Cell PRESS
Cancer Cell
Article

Dual Targeting of HSC70 and HSP72 Inhibits HSP90 Function and Induces Tumor-Specific Apoptosis

Marissa V. Powers,[1] Paul A. Clarke,[1] and Paul Workman[1,*]
[1]Signal Transduction and Molecular Pharmacology Team, Cancer Research UK Centre for Cancer Therapeutics, The Institute of Cancer Research, Haddow Laboratories, Sutton, Surrey SM2 5NG, UK
*Correspondence: paul.workman@icr.ac.uk
DOI 10.1016/j.ccr.2008.08.002

SUMMARY

Heat-shock protein 70 (HSP70) isoforms contribute to tumorigenesis through their well-documented anti-apoptotic activity and via their role as cochaperones for the HSP90 molecular chaperone. HSP70 expression is induced following treatment with HSP90 inhibitors, which may attenuate the cell death effects of this class of inhibitor. Here we show that silencing either heat-shock cognate 70 (HSC70) or HSP72 expression in human cancer cell lines has no effect on HSP90 activity or cell proliferation. However, simultaneously reducing the expression of both of these isoforms induces proteasome-dependent degradation of HSP90 client proteins, G1 cell-cycle arrest, and extensive tumor-specific apoptosis. Importantly, simultaneous silencing of HSP70 isoforms in nontumorigenic cell lines does not result in comparable growth arrest or induction of apoptosis, indicating a potential therapeutic window.

INTRODUCTION

The molecular chaperone heat-shock protein 90 (HSP90) is an exciting cancer drug target because it plays a key role in ensuring the correct conformation, stability, and activity of many well-defined oncogenic client proteins. These include kinases such as CRAF, CDK4, AKT, and ERBB2; sex hormone receptors; and other transcription factors (Whitesell and Lindquist, 2005; Workman, 2004). Natural product HSP90 inhibitors, e.g., the benzoquinone ansamycin antibiotic geldanamycin and its analog 17-allylamino-17-demethoxygeldanamycin (17-AAG, or tanespimycin), inhibit HSP90 ATPase activity, inducing degradation of clients by the ubiquitin-proteasome pathway (Mimnaugh et al., 1996). This causes simultaneous and combinatorial blockade of multiple oncogenic pathways regulating cell growth, differentiation, motility, and death (Workman, 2004; Whitesell and Lindquist, 2005).

Alongside client protein depletion, we have previously demonstrated increased expression of both the major constitutive (heat-shock cognate 70 [HSC70]) and inducible (HSP72) isoforms of the HSP70 multigene family by 17-AAG in vitro (Hostein et al., 2001; Maloney et al., 2007; Banerji et al., 2005a). 17-AAG also induces HSP70 isoforms and depletes HSP90 clients in peripheral blood mononuclear cells and tumors of treated patients (Banerji et al., 2005b; Goetz et al., 2005).

The HSP70 family includes at least eight members with diverse biochemical functions, including nascent protein folding, preventing denatured protein aggregation, and modulating assembly/disassembly of protein complexes (Young et al., 2004; Daugaard et al., 2007). Importantly, the HSP70 proteins also act as cochaperones for HSP90 (Dittmar and Pratt, 1997; Morishima et al., 2000). The two major cytoplasmic isoforms are HSC70 and HSP72. Generally, HSC70 is abundantly and ubiquitously expressed in nontumor tissues, whereas HSP72 is present at relatively low levels in the absence of stress (Daugaard et al., 2007). However, under stress, the expression of inducible HSP72 increases considerably via heat-shock factor 1 (HSF1) transcription factor activation. This differential expression pattern is commonly lost in cancer, with increased, constitutive

SIGNIFICANCE

HSP90 inhibitors are of interest because they simultaneously inhibit multiple oncogenic pathways. However, their capacity to induce cell death is reduced by induction of antiapoptotic HSP70 isoforms. We demonstrate that simultaneously targeting HSC70 and HSP72 phenocopies HSP90 inhibition by inducing the combinatorial degradation of several client oncoproteins. Furthermore, simultaneous silencing of HSC70 and HSP72 inhibits cancer cell proliferation and induces considerable tumor–specific apoptosis to a much greater extent than pharmacologic HSP90 inhibition. This is due to interference with the cochaperone role of HSP70 isoforms in the HSP90 chaperone system and abrogation of their antiapoptotic effects. We propose that simultaneous targeting of HSP70 isoforms may provide an attractive approach to exploit both oncogene addiction and chaperone dependence of cancer cells.

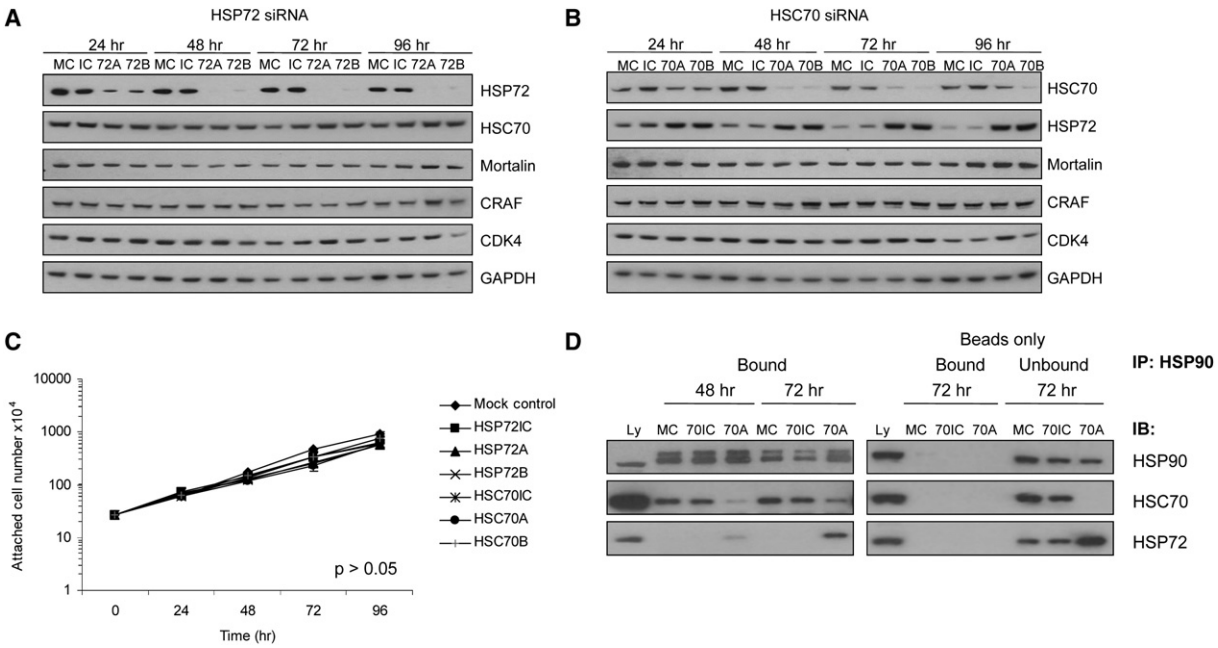

Figure 1. HSP72 or HSC70 Silencing Specifically Alters Expression of HSP70 Isoforms and Their Association with HSP90 but Does Not Affect HCT116 Cancer Cell Proliferation

(A and B) Immunoblots showing HSP70 isoform expression in HCT116 human colon cancer cells following transfection with active siRNAs targeting HSP72 (72A or 72B) (A) or HSC70 (70A or 70B) (B) or inverted control siRNAs (72IC or 70IC). Cells treated with siRNA buffer + transfection lipid (mock control [MC]) were included as an additional control. GAPDH was included as a loading control.

(C) HCT116 cell proliferation following silencing of HSP72 or HSC70. Transfection controls and siRNAs were used as described in (A). n = 3; error bars represent ±SEM. $p > 0.05$.

(D) Immunoprecipitation of HSP90 from HCT116 cells 48 or 72 hr after HSC70 silencing. The proteins shown were detected by immunoblotting. siRNAs were used as described in (A).

expression of HSP72 having been observed in several tumors (reviewed in Garrido et al., 2006).

The exact role of the HSP70 family in cancer remains to be elucidated. However, these proteins can contribute to tumor cell survival via multiple antiapoptotic functions (Mosser and Morimoto, 2004; Garrido et al., 2006). Increased expression of HSP70 family members, particularly HSP72, is also implicated in resistance to current cytotoxics (e.g., Sliutz et al., 1996).

Based on the increased expression of HSP70 isoforms in cancer, their essential role in the substrate-loading phase of the HSP90 molecular chaperone cycle, and their antiapoptotic properties, we hypothesized that their expression may be required to maintain HSP90 activity and support cancer cell survival. Also, through their antiapoptotic roles, we considered that increased HSC70 and HSP72 expression may reduce sensitivity to HSP90 inhibitors. Combinatorial modulation of these two HSP70 isoforms could therefore be doubly advantageous. Here we use a siRNA approach to evaluate the effect of silencing HSP72 and HSC70 in cancer and nontumorigenic cells, individually and simultaneously.

RESULTS

Silencing HSP72 or HSC70 Alters HSP70 Isoform Expression but Does Not Affect Cell Proliferation

Induction of HSP72 attenuates the sensitivity of human leukemic and prostate cancer cell lines to HSP90 inhibition (Guo et al., 2005; Gabai et al., 2005). We have confirmed HSP72 induction and have also identified increased expression of HSC70 following HSP90 inhibition (Maloney et al., 2007). This is of interest, as HSC70, like HSP72, is antiapoptotic (Mosser and Morimoto, 2004; Garrido et al., 2006).

To investigate how induction of HSC70 affects cellular sensitivity to 17-AAG, siRNA oligonucleotides were designed to selectively silence HSC70. In addition, to confirm whether increased sensitivity to HSP90 inhibition following restraint of HSP72 induction could be extended to the cell lines used here, we also designed siRNA oligonucleotides to specifically silence HSP72 expression. HSP72 or HSC70 was selectively silenced for up to 96 hr after transfection in HCT116 human colon carcinoma cells (Figures 1A and 1B). Specificity was shown by the lack of effect of control oligonucleotides that were identical to the active siRNAs apart from inversion of the two central base pairs, which are critical for silencing (Figures 1A and 1B).

