Systematic Identification of the HSP90 Regulated Proteome

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HSP90 is a central player in the folding and maturation of many proteins. More than two hundred HSP90 clients have been identified by classical biochemical techniques including important signaling proteins with high relevance to human cancer pathways. HSP90 inhibition has thus become an attractive therapeutic concept and multiple molecules are currently in clinical trials. It is therefore of fundamental biological and medical importance to identify, ideally, all HSP90 clients and HSP90 regulated proteins. To this end, we have taken a global and a chemical proteomic approach in geldanamycin treated cancer cell lines using stable isotope labeling with amino acids in cell culture and quantitative mass spectrometry. We identified >6200 proteins in four different human cell lines and ~1600 proteins showed significant regulation upon drug treatment. Gene ontology and pathway/network analysis revealed common and cell-type specific regulatory effects with strong connections to unfolded protein binding and protein kinase activity. Of the 288 identified protein kinases, 98 were downregulated upon geldanamycin treatment including >50 kinases not formerly known to be regulated by HSP90. Protein turnover measurements using pulsed stable isotope labeling with amino acids in cell culture showed that protein down-regulation by HSP90 inhibition correlates with protein half-life in many cases. Protein kinases show significantly shorter half lives than other proteins highlighting both challenges and opportunities for HSP90 inhibition in cancer therapy. The proteomic responses of the HSP90 drugs geldanamycin and PU-H71 were highly similar suggesting that both drugs work by similar molecular mechanisms. Using HSP90 immunoprecipitation, we validated several kinases (AXL, DDR1, TRIO) and other signaling proteins (BIRC6, ISG15, FLII), as novel clients of HSP90. Taken together, our study broadly defines the cellular proteome response to HSP90 inhibition and provides a rich resource for further investigation relevant for the treatment of cancer. Molecular & Cellular Proteomics 11: 10.1074/mcp.M111.016675, 1–14, 2012.

The protein HSP90 is a evolutionary conserved molecular chaperone that is abundantly and ubiquitously expressed in cells from bacteria to man. In concert with multiple cochaperones and other accessory proteins, its primary function is to assist in the proper folding of proteins and thereby helps to maintain the structural and functional integrity of the proteome (proteostasis). Over the past 30 years, more than 200 such “client” proteins have been identified using classical biochemical and biophysical methods (1–3). More recently, genome wide screens in yeast suggest that 10–20% of the yeast proteome may be regulated by HSP90 (1, 4). Therefore, not surprisingly HSP90 clients span a very wide range of protein classes (kinases, nuclear receptors, transcription factors etc.) and biological functions (signal transduction, steroid signaling, DNA damage, protein trafficking, assembly of protein complexes, innate immunity to name a few) (1, 2, 5). Because many HSP90 clients are key nodes of biological networks, HSP90 not only exercises important functions in normal protein homeostasis, but also in disease. Many HSP90 clients are oncogenes (EGFR, c.KIT, BCR-ABL etc.) that drive a wide range of cancers and whose cells have often become “addicted” to HSP90 function (1). The disruption of HSP90 function by small molecule drugs has therefore become an attractive therapeutic strategy and about a dozen of HSP90 inhibitors are currently undergoing clinical trials in a number of tumor entities and indications (2, 5, 6). Geldanamycin is the founding member of a group of HSP90 inhibitors that target the ATP binding pocket of HSP90 and block the chaperone cycle, which on the one hand leads to transcription factor activation and subsequent gene expression changes (e.g. HSF1) (7, 8) and, on the other hand, to proteasome mediated degradation of HSP90 substrates (5, 9). Experience from clinical trials shows that the efficacy and toxicity of HSP90 targeted therapy varies greatly between tumors suggesting that the current repertoire of client proteins and our understanding of drug mechanism of action is incomplete (10). To predict an individual patient’s responsiveness, it would thus be highly desirable to identify the complete set of HSP90 regulated proteins. Because HSP90 directly (e.g. by degradation) and indirectly (e.g. by induction of gene/protein expression) affects proteostasis, proteomic approaches are particularly attractive for studying e.g. the HSP90 interactome and the global effects of HSP90 inhibition on cellular systems. A number of proteomic approaches have been taken to explore the HSP90 regulated proteome including global proteome profiling using two-dimensional gels and mass spectrometry (11) as well as focused proteomic experiments utilizing immuno-
precipitation of HSP90 complexes and chemical precipitation using immobilized HSP90 inhibitors (12). These studies have identified some important new HSP90 clients but generally fail to provide a global view of HSP90 regulated proteome because the attained proteomic depth was very limited and many HSP90 interactions are too transient or of too weak affinity to be purified by these methods. Very recently, a report on the global proteomic and phosphoproteomic response of HeLa cells to the HSP90 inhibitor 17-dimethylaminoethoxy-17-demethoxygeldanamycin (17-DMAg) has appeared in the online version of Molecular and Cellular Proteomics (13) indicating that the cellular effects of HSP90 inhibition are much larger than previously anticipated.

