Published in final edited form as:

Exp Cell Res. 2007 November 1; 313(18): 3851–3858.

Akt shows variable sensitivity to an Hsp90 inhibitor depending on cell context

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Abstract

Hsp90 inhibitors are currently in clinical trials for cancer therapy based on their ability to promote proteasomal degradation of oncogenic protein kinases and nuclear receptors. Results from recent studies suggest that cancer cells are more sensitive to these inhibitors than cells from healthy tissues. We analyzed an immortalized cell line Ba/F3, for sensitivity to the Hsp90 inhibitor geldanamycin in the absence and presence of the oncogenic tyrosine fusion kinase NPM-ALK expressed from a retroviral vector. Our results showed that NPM-ALK expression makes Akt and Cdk4 more resistant to degradation in the presence of geldanamycin, and there was a slightly reduced amount of apoptosis. The mechanism underlying the effect of NPM-ALK on Akt stability was probed by comparison of the turnover of the kinase after translation inhibition and geldanamycin treatment. We observed that Akt was degraded more rapidly in the presence of GA than upon translation inhibition without NPM-ALK expression. This suggests that NPM-ALK protects the mature kinase. Furthermore, Akt failed to bind to the Cdc37 chaperone in cells expressing NPMALK, which also correlates with increased Akt stability.

Keywords

Hsp90; Akt; geldanamycin; chaperones

Introduction

Molecular chaperones promote protein folding and assembly inside cells. Hsp90 is a molecular chaperone that functions in the folding of many proteins involved in signal transduction, including nuclear receptors and protein kinases. Protein kinases fold in association with Hsp90 and several co-chaperones including Cdc37, which is thought to have some specificity for protein kinases over other Hsp90 clients. The relationship between Hsp90 and its kinase clients

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has been exploited recently for chemotherapeutic purposes. This is due to the rapid degradation of client protein kinases resulting from administration of Hsp90 inhibitors to cells. These inhibitors, including benzoquinoid ansamycins such as geldanamycin, inhibit Hsp90's ATPase activity which is essential for its chaperone function. Synthetic derivatives of geldanamycin (GA), such as 17-AAG, are in clinical trials for various types of cancer based on their ability to arrest cell growth by stimulating degradation of protein kinases important for growth and cell division [1-3].

Among the protein kinase clients of Hsp90 that have the most important clinical relevance are those that drive cell growth in their mutant or overexpressed form. These include several oncogenic kinases including ErbB2 (Her2/Neu), BCR-ABL, Flt3 and NPM-ALK [4]. Transcription factors that are targets of Hsp90 inhibitors include androgen receptors and estrogen receptors. In each case, treatment with GA or 17-AAG results in loss of chaperone function that leads to ubiquitination and degradation by the proteasome [5]. The ubiquitin ligase called Chip is thought to play a role in this process since it stimulates degradation of Hsp90 client proteins in the presence of GA [6-9]. However, GA can still promote degradation of a client kinase, ErbB2, even in Chip $-/-$ fibroblasts, albeit with reduced kinetics [6]. This suggests that Chip may function in ubiquitination of misfolded Hsp90 clients in association with another E3 ubiquitin ligase whose identity is unknown.

Recent studies have shown that degradation of Hsp90 client kinases in the presence of GA occurs by two distinct methods involving nascent kinase molecules and mature proteins that have already folded. For example, both ErbB2 and EGFR receptor (ErbB1) are susceptible to degradation in the presence of GA in their nascent chain forms. However, once folded, only ErbB2 remains susceptible while mature EGFR receptor is relatively insensitive to drug treatment [10]. The sequence motifs that mediate this differential sensitivity reside on a loop in the N-lobe of the kinase catalytic domain [11]. This loop, between the α -C helix and β 4 sheet has a glycine in ErbB2 that appears to promote binding of Hsp90 and Cdc37 and leads to enhanced GA sensitivity. Mutation of this glycine to aspartate (which is present in the same position in ErbB1) reduces chaperone binding and drug sensitivity. What is unclear is how many different kinases are sensitive to GA in both their mature and nascent chain forms. Analysis of 105 protein kinases showed that no sequence motifs positively correlate with sensitivity to GA [12], suggesting that the α -C β 4 loop structure that renders ErbB2 sensitive to drug treatment may not be a general phenomenon. In other studies, cancer cells were found to be more sensitive to GA than cells from healthy tissues [13,14]. Specifically, Hsp90 from cancer cells had a higher affinity for both ATP and GA. This was correlated with accumulation of Hsp90 in multi-chaperone complexes, perhaps driven by the large amounts of oncogenic client kinases. Conversely, recent studies showed that even purified Hsp90 was capable of adopting a high affinity conformation for both nucleotide and GA, illustrating the complexity of chaperone function in cancer and non-cancer cells [15]. In the current study, we began by analyzing how oncogenic kinase expression affected the sensitivity of other kinases, such as Cdk4 and Akt, to GA treatment.