Supporting their specificity, HSP72 siRNAs had no effect on HSC70 expression in HCT116 colon (Figure 1A) or A2780 human ovarian cancer cells (see Figure S1A available online). However, silencing HSC70, using two different siRNA oligonucleotides, caused concurrent HSP72 induction in HCT116 (Figure 1B), A2780 (Figure S1B), and U87MG human glioblastoma cells (data not shown). The control siRNA did not silence HSC70 or increase HSP72 expression (Figure 1B), demonstrating that induction of HSP72 was specific to silencing HSC70. Furthermore,

expression of the mitochondrial isoform of HSP70, mortalin, was unaffected following silencing of either HSP72 (Figure 1A) or HSC70 (Figure 1B).

Previous studies have produced conflicting data on the effects of silencing HSP70 isoforms on the growth and survival of tumorigenic cell lines (e.g., Nylandsted et al., 2000, 2002; Gabai et al., 2005). Interestingly, we found that individual silencing of either HSP72 or HSC70 had no significant effect on HCT116 ($p > 0.05$; Figure 1C) or A2780 cell proliferation (data not shown). Control siRNAs also had no effect ($p > 0.05$; Figure 1C).

Thus, we have identified a cellular response that induces HSP72 expression, potentially compensating for the reduced expression of HSC70. However, individual silencing of HSC70 or HSP72 had no effect on cell proliferation.

HSP72 Replaces HSC70 in the HSP90 Chaperone Complex following Silencing of HSC70 Expression

HSP70 isoforms are required for loading client proteins onto HSP90 (Dittmar and Pratt, 1997; Morishima et al., 2000). We therefore determined the effect of silencing HSC70 or HSP72 on HSP90 function, as determined by expression of the clients CRAF and CDK4. Interestingly, we found no effect on the expression of these clients up to 96 hr after individual silencing of HSP72 or HSC70 (Figures 1A and 1B).

To understand the lack of effect of individual HSP70 isoform silencing on HSP90 function, we analyzed HSP72 and HSC70 in the immunoprecipitated HSP90 cochaperone complex. HSC70, but not HSP72, was associated with HSP90 under control conditions (Figure 1D). Importantly, when HSC70 was silenced, not only was HSP72 induced, but this cochaperone replaced HSC70 in the HSP90 complex (Figure 1D).

Based on the lack of effect on HSP90 client protein expression (Figures 1A and 1B) together with the immunoprecipitation data (Figure 1D), we hypothesize that replacement of HSC70 by HSP72 in the HSP90 complex can prevent inhibition of HSP90 chaperone activity when HSC70 is silenced. Overall, these results demonstrate the ability of HSP90 to utilize separately either HSC70 or HSP72 as a likely mechanism to conserve chaperone activity.

Sensitivity to HSP90 Inhibitors Is Dependent on Specific HSP70 Isoforms in Cancer and Nontumorigenic Cells

The role of HSP72 in the sensitivity of human colon and ovarian cell lines to HSP90 inhibition is unknown, as are the effects of HSC70 on 17-AAG sensitivity. We investigated the effects of HSP72 or HSC70 silencing on the proliferation of HCT116 and A2780 cancer cell lines in response to 17-AAG. Consistent with our previous observations (Hostein et al., 2001; Maloney et al., 2007), 24 hr exposure of HCT116 and A2780 cells to 17-AAG decreased cell proliferation (Figures 2A and 2B). As seen in human leukemia and prostate cancer cell lines (Guo et al., 2005; Gabai et al., 2005), silencing of HSP72 expression before treatment significantly increased the effects of 17-AAG in HCT116 ($p < 0.05$; Figure 2A) and A2780 cells ($p < 0.05$; Figure 2B).

Restraining induction of HSP72 in response to 17-AAG may represent an interesting approach to enhancing tumor cell sensitivity to 17-AAG. However, it is necessary to determine the potential for selectivity between tumor and nontumorigenic cells. Therefore, we determined the effect of reducing HSP72 expression prior to 17-AAG in the nontumorigenic prostate epithelial

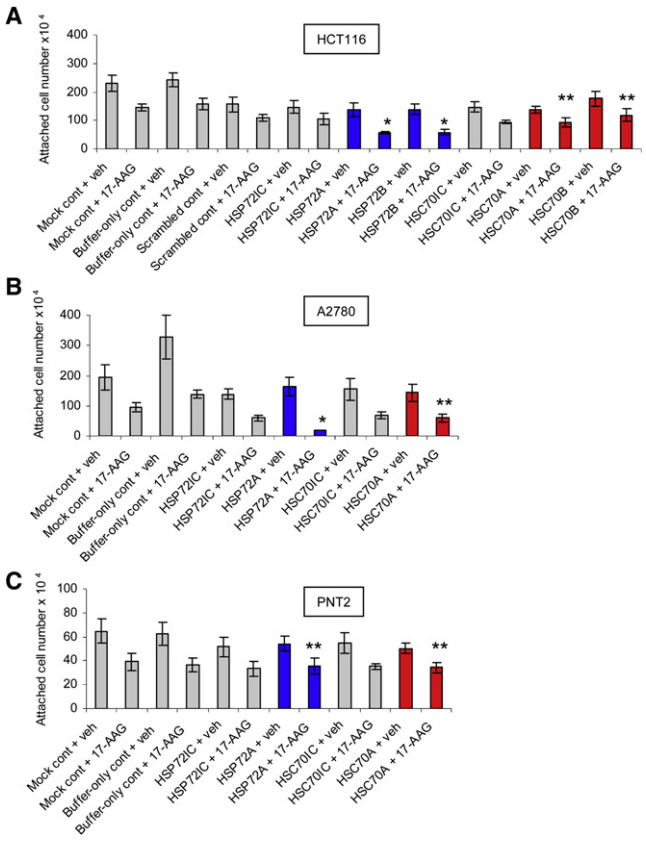

Figure 2. Silencing HSP72 Sensitizes HCT116 and A2780 Cancer Cells, but Not Nontumorigenic PNT2 Cells, to 17-AAG

HCT116 human colon cancer (A), A2780 human ovarian cancer (B), and PNT2 nontumorigenic human prostate epithelial cells (C) were transfected with siRNA against the individual isoforms of HSP70. Twenty-four hours after transfection, cells were exposed to 5 × IC_{50} 17-AAG (HCT116 117 nM, A2780 68 nM, PNT2 490 nM) or the equivalent volume of DMSO drug vehicle (veh) for 24 hr, after which adherent trypan blue-excluding viable cells were counted. Cells were transfected with scrambled control siRNA ([A] only), inverted control siRNA (72IC or 70IC), or active siRNA against HSP72 (72A or 72B, blue bars) or HSC70 (70A or 70B, red bars). Also included were transfection controls, which comprised cells treated with siRNA buffer only and a mock control. n = 3; error bars represent ±SEM. *$p < 0.05$, **$p > 0.05$ versus control siRNA-transfected cells.

cell line PNT2. Consistent with the results for the other cell lines studied here, exposure to 5 × IC_{50} 17-AAG reduced proliferation of PNT2 cells at 24 hr (Figure 2C). Importantly, while PNT2 cell proliferation was inhibited by 17-AAG treatment, the IC_{50} for this cell line was 4- to 7-fold higher than in HCT116 or A2780 cells, consistent with previous observations that nontumorigenic cell lines are generally less sensitive to HSP90 inhibition than tumorigenic cell lines (Kamal et al., 2003). Furthermore, no significant difference in the antiproliferative effect of 17-AAG was observed when HSP72 expression was reduced prior to treatment in the nontumorigenic PNT2 cell line (Figure 2C). These observations support the potential for a therapeutic differential when combining silencing of HSP72 with 17-AAG.

In contrast to HSP72, when HSC70 expression was silenced prior to 17-AAG treatment, there was no significant difference in the response of HCT116 colon cancer, A2780 ovarian cancer,

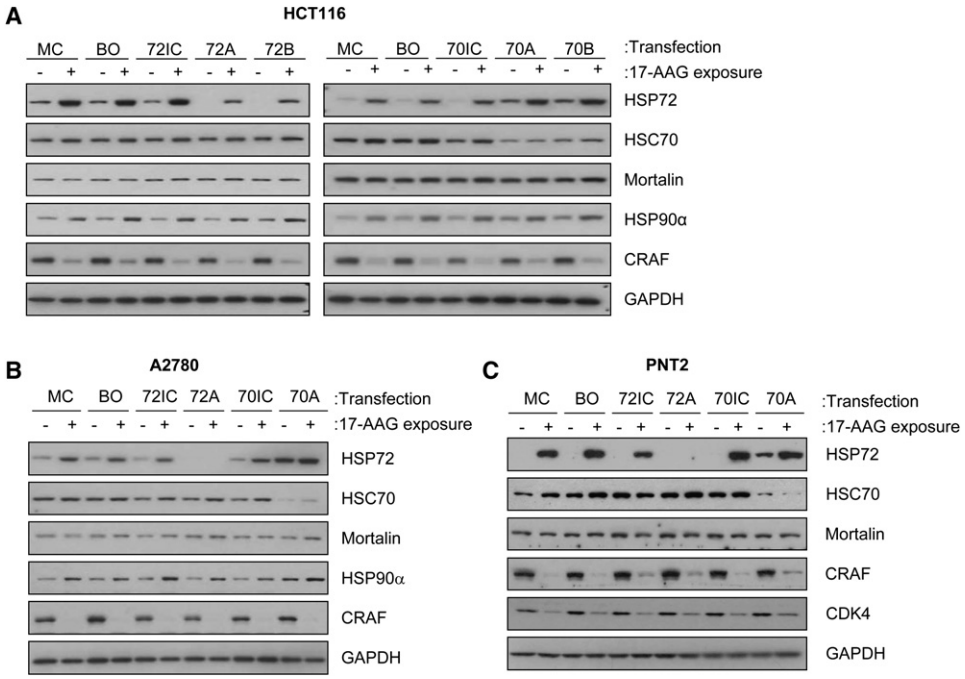

Figure 3. HSC70 or HSP72 Silencing Individually Does Not Affect 17-AAG-Induced Client Depletion in HCT116 and A2780 Cancer or Nontumorigenic PNT2 Cells

Immunoblots are shown. GAPDH was included as a loading control.

(A) HCT116 human colon cancer cells were treated with siRNA buffer + transfection lipid (MC) or siRNA buffer only (BO) or were transfected with control siRNA (72IC or 70IC) or siRNA targeting HSC70 (70A or 70B) or HSP72 (72A or 72B) for 4 hr prior to 24 hr exposure to 5 × IC_{50} 17-AAG (117 nM).