In this study, we have profiled the global response of the proteomes and kinomes of the four cancer cell lines K562, Colo205, Cal27, and MDAMB231 to the HSP90 inhibitor geldanamycin. Using a combination of stable isotope labeling in cell culture (14), core proteome profiling (15), chemical precipitation of kinases (16), and quantitative mass spectrometry in cell culture (14), core proteome profiling (15), chemical precipitation and pulldowns with immobilized geldanamycin, we identified >6200 proteins of which ~1600 proteins showed common as well as cell type specific regulation upon drug treatment. Bioinformatic analysis enabled a functional organization of this data into protein pathways, networks and complexes highlighting many known and novel aspects of HSP90 function. Protein turn-over measurements using pulsed stable isotope labeling with amino acids in cell culture (SILAC) (18, 19) showed that, for a significant number of proteins, the rate of HSP90 inhibition induced protein downregulation correlates with protein half-life and that protein kinases have significantly shorter half lives than other proteins with potentially important implications for HSP90 inhibition in cancer therapy. A comparison of the effects of geldanamycin and the phase I drug PU-H71 (20) suggests that both molecules work by similar molecular mechanisms. Using HSP90 immunoprecipitation and pulldowns with immobilized geldanamycin, we validated several kinases (AXL, DDR1, TRIO) and other signaling proteins (BIRC6, ISG15, FLI1), as novel bona fide clients of HSP90. Collectively, the data demonstrate the value of the global drug profiling approach and provides a rich resource for future investigation in HSP90 dependent biological processes.

EXPERIMENTAL PROCEDURES

SILAC Labeling and Cell Culture—CAL27 and MDAMB231 cells were cultured in Dulbecco’s modified Eagle’s medium (4.5 g/l glucose) medium, K562 and COLO205 cells were cultured in Roswell Park Memorial Institute 1640 medium. For SILAC labeling cells were grown in normal medium deficient in Arginine and Lysine (PAA, Pasching, Austria) supplemented with either stable isotope encoded amino acids in cell culture; PBS, phosphate buffered saline; REVIGO, Reduce and Visualize Gene Ontology; VSN, variance stabilization normalization.

1 The abbreviations used are: SILAC, stable isotope labeling with amino acids in cell culture; PBS, phosphate buffered saline; REVIGO, Reduce and Visualize Gene Ontology; VSN, variance stabilization normalization.
Mini Gel. Gel lanes containing separated immunocomplexes were cut into 12 slices and in-gel trypsin digestion was performed according to standard protocols. For GA-NHS affinity purification, the same cell lysate as immunoprecipitate were pre-incubated with either 25 μM Geldanamycin or 0.1% dimethyl sulfoxide for 1 h at 4 °C and Sepharose beads with the immobilized GA were subsequently added for another 1 h at 4 °C. The following steps were the same as immunoprecipitation mentioned above.

Liquid Chromatography Tandem MS (LC-MS/MS) Analysis—Nano flow LC-MS/MS was performed by coupling an Eksigent nanoLC-Ultra 1D+ (Eksigent, Dublin, CA) to a LTQ-Orbitrap XL ETD (Thermo Scientific, Bremen, Germany). Tryptic peptides were dissolved in 20 μl 0.1% formic acid and 10 μl was injected for each analysis. Peptides were delivered to a trap column (100 μm i.d. × 2 cm, packed with 5 μm C18 resin, Reprosil PUR AQ, Dr. Maisch, Ammerbuch, Germany) at a flow rate of 5 μl/min in 100% buffer A (0.1% formic acid in HPLC grade water). After 10 min of loading and washing, peptides were transferred to an analytical column (75 μm × 40 cm C18 column Reprosil PUR AQ, 3 μm, Dr. Maisch, Ammerbuch, Germany) and separated using a 210 min gradient from 2% to 35% of buffer B (0.1% formic acid in acetonitrile) at 300 nL/minute flow rate. The LTQ-Orbitrap was operated in data-dependent mode, automatically switching between MS and MS2. Full scan MS spectra were acquired in the Orbitrap at 60,000 resolution. Internal calibration was performed using the ion signal (Si(CH3)2O)6H+ at m/z 445.120025 present in ambient laboratory air. Tandem mass spectra were generated for up to eight peptide precursors in the linear ion trap for fragment by using collision-induced dissociation.

