inactivated fetal bovine serum (H9262). MCF-7 cells were transfected with hemagglutinin (HA)-tagged Akt and FLAG-tagged Cdc37 (cDNA was inserted in the BamHI site of pcMV5 vector) (25, 26) by standard calcium phosphate methods.

Protein Analysis—Cells were exposed to drug or Me2SO vehicle. Cells were lysed in Nonidet P-40 buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2.5 mM Na3VO4, 10 mM phenylmethylsulfonyl fluoride, and 10 μM each leupeptin, aprotinin, and soybean trypsin inhibitor) and cleared by centrifugation. Nonidet P-40-insoluble fractions were lysed in Nonidet P-40 buffer (50 mM Tris, pH 7.5, 1% Nonidet P-40, 150 mM NaCl, 2.5 mM Na3VO4, 10 mM phenylmethylsulfonyl fluoride, and 10 μM each leupeptin, aprotinin, and soybean trypsin inhibitor) and cleared by centrifugation. Nonidet P-40-insoluble fractions were lysed in 2% SDS in 50 mM Tris and boiled for 15 min. Protein concentration was determined by using the BCA reagent (Pierce). Samples were separated by 7–15% SDS-PAGE, transferred to nitrocellulose, immunoblotted, and detected by chemiluminescence using the ECL detection reagents (Amer sham Biosciences). Results were quantified with the Bio-Rad Gel Doc system.

Antibodies—Antibodies used were: Akt, P-Akt (Ser-473), S6K, P-PDK1 (Cell Signaling, Beverly, MA); Akt, p65 (PI3k), p90 RSK, PDK1 (Upstate Biotechnology, Lake Placid, NY); Akt1 (D-17), Akt2 (D-17), HER2 (c-18), PKA catalytic unit (Santa Cruz Biotechnology, Inc., Santa Cruz, CA); S6K (U. S. Biological, Swampscott, MA); Akt3 (Alpha Diagnostic, San Antonio, TX); PKCα, PKCβ, PKCε, and PKCζ (Pharmin gen, San Diego, CA); Cdc37 (Neomarker, Fremont, CA); Hsp90 (spa-35; Stressgen, Victoria, Canada); and FLAG, ubiquitin, HA (Sigma). Conjugated Akt (Cell Signaling), Cdc37 (Santa Cruz), and HA antibodies (Roche Molecular Biochemicals) were used for immunoprecipitation.

Metabolic Labeling—For pulse-labeling experiments, 2 million cells/10-cm plate were treated with drug or vehicle for the indicated times. For the last 2 h they were incubated with Dulbecco’s modified Eagle’s medium:F12 without cysteine or methionine, and new vehicle or drug was added as well. Then the cells were pulse-labeled for 30 min with 500 μCi of [35S]methionine (PerkinElmer Life Sciences) in the presence of vehicle or drug. For pulse-chase experiments 2 million cells/10-cm plate were incubated with 250 μCi of [35S]methionine for 12 h, cells were washed with phosphate-buffered saline, and normal medium, supplemented with vehicle or drug and 10 μg/ml lactalbumin (Sigma), was added for the indicated times.

Akt Activity Assay—Kinase activity was assayed using a Cell Signaling Akt kinase kit. Complexes were immunoprecipitated, washed twice with lysis buffer, and then twice with kinase buffer (25 mM Tris, pH 7.5, 5 mM β-glycerophosphate, 2 mM dithiothreitol, 0.1 mM Na3VO4, 10 mM MgCl2). 200 μM ATP and 1 μg of substrate (paramyosin fused to a GSK-3 cross-tide) were added, and assays were performed at 30 °C for 30 min. Reaction mixtures were separated by 15% SDS-PAGE, and the P-GSK3 reaction product was detected by immunoblotting. Paramyosin exists in two forms differing in size, and thus the reaction product appears as a doublet.