(B) A2780 human ovarian cancer cells following transfection with control or active siRNA as described in (A). Twenty-four hours after transfection, cells were exposed to 5 × IC_{50} 17-AAG (68 nM).

(C) PNT2 nontumorigenic human prostate epithelial cells following transfection with control or active siRNA as described in (A). Twenty-four hours after transfection, cells were exposed to 5 × IC_{50} 17-AAG (490 nM).

or nontumorigenic PNT2 cells to 17-AAG ($p > 0.05$; Figures 2A–2C). These results indicate that although both HSP72 and HSC70 are induced by 17-AAG and both can bind to HSP90, only depletion of HSP72 is able to enhance the sensitivity of tumor cells to this HSP90 inhibitor.

When HSP72 was silenced before 17-AAG treatment, induction of this isoform in HCT116 colon cancer cells was restrained following HSP90 inhibition to levels observed in vehicle-treated control populations (Figure 3A). In A2780 ovarian cancer (Figure 3B) and PNT2 nontumorigenic cells (Figure 3C), HSP72 induction by 17-AAG was undetectable by immunoblotting following HSP72 siRNA.

In all cell lines studied, silencing of HSC70 increased HSP72 expression prior to 17-AAG treatment. The extent of HSP72 induction after HSC70 silencing alone was similar to that observed in the corresponding siRNA controls treated with 17-AAG (Figures 3A–3C). Therefore, before treatment with 17-AAG, cells with reduced HSC70 expression had increased levels of antiapoptotic HSP72, which reduces the sensitivity of various tumor cell types to HSP90 inhibition (Figures 2A and 2B; Gabai et al., 2005; Guo et al., 2005). This observation explains the lack of increased cellular sensitivity to 17-AAG following HSC70 silencing in all cell lines studied (Figures 2A–2C).

In addition to client protein depletion and HSP70 induction, the molecular signature of HSP90 inhibition in A2780 ovarian cancer cells includes the induction of HSP90α, the inducible isoform (Maloney et al., 2007). The expected molecular changes, i.e., induction of HSP72 and HSP90α and depletion of the CRAF client, were seen following 17-AAG treatment in HCT116 and A2780 cancer cells (Figures 3A and 3B). The extent of client depletion and HSP90α induction by 17-AAG in A2780 and HCT116 cells following HSP72 silencing was similar to that in the corresponding controls treated with 17-AAG (Figures 3A and 3B), irrespective of an increase in antiproliferative effects being observed in these cells following HSP72 silencing and 17-AAG treatment (Figures 2A and 2B). We also found no difference in the level of client depletion by 17-AAG between HCT116 cells that had undergone the different transfections up to 72 hr after the initial treatment with 17-AAG (Figure S2). As there was no change in extent of HSP90 inhibition, we conclude that the increase in antiproliferative effects observed following HSP72 silencing and 17-AAG treatment was due to decreased HSP72 induction.

Importantly, an overall molecular profile similar to that observed in HCT116 and A2780 cancer cells in response to HSP72 silencing and 17-AAG treatment was also observed in PNT2 cells (Figure 3C), despite the lack of increased sensitivity to 17-AAG in response to HSP72 silencing in this nontumorigenic cell line (Figure 2C).

In all cell lines studied, we also found that silencing HSC70 prior to 17-AAG treatment had no effect on the molecular marker changes commonly associated with pharmacologic HSP90 inhibition (Figures 3A–3C).

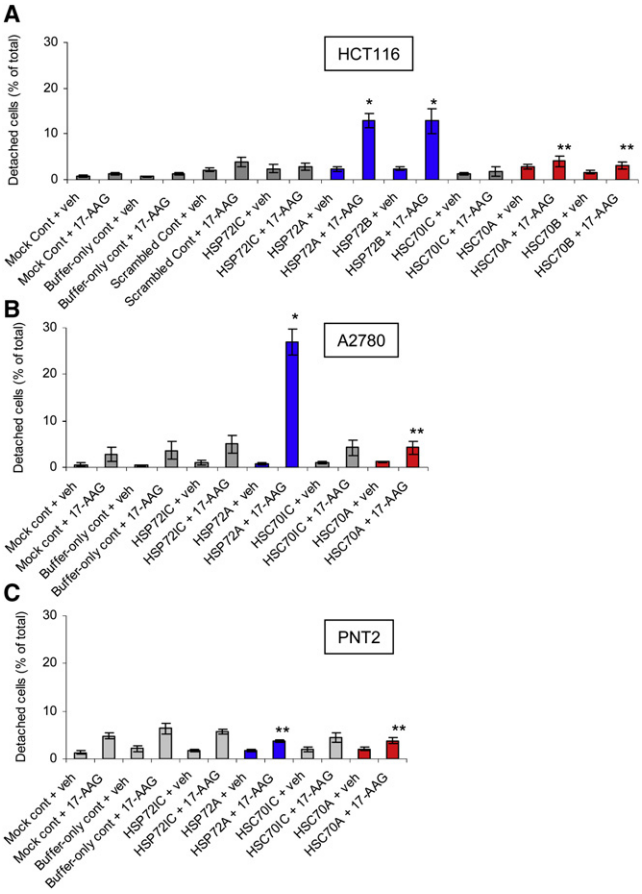

Figure 4. Silencing of HSP72 Increases Apoptosis in Response to 17-AAG in Tumor but Not Nontumorigenic Cells

HCT116 human colon cancer (A), A2780 human ovarian cancer (B), and nontumorigenic human PNT2 prostate epithelial cells (C) were transfected with siRNA against the individual isoforms of HSP70 for 4 hr. Twenty-four hours after transfection, cells were exposed to 5 × IC_{50} 17-AAG (HCT116 117 nM, A2780 68 nM, PNT2 490 nM) or the equivalent volume of DMSO drug vehicle (veh) for 24 hr, after which cell death was quantified. Transfection controls included cells treated with siRNA buffer only or with siRNA buffer + transfection lipid (mock control) or cells transfected with scrambled control siRNA ([A] only). Cells were transfected with inverted control siRNA (72IC or 70IC) or active siRNA against HSP72 (72A or 72B, blue bars) or HSC70 (70A or 70B, red bars). n = 3; error bars represent ±SEM. *p < 0.05, **p > 0.05 versus control siRNA-transfected cells.

Overall, the data in this section support the hypothesis that reducing HSP72 but not HSC70 expression can sensitize tumor cells, but not nontumorigenic cells, to 17-AAG by a mechanism that does not involve direct alteration of HSP90 chaperone function as measured by client protein expression.

Extent of Apoptosis Induced in Response to 17-AAG Is Dependent on Specific HSP70 Isoforms

We have previously demonstrated that detached HCT116 and A2780 cancer cells are apoptotic according to morphology, sub-G1 DNA content, and PARP cleavage (Hostein et al., 2001; Maloney et al., 2007). Little increase in apoptotic detached cells was observed in siRNA-transfected HCT116 and A2780 controls following 24 hr exposure to 17-AAG (Figures 4A and 4B). In addition, we saw no significant increase in apoptosis when HSC70 expression was silenced prior to 17-AAG treatment in any of the cell lines studied (p > 0.05; Figures 4A–4C). However, when HSP72 was silenced in HCT116 or A2780 cancer cells prior to 17-AAG treatment, a respective 5-fold (±1.1 SEM) and 6-fold (±0.3 SEM) increase in apoptosis was observed (p < 0.05; Figures 4A and 4B), supporting results in human leukemia and prostate cancer cell lines (Guo et al., 2005; Gabai et al., 2005). In contrast, we observed no significant increase in apoptosis in the nontumorigenic PNT2 epithelial cell line when HSP72 expression was silenced prior to 17-AAG treatment (p > 0.05; Figure 4C), reinforcing greater chaperone dependence in cancer cells.

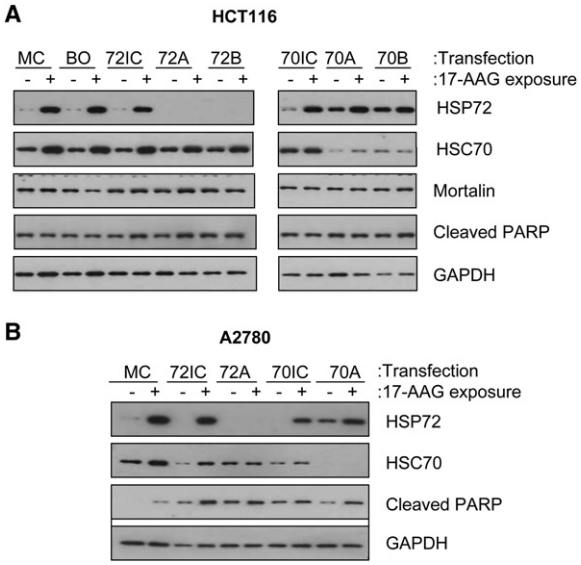

Figure 5. siRNA Completely Restrains HSP72 Induction in Response to 17-AAG Treatment in Detached, Apoptotic Cancer Cells

Immunoblots of HCT116 human colon cancer (A) and A2780 human ovarian cancer cells (B) following treatment with siRNA buffer + transfection lipid (MC) or siRNA buffer only (BO) or transfection with control siRNA (72IC or 70IC) or siRNA targeting HSC70 (70A or 70B) or HSP72 (72A or 72B) for 4 hr prior to 24 hr exposure to 5 × IC_{50} 17-AAG (HCT116 117 nM, A2780 68 nM). GAPDH was included as a loading control.

Silencing of HSP72 prior to 17-AAG prevented the induction of HSP72 in response to 17-AAG in detached HCT116 and A2780 cells (Figures 5A and 5B). Consistent with earlier observations (Figure 1; Figure 3), increased HSP72 expression following HSC70 silencing was also apparent prior to 17-AAG exposure in detached HCT116 and A2780 cells (Figure 5), supporting the hypothesis that the induction of this antiapoptotic protein attenuates apoptosis due to 17-AAG when HSC70 expression is silenced.

Detection of cleaved PARP, an apoptotic marker, further confirmed that detached HCT116 and A2780 cancer cells were apoptotic (Figures 5A and 5B). This qualitative method was complimentary to the quantitative assay, which demonstrated a significant increase in HCT116 and A2780 cancer cell death following silencing of HSP72 expression prior to 17-AAG treatment (Figures 4A and 4B). Cleaved PARP was also detectable in controls when equal amounts of protein from the detached population were analyzed (Figure 5). This is representative of the low background level of cell death for these cell lines, which was

considerably less than that seen in the relevant treated groups (Figures 4A and 4B).