Peptide and Protein Quantification and Identification—Raw MS spectra were processed by Maxquant (version 1.1.1.25) for peak detection and quantification (23). MS/MS spectra was searched against the IPI human database human (version 3.68, 87,061 sequences) by Andromeda (24) search engine enabling contaminants and the reversed versions of all sequences with the following search parameters: Carbamidomethylation of cysteine residues as fixed modifications and Acetyl (Protein N-term), Gin_pyro-Glu (N-Term Q), Glu_pyro-Glu(N-Term E), Oxidation (M), Phospho (ST), Phospho(Y) as variable modifications. Trypsin was specified as the proteolytic enzyme with up to 2 miss cleavages were allowed. The mass accuracy of the precursor ions was decided by the time-dependent recalibration algorithm of Maxquant, fragment ion mass tolerance was set to 0.6 Da. The maximum false discovery rate for proteins and peptides was 0.01 and a minimum peptide length of six amino acids was required.

Statistical Analysis—Statistical analysis of quantified proteins was performed using R (version 2.12.1) (25). Raw protein abundance values were first normalized using Variance Stabilization Normalization (VSN) (26). VSN is able to stabilize the variance across the entire intensity range and addresses the error structure in the data. The application of VSN has previously been shown to be beneficial for MS-based quantification (27, 28). To investigate the data distribution and ensure the appropriate application of statistical tools, normal quantile-quantile plots were created for all protein intensities in each cell line. Variance stabilization normalization was applied to the data and variance-mean dependences were visually verified (supplemental Figs. S2 and S3). Differential expression of paired samples was assessed with a moderated linear model using the limma package (29) in Bioconductor (30). Differences in protein expression between “treated” and “control” samples were estimated with the least squares linear model fitting procedure and tested for differential expression with moderated Student’s t-statistic via the empirical Bayesian statistics described in the limma package (29). We accepted or rejected the null hypothesis on the basis of p values computed for the omnibus B-statistic via limma at a specified significance level. p values were adjusted for multiple testing to control the false discover rate at 5%. For multiple testing adjustments, we calculated the false discover rate using the algorithm of Benjamini and Hochberg (31). p values, with appropriate multiple testing adjustment to control the false discovery rate at 5% allowed us to identify differentially expressed proteins.

GO Enrichment/Pathway Analysis/Complex Analysis—Classification and functional enrichment analysis of the identified proteins were performed using Database for Annotation, Visualization and Integrated Discovery (DAVID) Bioinformatics Database (32, 33) for the biological process (BP), molecular function (MF), and cellular component (CC). In order to make the functional categories more understandable, terms were clustered according to their functional similarity using REVIGO (34). Pathway membership of the identified proteins were analyzed by the Ingenuity Pathway Analysis (IPA) tool (Ingenuity Systems, Redwood City, CA, USA) for their functional significance and in the context of biological association networks. To investigate HSP90 targeting of many macromolecular complexes, we analyzed the list of significantly expressed proteins (adjusted p < 0.05) using Comprehensive Resource of Mammalian protein complexes (35), a database of manually curated and validated mammalian protein complexes.

Immunoblot Analysis—Anti-DDR1 and α-tubulin antibodies were purchased from Santa Cruz. For immunoblot analysis, cells were washed with cold phosphate-buffered saline and lysed in RIPA buffer. Protein concentration was determined by the Bradford assay. Fifty micrograms of lysate was mixed with an equal volume of 2 × NuPAGE® LDS sample buffer containing 10 mM dithiothreitol and biotin for 5 min at 95 °C. Proteins were subsequently separated by 4–12% NuPAGE gel and transferred onto to polyvinylidene difluoride membranes (Invitrogen, Darmstadt, Germany). Membranes were blocked for 1 h in blocking solution (2% bovine serum albumin in 1×Tris Buffered Saline, TBS, 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween-20) at room temperature and probed overnight at 4 °C with the respective primary antibody. Immunoreactivity was detected using IRDye® conjugated secondary antibody (LI-COR®, Nebraska) and visualized by Odyssey imaging system (LI-COR).