Animal Studies—Six-week-old athymic BALB/c female mice (National Cancer Institute, Frederick Cancer Center) were maintained in pressurized ventilated cages. Experiments were carried out under an IACUC-approved protocol, and institutional guidelines for the proper, humane use of animals in research were followed. Prior to tumor cell inoculation, 0.72 mg of SR 17B-estradiol pellets (Innovative Research of America, Sarasota, FL) were placed subcutaneously into the right flank. 1 × 107 MCF-7 cells were mixed at 1:1 with Matrigel (Collaborative Research, Bedford, MA) and injected subcutaneously. Mice were randomized to treatment or control groups and treated with 17-AAG or the egg phospholipid (EPL) vehicle alone. To analyze cellular markers, mice were sacrificed, and tumor tissue was homogenized in 2% SDS lysis buffer.

Immunofluorescence—Cells were plated on fibronectin-coated LabTek chamber slides (VWR, Willard, OH). After the experiment, cells were washed with phosphate-buffered saline and fixed with a 1:1 methanol:aceton mixture. Fixed cells were washed with distilled water and blocked with 5% bovine serum albumin in phosphate-buffered saline. Cells were incubated with primary antibody followed by fluorescein-conjugated secondary antibody (Molecular Probes, Eugene, OR). Nuclei were stained with 0.5 μg/ml of bis-benzimide (Hoechst 33258). Slides were visualized using confocal microscopy.

RESULTS

Treatment with Hsp90 Inhibitors Resulted in a Loss of Akt Protein—Hsp90 inhibitors cause a loss of D-cyclin expression by down-regulating a PI3 kinase/Akt-dependent pathway (23). This led us to investigate the mechanism by which Hsp90 inhibitors down-regulate this pathway. These drugs have no effect on PI3 kinase expression or activity (24). We tested whether Hsp90 inhibitors altered the expression of Akt protein. In a panel of over 30 primary, immortalized, and cancer cell lines, geldanamycin, 17-allylamino-17-deoxygeldanamycin (17-AAG), and radicil caused a decline in the level of Akt protein between 6 and 24 h after drug treatment (Fig. 1A and D, and data not shown). On average, levels of Akt were reduced by 80% at 24 h. The loss of Akt was both time- and concentration-dependent (Fig. 1A and B). In MCF-7 and SKBr-3 breast cancer cells, 10–50 nM 17-AAG was required to reduce Akt expression by 50% (Fig. 1B, data not shown). The levels of Akt1, Akt2, and Akt3 declined with similar kinetics (Fig. 1C).

Synthetic small molecules designed to bind to the ATP/ADP pocket of Hsp90 have been shown to have biological properties similar to those of 17-AAG and to deplete cellular levels of HER2. Their potency in degrading HER2 varies directly and linearly with their affinity for Hsp90 (27, 28). One such compound, PU24F-CI, caused an 80% reduction of Akt protein in MCF-7 and SKBr-3 cells after 24 h (Fig. 1D, data not shown). Loss of Akt, Raf-1, and HER2 occurred at 15 μM, a drug concentration corresponding to its binding affinity for Hsp90 (data not shown). Treatment with a compound structurally similar to PU24F-CI that does not bind the Hsp90 pocket had no effect on Akt protein levels (Fig. 1D, data not shown) (29).

In MCF-7 cells, 17-AAG caused a parallel decline in Akt protein, phosphorylation, and kinase activity. The loss of Akt expression correlated with dephosphorylation of its endogenous substrates, glycogen synthase kinase-3 (GSK-3) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP-1) (data not shown). Inhibition of upstream activation of Akt kinase with the PI3 kinase inhibitor, LY294002, or the epidermal growth factor receptor inhibitor, ZD1839, did not affect the levels of total Akt protein (24, 30).