Collectively, these data extend previous observations indicating increased apoptosis following combinatorial HSP72 silencing and HSP90 inhibition and in particular demonstrate a response that is related to the tumorigenic status of the cell.

Simultaneous Silencing of HSC70 and HSP72 Reduces Cell Proliferation, Depletes HSP90 Clients, and Enhances 17-AAG-Induced Apoptosis

To prevent induction of HSP72 following HSC70 siRNA, a simultaneous transfection targeting both HSC70 and HSP72 was performed in HCT116 cancer cells. In addition to the combination of two active siRNAs and the specific transfection controls described earlier, several combinations of one functional and one control siRNA were included to ensure that the use of two independent siRNA sequences was not inducing a nonspecific response. The molecular changes associated with the single siRNA transfections (Figure 3; Figure 5) were conserved when incorporated within the combinatorial transfection with a control siRNA (Figure 6A). These included siRNA-mediated restraint of HSP72 induction following treatment with 17-AAG (Figure 6A). This is important because it was necessary to decrease the concentration of each siRNA used in the dual transfection to keep the total siRNA concentration constant in both single and combinatorial transfections.

Combined silencing of HSP72 and HSC70 in HCT116 cells was effective in reducing HSC70 expression with no concomitant HSP72 induction (Figure 6A). Exposure to 17-AAG following the dual transfection caused a moderate induction of HSP72 and HSC70, but to levels much lower than observed in corresponding siRNA controls (Figure 6A).

When HSC70 and HSP72 expression were simultaneously silenced in HCT116 colon cancer cells in the absence of 17-AAG treatment, the HSP90 client proteins CRAF and CDK4 were depleted to an extent similar to that observed in siRNA controls treated with 17-AAG (Figure 6A). Moreover, CRAF and CDK4 were further reduced following the dual HSC70 and HSP72 siRNA transfection by cotreatment with 17-AAG, resulting in client levels below those observed in any other condition (Figure 6A). Expression of HSP90α was unaffected by the simultaneous reduction of HSC70 and HSP72.

Dual HSC70 and HSP72 silencing significantly decreased the number of attached, viable HCT116 cells ($p < 0.05$; Figure 6B). The antiproliferative effect of HSC70 and HSP72 silencing was equivalent to that observed in transfection controls treated with 17-AAG (Figure 6B). Furthermore, the inhibition of HCT116 cell proliferation following combined depletion of HSC70 and HSP72 was dramatically increased by 24 hr exposure to 5 × IC_{50} 17-AAG. Under these conditions, the number of attached, viable cells was significantly decreased ($p < 0.05$) to levels below those initially seeded (Figure 6B). This response was far greater than when HSC70 and HSP72 were silenced without 17-AAG treatment (Figure 6B).

The increased antiproliferative effect was not a consequence of the simultaneous transfection procedure, as a similar response was not observed in any of the relevant transfection controls (Figure 6B). In addition, the extent to which proliferation was inhibited following the simultaneous silencing of HSC70 and HSP72 and subsequent treatment with 17-AAG was far greater than when the expression of either of these isoforms was reduced alone (Figure 2A; Figure 6B), confirming our earlier hypothesis that the concurrent induction of HSP72 in response to HSC70 siRNA was indeed reducing the sensitizing effect of silencing HSC70 prior to 17-AAG.

Consistent with the observations described above for HCT116 cancer cells, simultaneous HSP72 and HSC70 silencing in nontumorigenic PNT2 cells also resulted in the degradation of the HSP90 clients CRAF and CDK4 (Figure 6C) and also enhanced their depletion in response to 17-AAG (Figure 6C). However, in contrast to HCT116 cancer cells, client protein depletion caused by dual HSC70 and HSP72 silencing was not accompanied by a significant reduction in proliferation of nontumorigenic PNT2 cells ($p > 0.05$; Figure 6D), nor did it significantly affect the response of this cell line to 24 hr exposure to 5 × IC_{50} 17-AAG ($p > 0.05$; Figure 6D).

In HCT116 cells, decreased proliferation following the simultaneous silencing of HSC70 and HSP72 was accompanied by a significant increase in cell death in the absence of 17-AAG treatment (Figure 6E). The mode of cell death was confirmed as apoptotic by detection of cleaved PARP (Figure 6F). The number of apoptotic HCT116 cells was increased 10-fold (±2.9 SEM) from 1.4% (±0.3% SEM) in corresponding siRNA controls to 13.1% (±2.2% SEM; $p < 0.02$) in cells that had undergone dual HSC70 and HSP72 silencing. In addition, simultaneous silencing of the two HSP70 isoforms increased HCT116 cell apoptosis in response to 17-AAG by 20-fold (±6.9 SEM) to 40.3% (±13.8% SEM) compared to only 2.4% (±0.7% SEM) in siRNA controls treated with 17-AAG ($p < 0.04$). In contrast, the same dual silencing of HSC70 and HSP72 in nontumorigenic PNT2 cells did not induce significant apoptosis in either the presence or absence of 17-AAG ($p > 0.05$; Figure 6E). This demonstrates that a nontumorigenic epithelial cell line can tolerate reduced expression of the two major HSP70 isoforms (Figure 6E) and is also able to withstand additional pharmacologic inhibition of HSP90 function as shown by client protein degradation (Figure 6C).

Our data reveal that by simultaneously silencing HSC70 and HSP72, it is possible to inhibit HSP90, as demonstrated by the depletion of HSP90 clients, and also to block proliferation of cancer cells to an extent similar to that seen with pharmacologic HSP90 inhibition. Moreover, apoptosis is much greater when HSP90 clients are depleted by HSC70 and HSP72 silencing compared to pharmacologic HSP90 inhibition. Also, we show that dual HSC70 and HSP72 silencing sensitizes cells to the apoptotic effects of 17-AAG to an extent far greater than when HSP72 is silenced alone. Importantly, we also reveal a differential tumor cell versus nontumorigenic cell response after silencing of HSP70 isoforms, which supports targeting this family for cancer therapy.

Kinetics of HSP90 Client Protein Depletion, Antiproliferative Effects, and Apoptosis upon Simultaneous HSP72 and HSC70 Depletion

Next, we investigated the detailed effects of dual HSC70 and HSP72 silencing, in the absence of cotreatment with 17-AAG, over a more prolonged time course. Dual silencing in HCT116 cancer cells caused maximum reduction of HSP70 isoform expression at 72 hr, with a slight recovery at 96 hr (Figure 7A). This was accompanied by a time-dependent decrease in

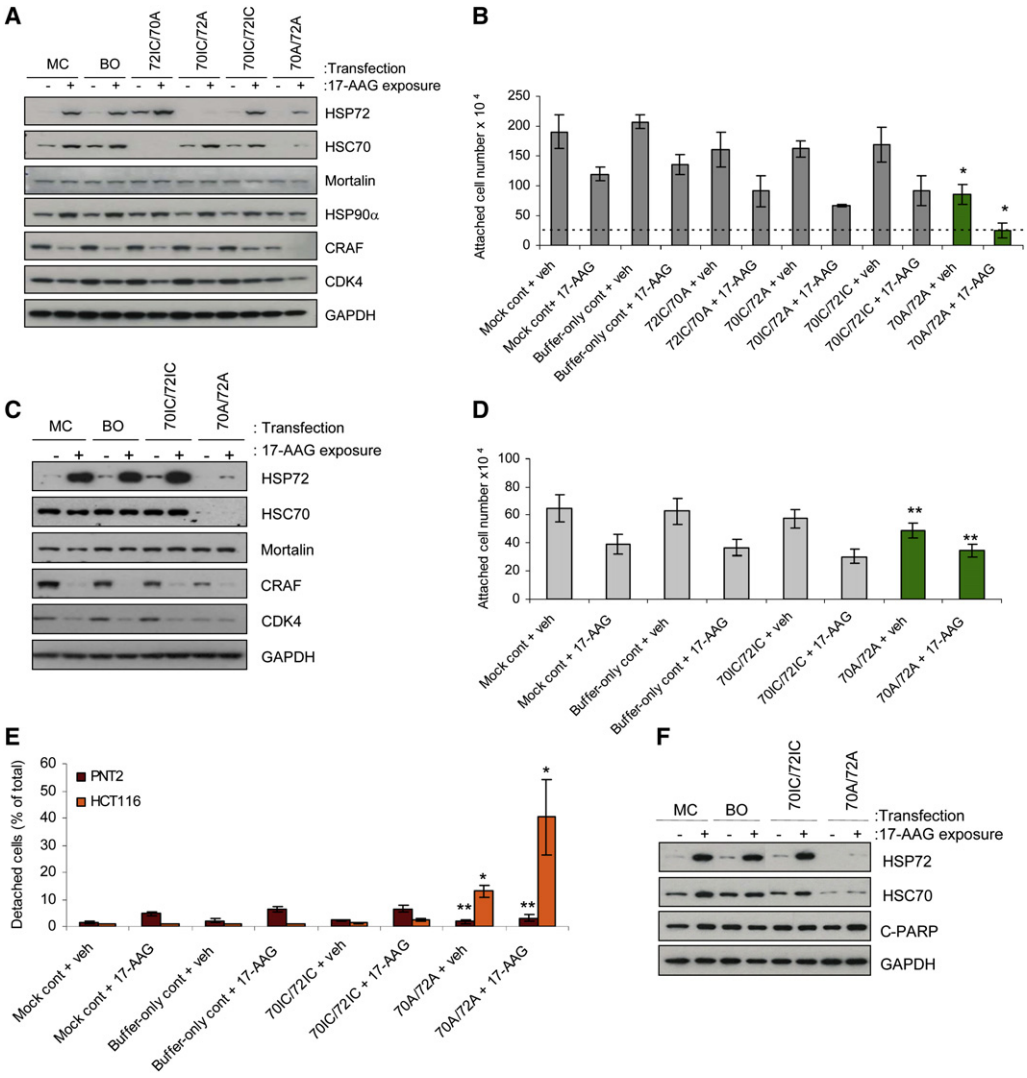

Figure 6. Dual Depletion of HSP72 and HSC70 Increases Tumor but Not Nontumorigenic Cell Apoptosis in Response to Pharmacologic HSP90 Inhibition

(A) HCT116 human colon cancer cells were transfected with a series of siRNA combinations involving control siRNAs (72IC and 70IC) and/or active siRNAs against HSC70 (70A) or HSP72 (72A). Twenty-four hours later, cells were exposed to 5 × IC_{50} 17-AAG (117 nM) for a further 24 hr. The efficacy of gene silencing was determined by immunoblotting. GAPDH was included as a loading control.