RESULTS

High Quality Map of HSP90 Regulated Proteins—The idea behind the experimental strategy taken (Fig. 1) was to compare the proteomes of cancer cell lines in response to HSP90 inhibition by the small molecule drug GA. Proteins that are significantly down-regulated upon drug treatment should represent HSP90 clients, interactors and more complex regulatory events whereas proteins up-regulated upon drug treatment should primarily represent the latter category. In practice, human cell lines were SILAC labeled, “light” cells were treated with geldanamycin (5–10 μM for 24 h, supplemental Fig. S1) and “heavy” cells were left untreated. Cell lysates were mixed 1:1 and subjected in parallel to protein separation by 1D-SDS-PAGE and to the enrichment of kinases using an affinity reagent called kinobeads (a set of unspecific kinase inhibitors immobilized on Sepharose beads, (16, 22)). Following digestion into tryptic peptides, protein identification and differential quantification was performed by LC-MS/MS on an LTQ-Orbitrap XL mass spectrometer and using the software package MaxQuant. Following the above strategy, we analyzed four human cancer cell lines from different origin in the human body (Cal27,
HSP90 Regulated Proteome

![Diagram of experimental strategy for the identification of the HSP90 regulated proteome.](image)

**Fig. 1. Experimental strategy for the identification of the HSP90 regulated proteome.** Cells are grown in “light” and “heavy” SIALC medium. Light cells are treated with the HSP90 inhibitor geldanamycin. Treated and untreated cells are combined and full lysates are either separated by 1D gel electrophoresis (left branch) or first subjected to purification of kinases using kinobeads (right branch). Following trypsin digestion, both samples are analyzed by LC-MS/MS and proteins are subsequently identified and quantified. Each experiment was performed in biological triplicates.

Each analysis was performed in biological triplicates to enable statistical analysis within and across cell lines. Collectively, 6283 proteins including 288 protein kinases were identified (Figs. 2A and 2B, supplemental Fig. S4 and S5, supplemental Tables S1 and S2) at a false discovery rate of <1%. For all subsequent data analysis and bioinformatic analysis, we only used proteins that were quantified in all three biological replicates of a particular cell line. Using these rigorous criteria, more than 1600 proteins including 117 protein kinases were significantly (p < 0.05) up- or down-regulated following geldanamycin treatment (Fig. 2C, supplemental Fig. S6, supplemental Tables S3 and S4). The mass spectra and extracted ion chromatograms shown in Fig. 2D give examples of three protein kinases that are down-regulated (left, DDR1), not regulated (middle, CK2A2), or up-regulated (right, AURKA). Depending on the cell line, between 20% (MDAMB231 cells) and 30% (K562 cells) of all proteins were significantly regulated and, in all cell lines, more proteins were down-regulated (52–65%) than up-regulated (36–48%; supplemental Fig. S7). This observation was far more pronounced for protein kinases for which 89–92% were down regulated upon drug treatment (supplemental Fig. S8).