In murine xenograft models, the maximally tolerated dose of 17-AAG given daily for 5 days ranged from 75 to 125 mg/kg. Treatment resulted in loss of HER2 expression in the tumors and inhibition of tumor growth (24). The effect of 17-AAG on Akt levels in xenograft tumors was determined after three daily treatments. Levels of Akt were reduced by 70% with 100
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Fig. 1. Hsp90 inhibitors induced loss of Akt protein expression. Levels of Akt were analyzed by immunoblotting. A, breast cancer cell lines MCF-7, MDA-468, BT-474, and SKBr-3 were treated with 1 \( \mu \text{M} \) 17-AAG for the indicated times. B, MCF-7 cells were treated with various concentrations of 17-AAG for 24 h. C, MCF-7 cells were treated with 1 \( \mu \text{M} \) 17-AAG over 24 h. D, MCF-7 cells were treated with 1 \( \mu \text{M} \) radicicol, 15 \( \mu \text{M} \) PU24F-CI, and 15 \( \mu \text{M} \) of the inactive compound 9-N-butylenamine (Ad-But) for the indicated times. E, mice bearing human MCF-7 xenografts were treated with 0, 50, or 100 mg/kg 17-AAG for 3 days and then sacrificed 12 h after the last treatment. The levels of p85 (PI3 kinase) were also determined.

mg/kg in MCF-7 xenografts (Fig 1E). Similar findings were observed in BT-474 xenografts (65% reduction with 100 mg/kg; data not shown). Under these conditions, there was no significant change in p85 (regulatory subunit of PI3 kinase) expression nor was any toxicity noted (Fig. 1E, data not shown). At these doses, 17-AAG inhibits tumor growth in MCF-7 and BT-474 xenografts (data not shown).

**Effect of 17-AAG on Akt Synthesis and Half-life**—A decline in steady state level of Akt could result from changes in the rates of its synthesis or degradation. Cells were metabolically labeled with \(^{35}\text{S}\)methionine following pretreatment with 17-AAG for different times. Treatment with 17-AAG had no significant effect on the incorporation of the labeled amino acid into Akt, indicating there was no change in the rate of its synthesis (Fig. 2A). Pulse-chase experiments were performed to assess the rate of Akt degradation. Cells were labeled with \(^{35}\text{S}\)methionine for 12 h and then chased with media containing vehicle or drug. The half-life of Akt was shortened from 36 to 12 h in cells exposed to 17-AAG (Fig. 2B). 17-AAG treatment did not cause a general increase in degradation of all proteins, but rather drug exposure resulted in a selective effect on Akt (Fig. 2B, lower panel).

Akt Degradation Induced by Hsp90 Inhibitors Is Proteasome-dependent—Protein degradation is catalyzed by various means including lysosome-, proteasome-, caspase-, and calpain-dependent pathways. To determine which pathway was responsible for the accelerated degradation of Akt, selective inhibitors were employed. Weak bases, such as ammonium chloride, raise vacuolar pH and inhibit lysosomal function (31). The peptide aldehyde protease inhibitors Proteasome Inhibitor I (Z-Ile-Glu (OtBu)-Ala-Leu-CHO) and MG-132 (Z-Leu-Leu-Leu-CHO) are reversible inhibitors of the proteasome (32–34). Z-VAD.FMK (benzoyloxycarbonyl-valinyl-alaninyl-aspartyl fluoromethyl ketone) is an irreversible inhibitor of caspases 1, 3, 4, and 7 (35). Calpeptin (Z-Leu-Nle-CHO) inhibits calpains, Ca\(^{2+}\) dependent cysteine proteases (36).

Inhibitors of the proteasome had no effect on Akt expression alone, but abrogated the effect of Hsp90 inhibitors (Fig. 3A). The loss of all three isoforms of Akt was prevented by proteasome inhibition (data not shown). In contrast, inhibitors of caspase, calpain, and lysosomal function had no effect (Fig. 3A). In cells treated with proteasome inhibitors and 17-AAG, the Akt protein was lost from the Nonidet P-40 soluble fraction and accumulated in an Nonidet P-40 insoluble cellular fraction (Fig. 3A). The Akt protein found in the Nonidet P-40 insoluble fraction was phosphorylated on Ser-473 (data not shown). The kinase activity of this protein could not be determined because of the requirement of SDS to solubilize the protein. The protected protein showed a faint laddering pattern typically observed for ubiquitinated proteins (Fig. 3A). Loss of Akt expression in cells treated with 17-AAG was first detectable at 9 h after drug treatment. In cells treated with the proteasome inhibitor and 17-AAG, accumulation in the insoluble fraction began at the same time (Fig. 3B). A similar effect has been reported for mutant p53 and Ral (37, 38).