Materials and Methods

Chemicals

Geldanamycin was purchased from Invivogen (San Diego, CA) and dissolved in 100% DMSO. The PI3 kinase inhibitor LY294002 and cycloheximide were obtained from Sigma-Aldrich (St. Louis, MO) and dissolved in DMSO and water respectively. Calyculin A, a phosphatase inhibitor was purchased from Cell Signaling.

Cell culture

Murine hematopoietic Ba/F3 cells were maintained in RPMI medium supplemented with 10% heat-inactivated fetal calf serum (FCS) (Gibco-BRL, Carlsbad, CA) and 1ng/ml mouse recombinant IL-3 (R&D Systems, Minneapolis, MN). Ba/F3 cells stably transfected with the MSCV retroviral vector [16] were cultured in the previously described medium with the addition of 1mg/ml G418 (Gibco). The SR-786 cell line was cultured in RPMI with 10% FCS. All the cell lines were incubated at 37°C in 5% CO₂ and were passaged when they reached a density of approximately 0.5 to 1 × 10⁶/ml. 24 hours before treatments the cells were transferred in medium without antibiotics. For the experiments shown in Fig. 3, the phosphatase inhibitor Calyculin A was added to a final concentration of 50 nM 30 minutes prior to cell harvesting. For the isolation of bone marrow cells, 2 healthy BALB/c mice were sacrificed by CO₂ asphyxiation followed by cervical dislocation. Bone marrow cells were isolated by flushing femurs and tibias with ice-cold PBS and cultured in RPMI with 10% FCS.

Viability and growth curve analysis

Cell viability was assayed by the trypan blue exclusion method. Growth curves after geldanamycin or LY294002 treatments were conducted using the CellTiter-Glo® Luminescent Assay of Promega according to the manufacturer's instructions.

Western blotting and antibodies

For each sample, 10⁶ cells were collected by centrifugation (1000rpm for 5min), washed once with ice-cold PBS and lysed in 100µl of lysis buffer containing 2% SDS, 20 mM HEPES [pH 7.5], 0.12 M NaCl, 1 mM EDTA, 10% glycerol, 2.5 mM glycerophosphate, 1mM phenylmethylsulfonyl fluoride, 10mM NaF and protease (Complete Mini, Roche Diagnostics, Indianapolis, IN) and phosphatase inhibitors (Phosphatase Inhibitors Cocktail 2, Sigma). Protein concentration was determined using the BCA reagent (Pierce, Rockford, IL). Samples of 20µg were analyzed in 10% SDS-polyacrylamide gels, transferred to PVDF membranes (Immobilon-P, Millipore, Bedford, MA) and blocked for 1h at room temperature with 5% nonfat dry milk in TBS buffer (20mM Tris HCl [pH 7.5], 0.5M NaCl). Incubation with the primary antibodies (usually diluted 1:1000 in antibody dilution buffer; 1× phosphate-buffered saline, 3% bovine serum albumin, 0.05% Tween-20 and 0.1% thimerosal) was done at room temperature for 2h or overnight at 4°C. After three washes with TBS supplemented with 0.05% Tween-20 the membranes were incubated with the appropriate secondary antibody (horseradish peroxidase-conjugated goat anti-mouse or anti-rabbit IgG, diluted 1:10,000 in antibody dilution buffer) for 2h at room temperature. After three more washes the blots were treated with the enhanced chemiluminescence reagent (Pierce) and exposed to x-ray film (Kodak) for detection. In addition, Western blots were quantified using a Licor Odyssey Infrared imaging system. Antibodies used were: Akt, Akt-1, Cdk4 (Cell Signaling, Beverly, MA), Cdc37, Hsp90 (SPA-830), Hsp70 (SPA-822) (Stressgen, Victoria, Canada). Secondary antibodies for use with the Licor system were IRDye 800CW conjugated goat anti-rabbit and IRDye 680 conjugated goat anti-mouse.