**HSP90 Regulated Proteome in Different Cell Lines**—In order to organize the extensive list of HSP90 regulated proteins, we applied several layers of bioinformatic analysis. First, gene ontology (GO) analysis revealed that the ~1600 regulated proteins across all four cell lines span a range of molecular functions and biological processes (Fig. 3A, supplemental Figs. S9–S13, supplemental Table S5). Expectedly, the most significant GO categories of the regulated proteins common to all four cell lines were unfolded protein binding (for up-regulated proteins) and kinase activity (for down-regulated proteins) thus validating the proteomic data given the well documented effect of HSP90 inhibition on proteins from these classes. Other common categories include among others “cell cycle” and “response to DNA damage.” For regulated proteins that are specific for a single cell line, the significant GO-categories are more diverse (supplemental Fig. S13, supplemental Table S5) likely representing the different biological backgrounds from which these cells are derived. For example, K562 cells are derived from progenitors of red blood cells and the GO-category “oxygen transport” only come up for this cell line. We next performed molecular pathway analysis in order to explore which cellular signaling pathways are affected by HSP90 inhibition. Again, expectedly, HSP90 inhibition affected multiple pathways and with strong differences between cell lines (Fig. 3B, supplemental Table S6). For example, the protein ubiquitination pathway is similarly up-regulated in all four cell lines reflecting the induction of degradation of HSP90 clients on HSP90 inhibition. Clathrin-mediated endocytosis signaling is a feature of many receptor-regulated processes and is increased in all solid cancer lines but not in the blood cell line K562. PPARα/RXRα activation is also implicated in several cancers and is increased in all cell lines but the colon line Colo205. For the down-regulated pathways, similar responses in e.g. the ERK/MAPK pathway are observed in all cell lines, which is not surprising given that many signaling events converge on this cascade. Ephrin receptor signaling is strongly down-regulated in all three solid tumor lines but not in the leukemia cell line likely because the latter cells do not require much capacity for cell-cell communication (a process in which Ephrin receptors are involved). Finally, down-regulation of mTOR signaling is much more pronounced in the colon cancer line Colo205 and the breast cancer line MDAMB231 compared with the other two cell lines. Collectively, the pathway analysis suggests that significant similarities and differences in the cellular response to HSP90 inhibition indeed do exist. Similarities and differences in the general signaling capacities of the different cell lines can also be observed when placing regulated proteins into molecular networks derived from the literature (supplemental Figs. S14 and S15, supplemental Table S7). Regulated networked proteins are often involved in the cell cycle, cell death, cellular growth, and proliferation, DNA replication, recombination, and repair. The identified networks around the tumor suppressor p53 from the breast cancer line MDAMB231 and the transcription factor complex NFκB from the head and neck line Cal27 are typical examples (supplemental Figs. S14 and S15). Given the
large number of regulatory proteins in these networks, it is not hard to imagine that HSP90 inhibition will exert pleiotropic effects on cancer (and normal) cells. HSP90 itself engages in many protein complexes and our data recapitulates much of the known binary interactions including many (mostly down-regulated) kinases (Fig. 3C, supplemental Fig. S16). Interestingly, by querying the Comprehensive Resource of Mammalian protein complex database (35), we also found that members of protein complexes sometimes appear to be co-regulated by HSP90 inhibition (supplemental Table S8). For example, core members of the kinase maturation complex 1 (HSP90a and b, CDC37, HSP70) were up-regulated upon drug treatment. We also detected six out of the seven members of the Arp2/3 complex (a cellular complex responsible for actin filament nucleation and branching) and all were up-regulated upon geldanamycin treatment. Examples for down-regulated protein complexes included the DNA-PK-Ku-eIF2-NF90-NF45 complex (DNA repair) the Nop56p-associated preribosomal ribonucleoprotein complex (ribosome biogenesis), the spliceosome (mRNA splicing), and the MNK1-eIF4F complex (protein translation initiation).

In order to investigate if the observed similarities and differences of HSP90 inhibition in different cell lines are mainly caused by differences in protein expression (or p value cutoff), we performed an “all against all” comparison of proteins quantified in the four different cell lines. When comparing the SILAC ratios of proteins upon HSP90 inhibition of Cal27 and K562 cells (Fig. 4A, supplemental Fig. S17 for all other combinations), many proteins with similar expression levels (as measured by MS intensity, inset of Fig. 4A) also show similar behavior in both cell lines (e.g. HSP70, DDR1, YES). There is also a significant number of regulated proteins that are exclusively expressed in one of the cell lines (e.g. BTK, FRK) and there are proteins that show different response to HSP90 inhibition despite similar overall expression (e.g. AURA, PLK1). Because fold change measurement are often not reliable measures of significance, we also compared the cell lines by the p values of protein changes on drug treatment and
obtained a similar overall picture (Fig. 4B, supplemental Fig. S18 for all other combinations). Although AURA is significantly changed in Cal27 cells, it is completely insignificant in K562 cells suggesting that the response of proteins to HSP90 inhibition does include a cell-type specific component. As mentioned previously, the GO analysis also revealed a very strong overrepresentation of protein kinases in the list of regulated proteins, ~90% of which are down-regulated by geldanamycin treatment. Mapping all identified protein kinases onto the phylogenetic kinome tree (Fig. 4C) shows that the majority of kinases are actually unaffected (blue marks) at the drug concentration and during the time of treatment used in this study. Drug regulated kinases were found in all the major kinase classes (red, yellow, and green marks) but some branches of the TK and AGC classes are particularly rich in regulated kinases. We identified a lot of kinases previously reported to be HSP90 clients (yellow marks) but our data also adds a further 51 down-regulated kinases not previously implicated in HSP90 regulation (red marks). Remarkably, and somewhat unexpectedly, there are 15 up-regulated kinases (green marks) including AURA, PERK, and AXL (see discussion section).