(B) Number of attached HCT116 cancer cells following simultaneous reduction of HSC70 and HSP72 followed by 24 hr treatment with 5 × IC_{50} 17-AAG (green bars). Cells remaining attached were assessed by trypan blue exclusion and compared to the number initially seeded (dotted line). Transfections were as described in (A). n = 3; error bars represent ±SEM. *$p < 0.05$ versus control siRNA-transfected cells.

(C) Immunoblot of nontumorigenic PNT2 human prostate epithelial cells transfected and treated for 24 hr with 5 × IC_{50} 17-AAG (490 nM). Transfections were as described in (A). GAPDH was included as a loading control.

(D) Number of attached nontumorigenic PNT2 cells after dual silencing of HSC70 and HSP72 followed by 24 hr treatment with 5 × IC_{50} 17-AAG (green bars). Cells remaining attached were quantified as described in (B). n = 3; error bars represent ±SEM. **$p > 0.05$ versus control siRNA-transfected cells.

(E) Percent detached HCT116 cancer and PNT2 nontumorigenic cells after simultaneous HSC70 and HSP72 silencing followed by 24 hr treatment with 5 × IC_{50} 17-AAG. Transfections were as described in (A). n = 3; error bars represent ±SEM. *$p < 0.05$, **$p > 0.05$ versus control siRNA-transfected cells.

(F) Immunoblot of detached HCT116 cells after simultaneous HSC70 and HSP72 silencing followed by 24 hr exposure to 5 × IC_{50} 17-AAG. Transfections were as described in (A). GAPDH was included as a loading control.

expression of the HSP90 clients CRAF, CDK4, and ERBB2 (Figure 7A). The kinetics of client depletion correlated with the pattern of reduction and recovery of HSP72 and HSC70 (Figure 7A).

The depletion of HSP90 client proteins observed after simultaneous silencing of HSC70 and HSP72 was accompanied by a significant, time-dependent inhibition of proliferation in the first 72 hr ($p < 0.05$; Figure 7B). Cell-cycle analysis of HCT116 cells that had remained adherent following the transfections demonstrated that the antiproliferative effects observed in Figure 7B following simultaneous HSC70 and HSP72 silencing were a consequence of a G1 arrest (Figure 7C).

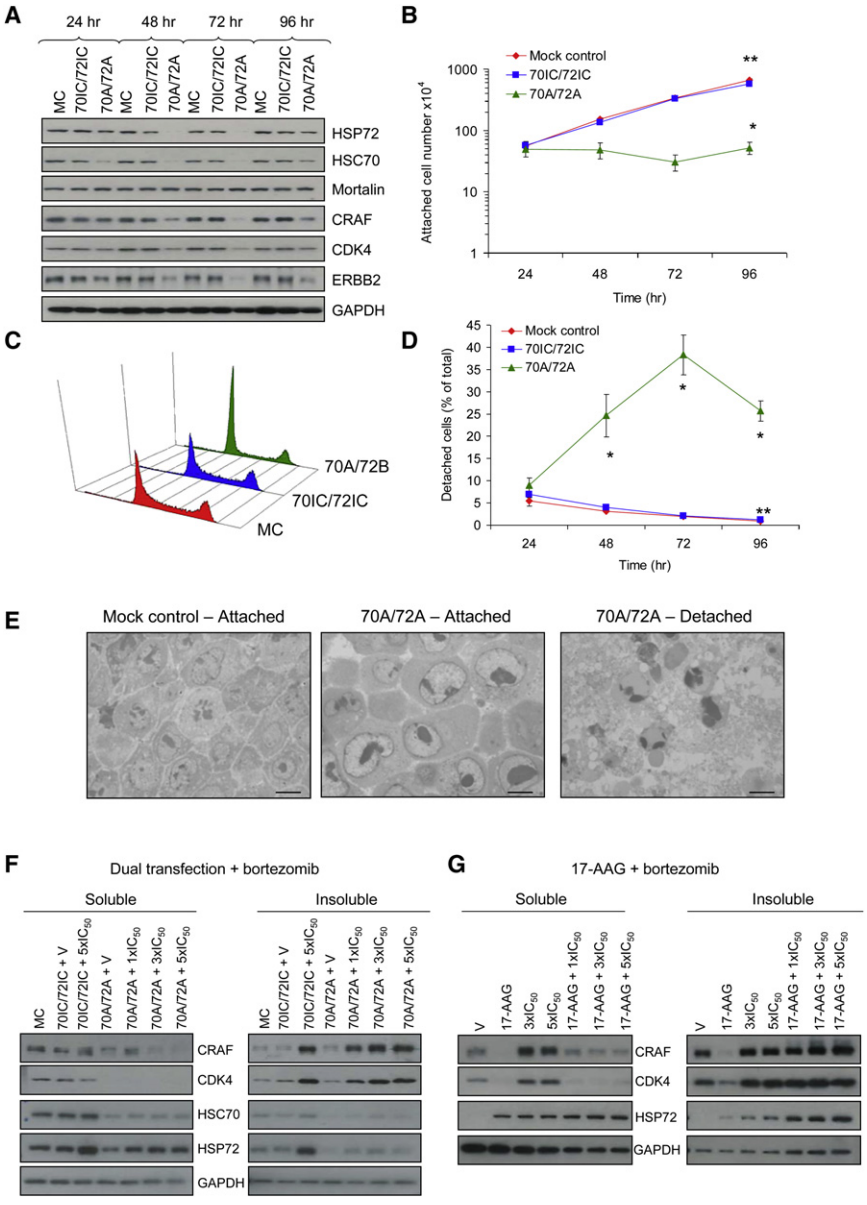

Figure 7. Dual HSC70 and HSP72 Silencing Induces Proteasome-Dependent Client Protein Depletion, Cell-Cycle Arrest, and Considerable Apoptosis

(A) HCT116 human colon cancer cells were treated with siRNA buffer + transfection lipid (MC) or transfected with a combination of HSP72 and HSC70 control siRNAs (72IC and 70IC) or the two active siRNAs against HSP72 and HSC70 (72A and 70A). Immunoblotting was used to determine expression of the proteins shown. GAPDH was included as a loading control.

(B) HCT116 cancer cells remaining attached after dual silencing were assessed by trypan blue exclusion. Transfections were as detailed in (A). n = 3; error bars represent ±SEM. *p < 0.05, **p > 0.05 versus control siRNA-transfected cells 96 hr after transfection.

(C) Cell-cycle analysis using propidium iodide staining of HCT116 cells 48 hr after dual HSP72 and HSC70 silencing. Transfections were as described in (A).

(D) Percentage apoptosis in HCT116 cancer cells following dual HSC70 and HSP72 silencing. Transfections were as described in (A). n = 3; error bars represent ±SEM. *p < 0.05, **p > 0.05 versus control siRNA-transfected cells 96 hr after transfection.

(E) Morphology of attached and detached HCT116 cancer cells using toluidine blue staining 72 hr after simultaneous HSP72 and HSC70 silencing. Transfections were as described in (A). Scale bars = 10 μm.

(F) Twenty-four hours after transfection, HCT116 colon cancer cells were exposed to increasing concentrations of the proteasome inhibitor bortezomib (IC_{50} = 13 nM ± 1.2 SEM) or DMSO vehicle (V) for 24 hr. Levels of the proteins shown were analyzed in the soluble and insoluble protein fractions by immunoblotting. GAPDH was included as a loading control. Transfections were as detailed in (A).

(G) HCT116 cancer cells were treated with bortezomib or DMSO vehicle at multiples of the IC_{50} for 1 hr prior to treatment with 5 × IC_{50} 17-AAG (117 nM) for a further 24 hr. Levels of the proteins shown were analyzed in the soluble and insoluble protein fractions by immunoblotting. GAPDH was included as a loading control.

Importantly, we conclude that combinatorial HSC70 and HSP72 silencing induced a significant, time-dependent increase in apoptosis in the absence of 17-AAG ($p < 0.05$; Figure 7D). Maximum apoptosis accounting for 38% (±4.5% SEM) of the total HCT116 cell population was observed 72 hr after the dual transfection, representing a 20-fold (±4.9 SEM) increase over siRNA controls. This correlates with the time at which maximum effects on HSC70 and HSP72 expression, HSP90 client protein depletion, and cell proliferation were observed (Figures 7A and 7B).

Following simultaneous HSP72 and HSC70 silencing, apoptotic morphology was seen in the detached HCT116 cell population, which exhibited reduced expression of both HSP70 isoforms (Figure 7E). This was in contrast to adherent cells, which displayed a nonapoptotic but slightly enlarged morphology as compared to the normal cell appearance in transfection controls (Figure 7E).

As with transfections using a single targeted siRNA (Figure 1C), we were careful to include relevant controls to ensure the specificity of the dual siRNA transfection. No significant difference was seen in HCT116 cell proliferation ($p > 0.05$; Figure 7B) over 96 hr, nor was any difference in cell-cycle distribution or apoptosis observed between any of the transfection controls (Figures 7C and 7D). In addition, all of the effects seen in HCT116 cells following the simultaneous silencing of HSC70 and HSP72 were reproduced following transfection with two different targeted siRNA oligonucleotides (data not shown).

Client Protein Depletion and Antiproliferative and Apoptotic Effects Correlate with Extent of Dual HSP72 and HSC70 Silencing in Cancer Cells

The results shown here so far demonstrate that the simultaneous and robust silencing of both HSP72 and HSC70, but not either

isoform alone, results in depletion of HSP90 clients, reduction of proliferation, and apoptosis in HCT116 colon cancer cells in the absence of 17-AAG. The more general applicability of these findings was examined in parallel experiments with A2780 ovarian and U87MG glioblastoma cell lines. Following the simultaneous silencing of both HSC70 and HSP72, proliferation of A2780 cells was significantly reduced ($p < 0.05$; Figure S3A) and apoptosis significantly increased at all time points up to 96 hr after the transfection ($p < 0.05$; Figure S3B), though not quite to the same extent seen in HCT116 cells (Figures 7B and 7D). In A2780 cells, HSC70 was silenced to levels below those in corresponding controls (Figure S3C). However, it was only possible to restrain the induction of HSP72 (in response to HSC70 siRNA) to the basal levels observed in corresponding controls (Figure S3C). Consistent with this, although reproducible depletion of the HSP90 clients CRAF and ERBB2 was observed in A2780 ovarian cancer cells (Figure S3C), the extent of depletion was less than in HCT116 cells (Figure 7A).