Co-immunoprecipitations

10⁷ cells treated with DMSO or geldanamycin were lysed in 600µl of Nonidet P-40 lysis buffer (0.1% NP-40, 20mM HEPES [pH 7.5], 0.12M NaCl, 1mM EDTA, 2.5mM glycerophosphate, 10mM NaF, 1mM sodium vanadate, 1mM phenylmethylsulfonyl fluoride and protease inhibitors). Cell lysates were cleared by centrifugation at 4°C for 15 min and 1µl of the extract was used for protein quantification by the Bradford assay. 500ug of the lysate in a total volume of 300µl were incubated with the appropriate antibody for 2h at 4°C and then 30µl of protein A/G PLUS agarose beads (Santa Cruz Biotechnology, Santa Cruz, CA) were added and further incubated for 30min. The resin was collected by low-speed centrifugation and washed 3 times

with the IP-lysis buffer. Proteins retained by the resin were solubilized in 25 μ l SDS sample buffer and the samples were resolved by denaturing SDS-PAGE as described above. Akt-1 (Cell Signaling) and Cdk4 Ab-1 (DCS-35) (Lab Vision Corporation, Fremont, CA) were used for immunoprecipitation.

Results

Ba/F3 is a pro-B cell line that is immortal but depends on the cytokine IL-3 for growth [17, 18]. For our studies, we utilized a retroviral infection system to generate stable cell lines expressing the oncogene NPM-ALK, which is a fusion kinase commonly found in anaplastic large cell lymphoma [19]. We treated the resulting cell lines with GA at different concentrations over a six-hour period and found that Akt and Cdk4 kinases began to disappear at concentrations above 50 nM GA in all three cell lines, including those with just the MSCV retroviral vector (Fig. 1A and B). Besides stimulating client kinase degradation, GA also stimulates induction of Hsp70 and other chaperones whose expression is regulated by heat shock factor [20]. In the parent Ba/F3 cell line, Hsp70 is induced at levels of GA that are comparable with those that stimulate client kinase degradation. However, in cells containing the retroviral vector, with or without the NPM-ALK oncogene, there was a marked reduction in Hsp70 induction after six hours (Fig. 1C). However, this represented a delay only, since robust Hsp70 induction was observed after 24 hours treatment (data not shown). These findings were compared with freshly prepared mouse primary bone marrow cells and with SR-786, an ALK-positive NPM-ALK expressing cancer cell line derived from a human patient with anaplastic large cell lymphoma [21]. The primary bone marrow cells were largely insensitive to GA treatment and we observed no degradation of Akt or induction of Hsp70 over a six hour period, even at 400 nM GA (Fig. 1E). By contrast, the SR-786 cancer cell line exhibited marked induction of Hsp70 and degradation of Cdk4. Akt was slightly more resistant to GA treatment although we did observe its disappearance at 400 nM of the drug (Fig. 1F).