**HSP90 Inhibition and Protein Turn Over**—In order to validate the results obtained above using quantitative mass spectrometry, we repeated the geldanamycin treatment of cells and used Western blotting to follow the quantities of a number of proteins over the course of 24 h (Fig. 5A). The Western blot data for all 10 proteins was congruent with the MS data showing strong reduction of EPHA2, BTK (both known HSP90 clients), andDDR1, moderate reduction of ERK1 and BCAR1 and strong induction of AURA and AXL. STAT3 protein levels were not affected by HSP90 inhibition but phosphorylation of

**Fig. 3. Bioinformatic analysis of the HSP90 regulated proteome part I.** A, Results of GO term enrichment analysis of all four cell lines (colored). B, Example results of Ingenuity Pathway Analysis (IPA) across the geldanamycin regulated proteomes of the four cell lines analyzed. The histogram bars indicate the degree (significance) of overrepresentation of a particular pathway in a particular cell line. C, Binary protein-protein interaction map of HSP90alpha and HSP90beta extracted by IPA from the list of geldanamycin regulated proteins. Orange edges and nodes indicate down-regulation, black ones indicate up-regulation upon drug treatment (see supplemental Fig. S16 for a high resolution map).
STAT3 was completely abolished within 3 h of drug treatment. These results show that the MS data can recapitulate known HSP90 clients and be interpreted in quantitative terms. The WB time course also showed that some proteins are much more rapidly removed from cells upon drug treatment than others. We note that the proteomic profiling and WB experiments cannot measure HSP90 inhibition induced client degradation directly but instead measure the effect of HSP90 inhibition on the global quantity of proteins in a cell. Because HSP90 inhibition should lead to the rapid degradation of newly synthesized HSP90 clients, we reasoned that the observed differences in kinetics of the loss of proteins from cells may be related to the individual protein turnover rate. We therefore conducted pulsed SILAC experiments without drug treatment in all four cell lines and enriched kinases using kinobeads (Fig. 5B), which allowed the determination of protein half lives from the kinetics of stable isotope incorporation into proteins (Fig. 5C, supplemental Tables S9 and S10). For example, a half-life of 7 h was determined for the protein kinase DDR1 (32 h for EGFR; Fig. 5C), which closely follows the time required to remove 50% of DDR1 from cells upon geldanamycin treatment (Fig. 5A). To generalize this analysis, we plotted the determined protein turnover of kinases against the level of kinase down-regulation by HSP90 inhibition (24 h pulsed SILAC versus 24 h GA treatment; Fig. 5D). The plot shows that protein turn over and GA induced protein removal from cells correlate for many proteins (blue area, e.g. DDR1, EPHA2, EGFR, AKT) suggesting that only a small pool of these proteins is in complex with HSP90 at any one time so that the rate of drug induced removal from cells is primarily
determined by the rate of their normal turnover. For proteins with different behavior (e.g., BTK, CSNK1D), more complex scenarios must be discussed (see discussion section). Interestingly, the determined half-lives of proteins ranged from a few to more than a hundred hours (Fig. 5E, supplemental Tables S9 and S10) and that protein kinases have generally much shorter half-lives than other protein classes suggesting that therapeutic HSP90 inhibition will have faster effect on kinase signaling pathways than other cellular activities.