The necessity of the simultaneous silencing of HSC70 and HSP72 expression was further exemplified using U87MG glioblastoma cells. In this cell line, it was not possible to restrain induction of HSP72 by HSC70 silencing, probably because of the very high level of induction of HSP72 in response to HSC70 siRNA (Figure S3D). Consistent with the lack of restraint of HSP72 induction, no change in expression levels of CRAF or CDK4 were observed in U87MG cells (Figure S3D).

Taking the results obtained in the three cancer cell lines together, it is clear that the effect of reducing the expression of HSC70 and HSP72 by siRNA on HSP90 client protein depletion directly correlated with the degree of HSC70 and HSP72 silencing achieved. We hypothesize that our earlier observation demonstrating that HSP72 can replace HSC70 in the HSP90 complex (Figure 1D) provides an explanation for why robust, simultaneous silencing of both HSP70 isoforms is necessary to observe an effect on HSP90 function.

HSP90 Client Proteins Are Degraded via the Ubiquitin-Proteasome Pathway following Combinatorial Silencing of HSP72 and HSC70

As with pharmacologic HSP90 inhibition, we hypothesized that the ubiquitin-proteasome pathway was involved in the depletion of HSP90 client proteins observed following the combined silencing of HSC70 and HSP72. To investigate this, HCT116 cells that had undergone siRNA-reduced expression of these isoforms were exposed for 24 hr to increasing concentrations of the proteasome inhibitors bortezomib (Figure 7F) or MG-132 (Figure S4).

Highly ubiquitinated proteins can aggregate and hence become insoluble in mild detergent (Mimnaugh and Neckers, 2005). Therefore, both detergent-soluble and -insoluble fractions of the cell extracts were analyzed. Loss of CRAF and CDK4 in the soluble fraction was accompanied by their accumulation in the insoluble fraction following combined depletion of HSC70 and HSP72 and exposure to bortezomib (Figure 7F) or MG-132 (Figure S4). The extent of accumulation was proportional to the concentration of bortezomib or MG-132 used, reflecting the degree of proteasome inhibition. Although a small amount of CRAF and CDK4 was visible in the insoluble fraction following transfection with the two control siRNAs, this was representative of the basal, proteasome-dependent turnover of these proteins, as no change in their levels was seen in the soluble fraction compared to the various controls (Figure 7F). These data confirm that reducing the expression of HSC70 and HSP72 simultaneously leads to the proteasome-dependent degradation of HSP90 client proteins, similar to that observed with direct inhibition of HSP90 by 17-AAG (Figure 7G).

Response to the Dual Silencing of HSC70 and HSP72 Exhibits Selectivity for Cancer versus Nontumorigenic Cells

To support the dual silencing of HSC70 and HSP72 as a therapeutic approach, its effects were investigated in nontumorigenic PNT2 prostate cells over 96 hr. Figure 8A demonstrates that 72 hr after the initial transfection, a robust reduction in HSC70 expression was achieved while the concomitant induction of HSP72 was completely prevented. This was accompanied by a time-dependent decrease in expression of the HSP90 clients CRAF, CDK4, and ERBB2 (Figure 8A). The magnitude of these effects was similar to that observed in HCT116 colon cancer cells (Figure 7A).

Although no significant antiproliferative effect was seen in PNT2 cells 48 hr after dual HSC70 and HSP72 silencing (Figure 6D), when the effects of the dual transfection were analyzed over a longer period, a significant inhibition of cell proliferation was observed 96 hr after the transfection ($p < 0.05$; Figure 8B). Importantly, despite achieving a similar level of silencing against the two HSP70 isoforms and client protein degradation comparable to that achieved in HCT116 cells (Figure 7A), the effect on PNT2 cell proliferation was considerably less than that observed in HCT116 cells (Figure 7B). At 96 hr after the combinatorial transfection, HCT116 cell proliferation was reduced by 91% (±2.0% SEM). In contrast, at the same time point, nontumorigenic PNT2 cell proliferation was inhibited by only 43% (±4.5% SEM; $p < 0.05$). Most importantly, PNT2 cells did not undergo significant apoptosis following simultaneous HSC70 and HSP72 silencing at any time point ($p > 0.05$; Figure 8C). This is in contrast to HCT116 cancer cells, which underwent extensive apoptosis accounting for 38% (±4.5% SEM) of the total cell population 72 hr after dual silencing ($p < 0.05$; Figure 7D). Finally, the population of adherent nontumorigenic PNT2 cells quantified in Figure 8C was confirmed as viable by the lack of PARP cleavage (Figure 8A).

To further explore the potential for therapeutic selectivity, the effects of dual HSC70 and HSP72 silencing were compared directly in HCT116 human colon cancer cells and in nontumorigenic CCD-18Co cells derived from normal human colon. Growth arrest was observed in CCD-18Co cells following the combinatorial silencing of HSC70 and HSP72, but to a much lesser extent than in HCT116 colon cancer cells (Figure S5). In addition, consistent with results for PNT2 cells, minimal cell death was observed in CCD-18Co cells compared to the extensive apoptosis seen in HCT116 colon cancer cells following the simultaneous silencing of HSP70 isoforms as determined by analysis of cleaved PARP (Figure S6A) and caspase-3 (Figure S6B).

Taken together, these results support the potential for combinatorial knockdown of HSP72 and HSC70 to exert greater

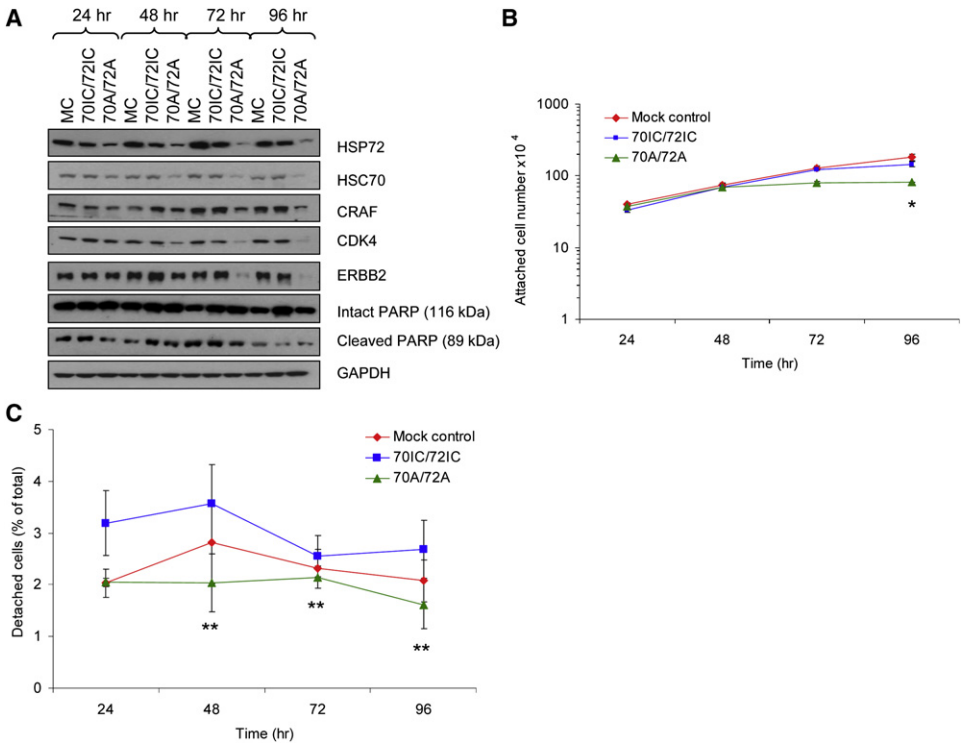

Figure 8. Dual HSC70 and HSP72 Silencing Leads to Client Protein Degradation and Modest Cell Proliferation Arrest, but Not Apoptosis, in a Nontumorigenic Cell Line

(A) PNT2 nontumorigenic human prostate epithelial cells were treated with siRNA buffer + transfection lipid (MC) or transfected with a combination of HSP72 and HSC70 control siRNAs (72IC and 70IC) or the two active siRNAs against HSP72 and HSC70 (72A and 70A). Immunoblotting was used to determine the levels of the proteins shown. GAPDH was included as a loading control.

(B) Quantification of nontumorigenic PNT2 cells remaining attached following dual HSC70 and HSP72 silencing. Transfections were as described in (A). n = 3; error bars represent ±SEM. *$p < 0.05$ versus control siRNA-transfected cells 96 hr after transfection.

(C) Percentage apoptosis in nontumorigenic PNT2 cells following simultaneous silencing of HSC70 and HSP72. Transfections were as described in (A). n = 3; error bars represent ±SEM. **$p > 0.05$ versus control siRNA-transfected cells up to 96 hr after transfection.

antiproliferative and apoptotic effects against cancer versus nontumorigenic cells.

DISCUSSION

HSP90 is an exciting therapeutic target in cancer because inhibition of this single protein causes the simultaneous degradation of multiple oncoproteins and combinatorial blockade of numerous oncogenic pathways (Workman, 2004; Whitesell and Lindquist, 2005). HSP70 molecular chaperones are of interest when considering modulation of HSP90 for several reasons. First, they function as cochaperones for HSP90 that are involved in the initial binding and transfer of client proteins onto HSP90 (Dittmar and Pratt, 1997; Morishima et al., 2000). Second, it is well established that expression of the major inducible isoform, HSP72, is increased following treatment with HSP90 inhibitors. In addition, HSP70 chaperones have a well-documented antiapoptotic function that is independent of their interaction with HSP90 (Mosser and Morimoto, 2004; Garrido et al., 2006). As a consequence, induction of HSP72 reduces the apoptotic effects of HSP90 inhibitors in human prostate and leukemia cell lines (Gabai et al., 2005; Guo et al., 2005). We have demonstrated that not only is HSP72 expression induced in response to HSP90 inhibition, but expression of the constitutive isoform of HSP70, HSC70, is also increased (Maloney et al., 2007). Here, we have used a siRNA approach to investigate the effects of specifically silencing the expression of HSC70 and/or HSP72 in three human cancer cell lines and two nontumorigenic human cell lines.