Further studies addressed whether prolonged GA treatment affected client kinase disappearance in the Ba/F3 cell line with or without NPM-ALK expression. Using a 24-hour time period of treatment, we observed that Cdk4 and Akt were largely absent from the Ba/F3 cells alone or with the MSCV control vector at 100 nM GA or higher concentrations (Fig. 2A). When NPM-ALK was expressed, both Akt and Cdk4 were relatively resistant to degradation at 100nM GA with approximately 50% and 40% remaining respectively (Fig. 2B). Even at 200nM GA there existed residual Akt in the cells expressing NPM-ALK (Fig. 2A). In a time course experiment, we tested whether Akt was degraded at the same rate in the three cell lines. As expected, we observed that Akt was degraded at a reduced rate in the cells that expressed NPM-ALK. Furthermore, a similar rate effect for all three cell lines was observed for active Akt, although it disappears more quickly than the total Akt protein (analysed with anti-phospho Ser473; Fig. 3). Analysis of PARP cleavage as a measure of apoptosis revealed a reduced amount in cells expressing NPM-ALK at 100nM GA up to 24 hours (Fig. 3C). Cells expressing NPM-ALK exposed to higher concentrations of GA did have cleaved PARP in a similar amount to the cells without NPM-ALK (data not shown). These combined data suggest that Akt is no more active in cells expressing NPM-ALK, but it has increased stability in the presence of GA, and the cells display a reduced level of apoptosis.

Next, we addressed the functional consequences of having GA-resistant Akt present in Ba/F3 cells expressing NPM-ALK. Cell viability measurements revealed that cells were indeed more resistant to GA treatment, however, this was so for the cells containing just the MSCV vector. At 100 nM of GA the parent Ba/F3 cell line was reduced to 20% viability at 24 hours, whereas cells with MSCV were over 60% viable under the same conditions. Cell growth assays were consistent with this conclusion, since Ba/F3 cells were growth-inhibited to a greater extent than cells containing MSCV (Fig. 4A and 4B). To determine whether Akt itself was a

contributing factor to this change in cell growth/viability in the presence of GA, we performed growth assays in the presence of the PI3 kinase inhibitor LY294002. As shown in Fig. 4C, all cells were equally sensitive to this drug independently of having MSCV integrated or NPM-ALK expressed. When both drugs are combined, the cells have greatly reduced viability but all die at a similar rate. Therefore, having increased amount of Akt appears to reduce the amount of apoptotic cell death, but overall viability is unchanged. The role of MSCV integration in making the cells more viable in the presence of GA remains unclear, although we did observe a slightly enhanced amount of total glutathione (data not shown). This may account for resistance of the MSCV cells to geldanamycin based on a previous study which showed a correlation between increased glutathione levels and resistance of cells to Hsp90 inhibitors [22].

We next investigated the mechanism underlying how Akt might change to become more GA resistant. These studies compared how GA treatment affected kinase levels relative to the effects of cycloheximide, the translation inhibitor. The rationale for this approach was to distinguish between the effects of GA on newly translated kinases versus those on folded molecules. If GA treatment had a similar effect on kinase levels as does cycloheximide, then it is likely that only newly synthesized kinase molecules are affected by the drug. As shown in Fig. 5A, there is a marked and rapid reduction in Akt levels after 2 hours treatment with GA in the Ba/F3 parent cells. In the presence of cycloheximide there is a relatively reduced rate of degradation, indicating that GA promotes rapid degradation of the mature kinase. Interestingly, cycloheximide antagonizes GA-induced degradation (especially at the 2 hour time point). Such differences between the rate of Akt degradation in cycloheximide versus GA treated cells were not as marked for Ba/F3 cells containing MSCV or expressing NPM-ALK. Indeed, in the NPM-ALK expressing cells there is very little difference suggesting that all Akt degradation in the presence of GA is that of the nascent chain. Similar results were recorded for Cdk4, where a marked reduction in the rate of its degradation was observed in cells expressing NPM-ALK compared to the MSCV control.

Previous studies have shown that Hsp90 and Cdc37 can interact with Akt even after folding [23]. However, it seems unclear from the studies described above, whether this population is degraded in the presence of GA. We investigated by immunoprecipitation whether NPM-ALK expression affected binding of either chaperone to mature Akt or Cdk4 (Fig. 6). Cdc37 was markedly absent on Akt from cells expressing NPM-ALK, whereas Cdk4 had similar amounts of Cdc37 from both cell lines. Interestingly, Cdc37 migrates as two bands on the SDS-PAGE [24], and both are represented at an apparent 1:1 ratio in the Akt immunoprecipitates. By contrast, only the more slowly migrating form of Cdc37 co-immunoprecipitates with Cdk4. The difference between these two forms of Cdc37 remains to be established. GA treatment caused Cdc37 dissociation from both kinases in all cell lines. However, Hsp90 remained associated with each kinase after treatment.