The location of the protected Akt protein was determined by immunofluorescence. In untreated SKBr-3 cells, Akt protein was activated and co-localized with HER2 at the plasma membrane (Fig. 4, top row). 17-AAG treatment for 12 h led to depletion of both Akt and HER2 protein and morphological changes consistent with epithelial differentiation, including flattening of the cells (Fig. 4, second row (21)). HER2 protein is depleted following exposure to 17-AAG after 2–4 h, whereas complete Akt depletion requires 24 h (24). The proteasome inhibitor alone had no effect on the localization of Akt or HER2 (Fig. 4, third row). In cells that were treated with the combination of 17-AAG and the proteasome inhibitor, the protected Akt protein accumulated in perinuclear vesicles. The protected HER2 protein accumulated in similar structures and co-localized with Akt (Fig. 4, fourth row).

The majority of proteins degraded in the proteasome are first modified by a polyubiquitin chain, which serves as a recognition signal for targeting to the proteasome (39). Indeed, we found that treatment of cells with the proteasome inhibitor alone or in combination with 17-AAG resulted in the formation of polyubiquitinated, higher molecular weight forms of Akt (Fig. 3C).

**Akt Associated with Cdc37 and Hsp90**—These results suggest that Hsp90 function is required for Akt stability. We found that endogenous Akt was present in a complex with Hsp90 and the co-chaperone Cdc37. Akt, Cdc37, and Hsp90 were detected in both Akt and Cdc37 immunoprecipitates (Fig. 5A). Cdc37 was found to be associated with all three Akt kinases (data not shown). As previously reported, Raf was also found to be associated with Cdc37 and Hsp90, whereas PKC and PKA were not (Fig. 5A, data not shown) (40). In MCF-7 cells, cotransfection experiments with FLAG-tagged Cdc37 and HA-tagged Akt showed the association of Akt and Hsp90 required the presence of Cdc37 (Fig. 5D). Although complexes containing endogenous Akt, Cdc37, and Hsp90 can be demonstrated, HA-Akt was found in a complex with Hsp90 only when Cdc37 was overexpressed.

More than 99% of Cdc37 protein could be immunoprecipitated by Cdc37 antibody and no Cdc37 was detected in supernatants (Fig. 5B, lane 3 versus lane 4). These immunoprecipitates contained one-third the amount of Akt as compared with the supernatant, suggesting that at least 33% of total Akt was...
complexed to Cdc37 (Fig. 5B, lane 3 versus lane 4). A similar percentage of Raf was found in the Cdc37 complex (data not shown). Treatment with 17-AAG did not disrupt the Akt-Cdc37 complex. The complex was maintained until 24 h post-treatment when Akt protein expression is lost (Fig. 6B). Treatment of cells with proteasome inhibitors also did not disrupt the complex (data not shown).

Immunoprecipitates of Cdc37 contained a kinase capable of phosphorylating GSK-3 in vitro (Fig. 6A). Inhibitors of PI3k blocked this kinase activity (Fig. 6A). Furthermore, the kinase activity fell during a 24-hour exposure to 17-AAG in parallel with loss of Akt protein from the Cdc37 complex (Fig. 6B). These data suggest that the Akt protein present in the Cdc37-Hsp90 complex is catalytically active and responsible for the GSK-3 kinase in the complex.

Neither Akt stimulation by epidermal growth factor (EGF) nor inhibition by serum starvation changed the amount of Akt present in the Cdc37-Hsp90 complex (Fig. 5C). Phosphorylation and activity of Akt were induced by EGF whether or not it was present in the Cdc37 complex (Figs. 5C and 6). In order to compare activation of Akt kinase in the Cdc37 bound and unbound states, Cdc37 was immunoprecipitated. Then Akt was immunoprecipitated from the Cdc37-cleared supernatants. Akt kinase assays were performed on the Cdc37 immunoprecipitates and compared with assays of Akt immunoprecipitates of the supernatant. In response to EGF, kinase activity rose and fell in parallel in both the Cdc37-bound and unbound fractions (Fig. 6C). There was no appreciable difference in specific activity and kinetics of induction and inactivation (Fig. 6C, data not shown).