Results presented here demonstrate that silencing HSC70 or HSP72 individually has no effect on proliferation of HCT116 colon cancer or A2780 ovarian cancer cells. The lack of effect on proliferation following specific silencing of these isoforms individually contrasts with reports that continued expression of HSP72 and/or HSC70 is necessary for the viability of tumor cells (Nylandsted et al., 2000, 2002). However, the degree of dependence on HSP70 isoform expression was previously shown to vary between cell lines, and the previous studies did not include the cell lines used here. In agreement with our findings, Gabai et al. (2005) also found that human prostate cancer cell lines retain viability when only HSP72 expression is reduced.

Confirming previous observations in other cancer cell types, we showed that silencing HSP72 prior to treatment with 17-AAG increases the apoptotic effects of this HSP90 inhibitor in both HCT116 colon and A2780 ovarian carcinoma cells (Figures 4A and 4B). These studies and previously published observations support the modulation of HSP72 expression and/or

function as a strategy to enhance the efficacy of HSP90 inhibitors (Gabai et al., 2005; Guo et al., 2005). Here we have also shown that preventing HSP72 induction in response to 17-AAG did not increase apoptosis in the nontumorigenic PNT2 prostate epithelial cell line (Figure 4C). This further supports the targeting of HSP72 as a mechanism to increase the response to HSP90 inhibitors and potentially to enhance the therapeutic index.

In contrast to HSP72, we have shown that restraining the 17-AAG-mediated induction of HSC70 has no effect on apoptosis induced by HSP90 inhibition (Figures 4A–4C). This was explained by the concurrent induction of HSP72 expression following silencing of HSC70 (Figure 1B). The degree to which HSP72 expression was increased was comparable to that following 24 hr exposure to pharmacologically relevant concentrations of 17-AAG. Therefore, even prior to treatment with 17-AAG, cells which had reduced expression of HSC70 were already exposed to increased expression of antiapoptotic HSP72. To counteract this, we simultaneously reduced the expression of both HSC70 and HSP72 by combinatorial siRNA. Using this approach, we revealed that dual silencing of HSC70 and HSP72 considerably increased the apoptotic response to 17-AAG (Figure 6E). Considering the findings made by ourselves and others on the effects of HSP72 induction on the cell death response to 17-AAG (see above), we hypothesize that the increased sensitization observed after the combinatorial silencing of HSP70 isoforms is at least in part due to restraining the induction of antiapoptotic HSP72.

With respect to the functional role of HSP70 isoforms in the HSP90 chaperone, we found that silencing of individual HSP70 isoforms had no effect on the cellular chaperone activity of HSP90, as determined by the unaltered expression of the commonly studied HSP90 client proteins CRAF and CDK4 (Figures 1A and 1B). Importantly, our coimmunoprecipitation experiments demonstrated that HSP72 replaces HSC70 in the HSP90 complex (Figure 1D). This suggests that reduced expression of HSC70 and its subsequent loss from the HSP90 complex may not affect the activity of HSP90 because of a functional replacement of HSC70 by HSP72. This hypothesis was reinforced by our discovery that simultaneous combinatorial silencing of HSC70 and HSP72 expression was able to phenocopy the effects of pharmacologic HSP90 inhibition by inducing ubiquitin-proteasome-dependent degradation of the HSP90 clients CRAF, CDK4, and ERBB2 without affecting the expression of HSP90 itself (Figure 6A; Figures 7A and 7F). Client protein depletion occurred to a different extent in HCT116 colon cancer, A2780 ovarian cancer, U87MG glioblastoma, and nontumorigenic PNT2 prostate cell lines. The magnitude of HSP90 client depletion was greater in HCT116 and PNT2 cells compared to A2780 cells, with no effect seen in U87MG cells. The extent to which client proteins were depleted was related to the extent to which HSC70 and HSP72 expression could be silenced by the dual siRNA. Consistent with this, another recent study was unable to simultaneously reduce the expression of HSC70 and HSP72 below basal protein levels in SKBr3 breast cancer cells, and, as our results would predict, no effect on ERBB2 was seen (Havik and Bramham, 2007).

Decreased expression of HSP90 clients following the silencing of HSC70 and HSP72 was accompanied by an increase in apoptosis in HCT116 cancer cells, to a much greater extent than seen with 17-AAG concentrations that achieved a similar depletion of clients (Figure 6E). The kinetics of client protein depletion and recovery, and also of induction of apoptosis, exactly followed those of HSC70 and HSP72 reduction and recovery after siRNA silencing (Figures 7A and 7D). The specificity of the combinatorial HSP72 and HSC70 dual siRNA silencing effect was demonstrated by the use of various controls, including sequence-specific control siRNAs, which collectively support the view that the observed changes were a direct and specific consequence of reducing the expression of HSC70 and HSP72.

The degree of proliferative arrest and apoptosis induced in the nontumorigenic human prostate epithelial cell line PNT2 following the simultaneous combinatorial silencing of HSC70 and HSP72 was far less than that observed in HCT116 colon carcinoma cells. This difference was observed despite achieving a similar level of silencing of the two HSP70 isoforms and a comparable level of HSP90 client depletion in the cancer and nontumorigenic cells. This differential effect of combinatorial HSP72 and HSC70 silencing on cancer cell proliferation and apoptosis was also reproduced when the effects were compared in HCT116 colon cancer cells and the nontumorigenic cell line CCD-18Co derived from normal human colon. Kamal et al. (2003) proposed a model to explain the differential responses to pharmacologic HSP90 inhibitors observed in tumor versus normal cells. In this model, HSP90 from tumor cells exists in a superchaperone complex that is highly sensitive to pharmacologic inhibition. Conversely, HSP90 from normal cells is present predominantly in an uncomplexed state that is relatively insensitive to HSP90 inhibitors. It was speculated that this differential might indicate the complete usage of HSP90 by cancer cells to ensure the correct folding, localization, and function of large quantities of mutated or overexpressed oncoproteins (Kamal et al., 2003). This is of importance because malignant cells are hypothesized to be ''addicted'' to the expression of certain oncoproteins to ensure their continued growth and survival (Weinstein, 2002; Workman et al., 2007). When considering the response of both tumor and nontumorigenic cells to the effects of the combinatorial reduction of HSP70 isoforms, it is important to consider that the effects could be attributed to exploitation of oncogene addiction by inhibition of HSP90 function (exemplified by degradation of client proteins) together with a decrease in the antiapoptotic effects of the silenced HSP70 isoforms. Tumor cells may be more reliant on the antiapoptotic effects of HSP70 isoforms due to the deregulated malignant phenotype, a suggestion reinforced by the overexpression of both HSP72 and HSC70 in several tumor types (reviewed in Garrido et al., 2006). This is also consistent with the superchaperone usage model described above (Kamal et al., 2003).

An exciting implication of our observations is the attractiveness of combinatorial targeting of HSC70 and HSP72 as an alternative means to achieve HSP90 inhibition, with the added advantage of avoiding the antiapoptotic effects of HSP70 isoform induction that limit the use of current pharmacologic inhibitors. The siRNA approach was used here as a tool to silence the expression of HSC70 and HSP72. It is possible that RNA interference may be developed as a potential therapeutic approach (Kim and Rossi, 2007). An alternative is the development of small-molecule drugs that inhibit the activity of HSC70 and HSP72. 15-deoxyspergualin and its analogs modulate the

activity of HSC70 (Brodsky, 1999; Fewell et al., 2001). Also, the benzylidene lactam KNK437 and the diterpene triepoxide triptolide block the induction of heat-shock proteins such as HSP70 in response to stress (Yokota et al., 2000; Westerheide et al., 2006). However, both of these compounds inhibit heat-shock protein expression by targeting HSF1 transcription factor activity (Yokota et al., 2000; Westerheide et al., 2006). Therefore, they not only inhibit the expression of HSC70 and HSP72 but also block expression of numerous genes regulated by HSF1. Further work is required to identify compounds that could specifically inhibit both HSC70 and HSP72 chaperones simultaneously to produce the promising molecular and cellular phenotype expected from the results presented here.

In conclusion, we have shown that HSP72 can functionally substitute for HSC70 in the HSP90 complex to maintain the activity of this chaperone protein. We have also demonstrated that dual silencing of both HSC70 and HSP72 produces a molecular signature of ubiquitin-proteasome-dependent depletion of clients that is identical to that seen with pharmacologic HSP90 inhibitors. In addition, extensive apoptosis was observed in cancer cells following dual silencing that could be attributed to the abrogation of the antiapoptotic properties of HSC70 and HSP72 alongside client protein depletion. The extent of apoptosis observed after combined HSP72 and HSC70 silencing was far greater than is commonly seen with pharmacologic HSP90 inhibitors, encouraging the therapeutic targeting of HSP70 isoforms in their own right. Importantly, the simultaneous silencing of HSP72 and HSC70 in nontumorigenic cell lines did not result in comparable growth arrest or significant apoptosis, indicating the potential for a therapeutic window. We propose that combinatorial targeting of HSP72 and HSC70 may provide an attractive approach to exploit both oncogene addiction and chaperone dependence in cancer cells.

EXPERIMENTAL PROCEDURES

Tissue Culture and Cell Growth Inhibition

HCT116 human colon adenocarcinoma, A2780 human ovarian carcinoma, U87MG glioblastoma, CCD-18Co nontumorigenic colon cell, and PNT2 human prostate epithelial cell lines were cultured as described in Supplemental Experimental Procedures. Viability of cells was assessed by trypan blue staining and counting on a hemocytometer. Cell growth inhibition was measured using the sulforhodamine B (Sigma) assay (Sharp et al., 2007).

Transfection with siRNA Oligonucleotides

siRNAs (Dharmacon RNA Technologies) were designed against the open reading frame of HSP72 (*HSPA1A*; accession number NM_005345) or HSC70 (*HSPA8*; accession number NM_006597). Two active sequences were used for studies against HSP72 (designated HSP72A or HSP72B) or HSC70 (designated HSC70A or HSC70B). Transfection controls were the relevant population of cells treated with siRNA universal buffer alone (buffer-only control; Dharmacon RNA Technologies), cells treated with siRNA buffer with Oligofectamine (mock control), or cells transfected with a scrambled control siRNA sequence (Dharmacon RNA Technologies). We also used inactive control siRNAs that were identical in sequence to the HSP72A and HSC70A siRNAs apart from inversion of the two central bases. Sequences for control and active siRNAs as well as transfection details are described in Supplemental Experimental Procedures.

Immunoblotting and Immunoprecipitation

Protein extraction, immunoblotting, immunoprecipitation, and antibodies used are described in Supplemental Experimental Procedures.