Discussion

Cancer cells in general have been shown to have a higher sensitivity to the hsp90 inhibitor GA, compared with normal tissue [13]. Although the underlying basis for this is unclear, it has been proposed that oncogenic protein kinase expression may affect drug sensitivity toward cancer cells [14]. Thus, primary cells are relatively insensitive while cancer cells are very sensitive and can be killed by GA at an IC_{50} of less than 50 nM. In our studies, we noted that Ba/F3 cells, which are immortal but not transformed, also had high sensitivity to GA, with an IC_{50} that was less than 50 nM (Fig. 2 and data not shown). In addition we observed rapid degradation of Akt and Cdk4 kinases. By contrast, primary bone marrow cells were insensitive to GA and Akt levels were unchanged after drug treatment. Expression of the oncogenic tyrosine kinase NPM-ALK led to reduced sensitivity of Akt that was evident at 24 hours after geldanamycin

treatment (Fig. 2). Comparison of phospho-Ser473 Akt in cells with and without NPM-ALK expression revealed no significant changes in Akt activity among the cell lines, suggesting that activity per se is not responsible for changes in Akt stability. Note that NPM-ALK expression is associated with increased Akt activity via a direct activation of PI3 kinase [25,26], although IL-3 was always included in our experiments which itself activates Akt [27].

We noted that Akt was especially sensitive to degradation in Ba/F3 cells in the presence of geldanamycin when compared to the translation inhibitor, cycloheximide, after 2-hours treatment. This also occurred in Ba/F3 cells that have MSCV integrated although to a lesser extent, whereas no difference in Akt decay was observed when NPM-ALK was expressed (Fig. 5). Similarly, NPM-ALK expression also stabilized Cdk4 when cells were exposed to geldanamycin. The sensitivity of Akt and Cdk4 to geldanamycin in the Ba/F3 cells was completely inhibited at early time points by co-incubation with cycloheximide. The reason for this is unknown but could point to a relationship between geldanamycin-dependent degradation and continued translation.

Ba/F3 cells expressing NPM-ALK exhibited reduced degradation of Akt at early time points (Fig. 5C) compared to the parent cell line. We propose that this decrease reflects increased stability of the mature form of Akt, while the nascent chain is still susceptible to degradation. This is because Akt was degraded at a similar rate in the presence of geldanamycin or cycloheximide in these cells. The hypothesis that mature Akt is more stable in cells expressing NPM-ALK is supported by our finding that Cdc37 failed to bind to Akt in these cells (Fig. 6). Since Cdc37 bound to Cdk4 in the same cells, these data suggest that NPM-ALK is having a specific effect on Akt. This conclusion is based on the notion that Cdc37 only binds to partially unfolded kinase molecules. However, we note that previous studies have observed enzymatically active preparations of Akt to contain Cdc37 [23]. Therefore it is also possible that NPM-ALK affects expression of an Akt binding protein that displaces Cdc37.

We tested whether NPM-ALK affected cell growth and apoptotic pathways in Ba/F3 cells exposed to geldanamycin. We observed decreased levels of apoptosis in cells expressing NPM-ALK up to 24 hours after 100 nM geldanamycin treatment (Figure 3), although higher concentrations of the drug did promote apoptotic PARP cleavage (not shown). However, we observed a strong effect of the MSCV vector alone on cell viability in the presence of geldanamycin (Fig. 4), making it difficult to address the specificity of NPM-ALK expression. Nevertheless, inhibition of PI3 kinase with LY294002 abolished this differential effect of MSCV integration, suggesting that the vector effects are not mediated via Akt. We also noted synergy between geldanamycin and LY294002 on cell viability independently of NPM-ALK expression. Similar findings for this synergy have been reported previously [28].