The Effect of Hsp90 Inhibitors on Other Serine Kinases—Akt belongs to the AGC family of protein kinases, which includes cyclic AMP-dependent protein kinase (PKA), cyclic GMP-dependent kinase, protein kinase C (PKC), p70 S6 kinase (S6K), p90 ribosomal S6 kinase (RSK), phosphoinositide-dependent kinase 1 (PDK1), and serum- and glucocorticoid-regulated kinase (SGK). The catalytic domains of these kinases are closely related. As Cdc37 binds to the catalytic domain of kinases, we evaluated the effects of Hsp90 inhibitors on other members of the AGC family. Hsp90 inhibitors did not alter the expression levels of any other member of the family except PDK1 (Fig. 7, A and B, data not shown). 17-AAG and radicicol did deplete...
levels of PDK1 and Raf in a fashion similar to Akt (Fig. 7B, data not shown). The degree of phosphorylation of PDK1 at serine-241, a site essential for PDK1 activity, declined in parallel with the fall in its protein expression (Fig. 7B). Additionally, inhibitors of the 20 S proteasome, but not other protease inhibitors, prevented the loss of PDK1 protein and caused it to accumulate in a Nonidet P-40-insoluble cellular fraction (Fig. 7C).

**DISCUSSION**

Hsp90 is an abundant chaperone that plays a variety of roles in cellular physiology. Hsp90 contains a highly conserved ATP
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Fig. 5. Akt associated with Cdc37 and Hsp90. A, MCF-7 lysates were immunoprecipitated with Akt and Cdc37. Westerns were immunoblotted for Akt, Cdc37, Hsp90, and PKA. B, immunoprecipitations (IP) and their supernatants were immunoblotted for Akt and Cdc37. Lane 1, IgG; lane 2, supernatant from IP Cdc37; lane 3, IP Cdc37; lane 4, supernatant from IP Cdc37. C, MCF-7 cells were grown in media, serum-starved, or starved and stimulated with 32 nM EGF for 5 min. Cdc37 immunoprecipitates were analyzed for Akt, P-Akt, Cdc37, and Hsp90. D, MCF-7 cells were transfected with HA-Akt and FLAG-Cdc37. HA immunoprecipitations were immunoblotted for FLAG, HA, and Hsp90.

binding pocket in its amino-terminal domain that is required for its function (14). Several natural products, including the ansamycin antibiotics, bind to this pocket and inhibit Hsp90 function (15). These drugs induce the degradation of Hsp90 clients such as Raf and HER2 and have been used as probes of the biochemical and cellular functions of this chaperone (1–3).

Pharmacological inhibition of Hsp90 function by these antibiotics has selective and specific effects on cancer cells. The RB-dependence of the G1 arrest induced by ansamycins suggests that Hsp90 selectively regulates pathways responsible for activation of cyclin D/cdk4 kinase. This is contrary to the usual view of Hsp90 as a general housekeeping protein regulating many processes. In fact cdk4 is an Hsp90 client and is degraded in cells exposed to drug (5). Furthermore, although D-cyclins are not direct targets of ansamycins, their expression declines in treated tumor cell lines. This decline is due to down-regulation of a PI3 kinase/Akt dependent pathway required for the efficient translation of cyclin D1 and D3 mRNA (23).

Hsp90 inhibitors down-regulate the PI3k/Akt pathway in part by causing a decline in Akt protein expression (24, 41). In this paper, we have determined the mechanism of this effect. In untreated cells, at least 30% of Akt is found in a complex with Hsp90 and Cdc37. Immunoprecipitates of Cdc37 contained a kinase capable of phosphorylating GSK-3. This kinase activity was abolished in cells exposed to the PI3 kinase inhibitor LY294002. Moreover, this activity was down-regulated in cells exposed to 17-AAG, in parallel with the loss of Akt protein in Cdc37 immunoprecipitates. Thus, the activity likely represents Akt kinase, which is therefore active when present in the Cdc37-Hsp90 complex.