Electrochemiluminescence Immunoassay

A Mesoscale Discovery system was used to measure levels of caspase-3 activation and PARP cleavage (Raynaud et al., 2007).

Proteasome Inhibition and Preparation of Detergent-Insoluble Fractions

Concentrations of proteasome inhibitors used and preparation of detergent-soluble and -insoluble fractions are described in Supplemental Experimental Procedures.

Morphological Analysis

Cells were harvested 72 hr after transfection, washed once in PBS (BDH), fixed, and embedded (Ronen et al., 1999). For light microscopy, 1.0 μm sections were cut, dried onto microscope slides, stained with toluidine blue (TAAB Laboratories), and viewed under a Leitz Diaplan microscope. Images were recorded using a Leica DFC320 digital camera.

Flow Cytometry

Cell-cycle distribution was analyzed by flow cytometry using propidium iodide (Hostein et al., 2001).

SUPPLEMENTAL DATA

The Supplemental Data include Supplemental Experimental Procedures, Supplemental References, and six figures and can be found with this article online at http://www.cancercell.org/cgi/content/full/14/3/250/DC1/.

ACKNOWLEDGMENTS

We thank our colleagues in the Signal Transduction and Molecular Pharmacology Team for valuable discussions, J. Titley for flow cytometry, and N. Perusinghe for microscopy. This work was supported by Cancer Research UK Program Grants no. C309/A2187 and C309/A8274. P.W. is a Cancer Research UK Life Fellow. M.V.P. was funded by a studentship from The Institute of Cancer Research.

The authors are employees of The Institute of Cancer Research, which has a commercial interest in HSP90 inhibitors. The authors have been involved in a funded research collaboration on HSP90 inhibitors with Vernalis Ltd., and intellectual property arising from the program has been licensed to Vernalis and Novartis. P.W. has been a consultant to Novartis.

Received: February 6, 2006
Revised: May 28, 2008
Accepted: August 8, 2008
Published: September 8, 2008

REFERENCES

Banerji, U., Walton, M., Raynaud, F., Grimshaw, R., Kelland, L., Valenti, M., Judson, I., and Workman, P. (2005a). Pharmacokinetic-pharmacodynamic relationships for the heat shock protein 90 molecular chaperone inhibitor 17-allylamino, 17-demethoxygeldanamycin in human ovarian cancer xenograft models. Clin. Cancer Res. *11*, 7023–7032.

Banerji, U., O'Donnell, A., Scurr, M., Pacey, S., Stapleton, S., Asad, Y., Simmons, L., Maloney, A., Raynaud, F., Campbell, M., et al. (2005b). Phase I pharmacokinetic and pharmacodynamic study of 17-allylamino, 17-demethoxygeldanamycin in patients with advanced malignancies. J. Clin. Oncol. *23*, 4152–4161.

Brodsky, J.L. (1999). Selectivity of the molecular chaperone-specific immunosuppressive agent 15-deoxyspergualin: modulation of Hsc70 ATPase activity without compromising DnaJ chaperone interactions. Biochem. Pharmacol. *57*, 877–880.

Daugaard, M., Rohde, M., and Jaattela, M. (2007). The heat shock protein 70 family: Highly homologous proteins with overlapping and distinct functions. FEBS Lett. *581*, 3702–3710.

Dittmar, K.D., and Pratt, W.B. (1997). Folding of the glucocorticoid receptor by the reconstituted Hsp90-based chaperone machinery. The initial

hsp90.p60.hsp70-dependent step is sufficient for creating the steroid binding conformation. J. Biol. Chem. *272*, 13047–13054.

Fewell, S.W., Day, B.W., and Brodsky, J.L. (2001). Identification of an inhibitor of hsc70-mediated protein translocation and ATP hydrolysis. J. Biol. Chem. *276*, 910–914.

Gabai, V.L., Budagova, K.R., and Sherman, M.Y. (2005). Increased expression of the major heat shock protein Hsp72 in human prostate carcinoma cells is dispensable for their viability but confers resistance to a variety of anticancer agents. Oncogene *24*, 3328–3338.

Garrido, C., Brunet, M., Didelot, C., Zermati, Y., Schmitt, E., and Kroemer, G. (2006). Heat shock proteins 27 and 70: anti-apoptotic proteins with tumorigenic properties. Cell Cycle *5*, 2592–2601.

Goetz, M.P., Toft, D., Reid, J., Ames, M., Stensgard, B., Safgren, S., Adjei, A.A., Sloan, J., Atherton, P., Vasile, V., et al. (2005). Phase I trial of 17-allylamino-17-demethoxygeldanamycin in patients with advanced cancer. J. Clin. Oncol. *23*, 1078–1087.

Guo, F., Rocha, K., Bali, P., Pranpat, M., Fiskus, W., Boyapalle, S., Kumaraswamy, S., Balasis, M., Greedy, B., Armitage, E.S., et al. (2005). Abrogation of heat shock protein 70 induction as a strategy to increase antileukemia activity of heat shock protein 90 inhibitor 17-allylamino-demethoxy geldanamycin. Cancer Res. *65*, 10536–10544.

Havik, B., and Bramham, C.R. (2007). Additive viability-loss following hsp70/hsc70 double interference and Hsp90 inhibition in two breast cancer cell lines. Oncol. Rep. *17*, 1501–1510.

Hostein, I., Robertson, D., DiStefano, F., Workman, P., and Clarke, P.A. (2001). Inhibition of signal transduction by the Hsp90 inhibitor 17-allylamino-17-demethoxygeldanamycin results in cytostasis and apoptosis. Cancer Res. *61*, 4003–4009.

Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M.F., Fritz, L.C., and Burrows, F.J. (2003). A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature *425*, 407–410.

Kim, D.H., and Rossi, J.J. (2007). Strategies for silencing human disease using RNA interference. Nat. Rev. Genet. *8*, 173–184.

Maloney, A., Clarke, P.A., Naaby-Hansen, S., Stein, R., Koopmann, J.O., Akpan, A., Yang, A., Zvelebil, M., Cramer, R., Stimson, L., et al. (2007). Gene and protein expression profiling of human ovarian cancer cells treated with the heat shock protein 90 inhibitor 17-allylamino-17-demethoxygeldanamycin. Cancer Res. *67*, 3239–3253.

Mimnaugh, E.G., Chavany, C., and Neckers, L. (1996). Polyubiquitination and proteasomal degradation of the p185c-erbB-2 receptor protein-tyrosine kinase induced by geldanamycin. J. Biol. Chem. *271*, 22796–22801.

Mimnaugh, E.G., and Neckers, L.M. (2005). Measuring ubiquitin conjugation in cells. Methods Mol. Biol. *301*, 223–241.

Morishima, Y., Murphy, P.J., Li, D.P., Sanchez, E.R., and Pratt, W.B. (2000). Stepwise assembly of a glucocorticoid receptor.hsp90 heterocomplex resolves two sequential ATP-dependent events involving first hsp70 and then hsp90 in opening of the steroid binding pocket. J. Biol. Chem. *275*, 18054–18060.

Mosser, D.D., and Morimoto, R.I. (2004). Molecular chaperones and the stress of oncogenesis. Oncogene *23*, 2907–2918.

Nylandsted, J., Brand, K., and Jaattela, M. (2000). Heat shock protein 70 is required for the survival of cancer cells. Ann. N Y Acad. Sci. *926*, 122–125.

Nylandsted, J., Wick, W., Hirt, U.A., Brand, K., Rohde, M., Leist, M., Weller, M., and Jaattela, M. (2002). Eradication of glioblastoma, and breast and colon carcinoma xenografts by Hsp70 depletion. Cancer Res. *62*, 7139–7142.

Raynaud, F.I., Eccles, S., Clarke, P.A., Hayes, A., Nutley, B., Alix, S., Henley, A., Di-Stefano, F., Ahmad, Z., Guillard, S., et al. (2007). Pharmacologic characterization of a potent inhibitor of class I phosphatidylinositide 3-kinases. Cancer Res. *67*, 5840–5850.

Ronen, S.M., DiStefano, F., McCoy, C.L., Robertson, D., Smith, T.A., Al-Saffar, N.M., Titley, J., Cunningham, D.C., Griffiths, J.R., Leach, M.O., et al. (1999). Magnetic resonance detects metabolic changes associated with chemotherapy-induced apoptosis. Br. J. Cancer *80*, 1035–1041.

Sharp, S.Y., Boxall, K., Rowlands, M., Prodromou, C., Roe, S.M., Maloney, A., Powers, M., Clarke, P.A., Box, G., Sanderson, S., et al. (2007). In vitro biological characterization of a novel, synthetic diaryl pyrazole resorcinol class of heat shock protein 90 inhibitors. Cancer Res. *67*, 2206–2216.

Sliutz, G., Karlseder, J., Tempfer, C., Orel, L., Holzer, G., and Simon, M.M. (1996). Drug resistance against gemcitabine and topotecan mediated by constitutive hsp70 overexpression in vitro: implication of quercetin as sensitiser in chemotherapy. Br. J. Cancer *74*, 172–177.

Weinstein, I.B. (2002). Cancer. Addiction to oncogenes–the Achilles heal of cancer. Science *297*, 63–64.

Westerheide, S.D., Kawahara, T.L., Orton, K., and Morimoto, R.I. (2006). Triptolide, an inhibitor of the human heat shock response that enhances stress-induced cell death. J. Biol. Chem. *281*, 9616–9622.

Whitesell, L., and Lindquist, S.L. (2005). HSP90 and the chaperoning of cancer. Nat. Rev. Cancer *5*, 761–772.

Workman, P. (2004). Combinatorial attack on multistep oncogenesis by inhibiting the Hsp90 molecular chaperone. Cancer Lett. *206*, 149–157.

Workman, P., Burrows, F., Neckers, L., and Rosen, N. (2007). Drugging the cancer chaperone HSP90: combinatorial therapeutic exploitation of oncogene addiction and tumor stress. Ann. N Y Acad. Sci. *1113*, 202–216.

Yokota, S., Kitahara, M., and Nagata, K. (2000). Benzylidene lactam compound, KNK437, a novel inhibitor of acquisition of thermotolerance and heat shock protein induction in human colon carcinoma cells. Cancer Res. *60*, 2942–2948.

Young, J.C., Agashe, V.R., Siegers, K., and Hartl, F.U. (2004). Pathways of chaperone-mediated protein folding in the cytosol. Nat. Rev. Mol. Cell Biol. *5*, 781–791.