Our findings are consistent with oncogenic expression resulting in increased robustness of cell survival pathways and that these decrease sensitivity of cells to Hsp90 inhibitors. On the other hand, our findings support the hypothesis that individual kinase sensitivity can be modulated by oncogene expression. This is consistent with recent findings suggesting that Zap70 and PDGF receptor show variable sensitivity to the drug depending on cell context [29-31].

Acknowledgements

This work was supported by a grant from the NIH (GM 70596).

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Figure 1. Degradation of kinases and induction of Hsp70 after 6 hours treatment with different concentrations of GA

After 6 hours of treatment with the indicated concentrations of GA, 20 μ g of total protein from each cell line were fractionated by SDS-PAGE and the blots were analyzed with antibodies specific for Akt-1 (A), Cdk4 (B), Hsp70 (C) and GAPDH (D). (E) Freshly isolated primary bone marrow cells were cultured in presence of GA and 20 μ g of total protein extract were used in Western analysis for Akt-1 and Hsp70. (F) 20 μ g of total protein extract from a human cancer cell line (SR-786) treated with GA analyzed for Akt-1, Cdk4, Hsp70 and GAPDH.

Figure 2. Effects of prolonged exposure to GA

The three cell lines, Ba/F3, MSCV and NPM-ALK were treated with different concentrations of GA for 24 hours (A). 20 μ g of total protein from each cell line were fractionated by SDS-PAGE and probed for Akt, Cdk4 and GAPDH. (B) Akt and Cdk4 are more resistant to geldanamycin in cells expressing NPM-ALK. The bars show the remaining amounts of Akt and Cdk4 after treatment with 100nM GA for 24 hours in the three cell lines, in 5 and 4 independent experiments respectively. Error bars represent SE.

Figure 3. Time course analysis of treatment with 100nM GA

Ba/F3, MSCV and NPM-ALK expressing cell lines were treated with 100nM GA for 0, 6, 12 and 24 hours. 30min before harvesting the cells were treated with the phosphatase inhibitor calyculin A. 20µg of total protein were used in SDS-PAGE analysis to detect Akt, pAkt (Ser473) and PARP cleavage (arrowed).

Figure 4. Cell viability and growth analysis

One million cells from Ba/F3, MSCV and NPM-ALK expressing cell lines were treated with 100nM of GA for 0, 12 and 24 hours. (A) Cell viability assay (trypan blue exclusion) for Ba/F3 (white bars), MSCV (grey bars) and NPM-ALK expressing cells (black bars). (B) Growth rate of Ba/F3 (diamond), MSCV (open triangle) and NPM-ALK (filled circle) expressing cell lines with DMSO (black lines) or 100nM GA (dashed lines). (C) Cells were treated for up to 24 hours with 20 μ M of the PI3 kinase inhibitor LY294002 (LY) or the combination of 100nM GA and 20 μ M LY294002 (LY+GA). The bars correspond to Ba/F3 (white bars), MSCV (grey bars) and NPM-ALK (black bars) and represent the mean from three independent experiments. Thin bars indicate the SD.

Figure 5. Akt and Cdk4 half-life in Ba/F3 cells with and without NPM-ALK expression

One million cells from Ba/F3 (A), MSCV (B) and NPM-ALK (C) cell lines were subjected to 50µg/ml cycloheximide (CHX) for 30min and then were incubated with or without 100nM GA for 2, 4 and 6 hours. The blots were probed for Akt, Cdk4 and Hsp70. The bands for Akt and Cdk4 were quantified and normalized at 0 time point. The bars represent the average of three different experiments and correspond to CHX treatment (white bars), CHX and GA (grey bars) and GA alone (black bars). Error bars indicate the SE.

Figure 6. Differential binding of Cdc37 to Akt

500μg of total protein extract were used for immunoprecipitation of Akt-1 (A) and Cdk4 (B) from the Ba/F3 cell line (lanes 1, 4), the MSCV cell line (lanes 2, 5) and the NPM-ALK (NA) cell line (lanes 3, 6) after incubation with DMSO (lanes 1, 2, 3) or GA (lanes 4, 5, 6) for 24h. Western blot analysis performed for Akt, Hsp90 and Cdc37. Lane 7, represents 20μg whole cell lysate.