Occupancy of the Hsp90 pocket by either of two natural products or by a designed synthetic compound led to the degradation of all three Akt kinases in the proteasome. This was manifested by a reduction in Akt half-life from 36 to 12 h and an 80% decline in Akt protein expression after 24 h of drug treatment. In xenograft models, Akt expression was depleted at doses that have anti-tumor activity, though it is not possible to know whether the anti-tumor effect is due to reduction in Akt expression or reduction of other Hsp90 client proteins. The reduction in Akt suggests that the association of Akt with a functional Hsp90-Cdc37 complex is required for Akt stability. 17-AAG did not decrease the level of Akt in the Cdc37 complex for at least nine to twelve h, until Akt became associated with the proteasome. The inference from these results is that binding of the drug to Hsp90 caused instability of the Akt in the complex. We cannot rule out from these data that binding of drug to free Hsp90 prevents its binding to Akt and that the free Akt is unstable with a half-life of 12 h.

Ansamycins have been shown to destabilize a family of Hsp90 client proteins. In some cases, they have been shown to prevent the binding of the target protein (Raf, v-src) to Hsp90 (26, 42). In contrast, in other systems, geldanamycin was shown to arrest maturation of Hsp90-bound Raf and steroid receptors without disrupting the complex (4, 43). We have previously shown in an in vitro model of Hsp90-dependent refolding of denatured luciferase that occupancy of the Hsp90 pocket by drug prevents the ATP-dependent release of Hsp90 and other chaperones from the refolded protein and leads to the ubiquitin-dependent degradation of the latter (18). It is possible that both mechanisms are operative. Binding of ansamycins to Hsp90 in the chaperone complex prevents target protein refolding. High concentrations of ansamycin that saturate free Hsp90 may prevent its binding to unfolded substrates.

The mechanisms through which the Hsp90-bound Akt is targeted to the proteasome after drug treatment is unknown. Degradation was associated with the ubiquitination of Akt. Ubiquitination has been shown to be necessary for ansamycin-induced degradation in in vitro models of protein refolding and for induction of IGF-1 receptor degradation in cells (44). The E3-ubiquitin ligase involved in induction of Akt degradation process has not yet been identified.

The induction of Akt degradation by Hsp90 inhibitors sug-
suggests that its stability is dependent upon a functional Cdc37-Hsp90 complex. The complex may have other functions as well. Sessa and co-workers have shown Akt phosphorylates eNOS and this reaction is enhanced by Hsp90. They show Hsp90 may act as a scaffold facilitating Akt/eNOS interaction (45). A similar Hsp90 scaffold function may exist for Akt and PDK1. The Akt in the Cdc37-Hsp90 complex was active and phosphorylated. The specific activity of Akt in the complex was similar to that of unbound Akt. In contrast, the activity of Raf has been shown to be enhanced by Cdc37 (26). Furthermore,
Inhibition of Akt by ansamycins would both cause the growth arrest of the tumor and sensitize it to agents that cause apoptosis. Akt inhibition leads to loss of D-cyclin expression, which associated with sensitization of tumor cells to taxanes, and to radiation in tissue culture and animal models (24, 62). Clinical trials of T-4AAG are now in progress to test whether inhibition of Akt expression can be accomplished in patients.

The G1 arrest induced by ansamycins occurs only in cells that express wild type RB and is associated with down-regulation of cyclin Dcdk4 kinase activity. Cd4k is a direct target of ansamycins, whereas D-cyclin expression is dependent on PDK1 activation of Akt, two other direct targets (5, 23). It is of interest that Akt and cd4k both associate with a Cdc37-Hsp90 complex. Furthermore, in transgenic mice Cdc37 collaborates with both cyclin D1 and Myc to enhance tumorigenesis (63). Cdc37-Hsp90 may act to integrate pathways required for progression through early G1 phase, and its dysregulation may play a role in tumorigenesis.

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