**Pharmacological Intervention with HSP90 Regulated Pathways**—Geldanamycin is a very effective HSP90 inhibitor but also a quite toxic compound. Therefore, other HSP90 inhibitors have been developed with better drug-like properties. We hence asked the question if there are strong differences in specificity between geldanamycin and the fairly novel HSP90 inhibitor PU-H71 that is currently in phase I clinical trials. To effect this, we repeated the kinobead profiling experiment using both drugs (Fig. 6A). The GA and PU data sets show a very high degree of correlation ($R^2 > 0.9$) suggesting that the mechanism by which these drugs inhibit HSP90 is similar. We next asked if global HSP90 inhibition by GA can be meaningfully combined with more targeted therapeutic approaches in cancers that are primarily driven by a specific oncogene (exemplified here by the head and neck cancer line Cal27 that is growth dependent on EGFR (36)). HSP90 inhibitors are quite toxic so reducing their dose would be clinically beneficial. Treating Cal27 cells with the highly selective EGFR inhibitor lapatinib or GA showed that a combination of the two drugs (fixed dose of 25 nM GA) is more effective than either drug alone (Fig. 6B) suggesting that such combination treatments...
might be effective (see also discussion). The molecular pathway analysis presented above (Fig. 3B) showed that mTOR signaling in Cal27 cells was strongly negatively affected by HSP90 inhibition, which would potentially represent another avenue for treating these cancer cells. To validate this hypothesis we used the potent dual PI3K/mTOR inhibitor PF-04691502, which very effectively killed Cal27 cells (and more so than the rather unspecific pan-SRC family inhibitor dasatinib). Given that the cells are growth dependent on EGFR and respond to lapatinib, this data suggests that EGFR signals into the PI3K/mTOR pathway however in an AKT independent fashion because the selective AKT inhibitor MK-2262 failed to kill the same cells (Fig. 6C). This experiment showed that the proteomic profiling data and subsequent bioinformatic analysis actually provide meaningful information that can be biologically and mechanistically interpreted.

**Preliminary Validation of Novel HSP90 Regulated Proteins**—Our global proteome profiling data does not distinguish between HSP90 regulated proteins, HSP90 client proteins, or HSP90 interactors. We therefore performed a series of affinity purification experiments to identify proteins that physically interact with HSP90. Immunoprecipitation using HSP90 antibodies and DDR1 Western blot detection showed that DDR1 is an interactor of HSP90 (and CDC37; Fig. 7A). Together with the strong regulation of DDR1 in response to geldanamycin treatment (Figs. 4A and 5A) we conclude that DDR1 is also a HSP90 client. Further HSP90 co-IP experiments using SILAC labeled Cal27 and MDAMB231 cells together with quantitative mass spectrometry (in biological triplicate) revealed ~50 significantly enriched proteins including known and potential novel HSP90 interactors (Fig. 7B, supplemental Tables S11 and S12). As a complementary approach to HSP90 IPs, we additionally performed pull-down experiments using immobilized geldanamycin from SILAC labeled MDAMB231 cells (in biological triplicate). Again, the quantitative MS analysis identified about 40 significantly drug-enriched proteins including several known HSP90 interactors (supplemental Tables S13 and S14). Finally, we compared the obtained list of physical HSP90 interactors with the 1600 geldanamycin regulated proteins in order to examine which of these proteins may qualify as HSP90 clients. About 50 such proteins were identified that are currently our best validated set of novel HSP90 client proteins (Fig. 7C, supplemental Table S15).

**DISCUSSION**

We have studied the global effects the HSP90 inhibitor geldanamycin on the proteomes of four different cancer cells. This not only allowed us broadly to define the HSP90 regu-
lated proteome but also enabled the identification of common as well as cell-type specific drug effects which will serve the community as a valuable starting point for further biological investigation. We identified 5400 proteins including almost 300 protein kinases and rigorous statistical analysis of the quantitative data showed that the expression levels of a rather unexpectedly high number of 1600 proteins was affected by the drug. Work in yeast has suggested that up to 10–20% of all proteins may be regulated by HSP90 (1, 4) and we find that this is also broadly the case in human cancer cells. Bioinformatic classification of the proteomic data faithfully recapitulated the well-known major effects of HSP90 inhibition on the up-regulation of the protein folding machinery and the strong down-regulation of kinase activity in the cell. Interestingly, bioinformatics also revealed similarities and differences between cancer cell types on the levels of signaling pathways, protein networks, and individual proteins, which has broad implications for the mode of action of the drug in these cells. This is likely also of clinical relevance because the efficacy of HSP90 inhibition varies a lot between tumor entities (10). In fact, up- as well as down-regulation of proteins in response to drug treatment could both be inhibiting (desired) and promoting (undesired) cancer cell survival and proliferation. Reduction in kinase activity will generally lead to cell death in cancer cells but the observed up-regulation of the receptor tyrosine kinase AXL may do the opposite (37). At the same time, we find that the tumor suppressor p53 is down-regulated in MDAMB231 cells, which might have adverse effects particularly if the drug is systemically used over extended periods of time (38). Likewise, the up-regulation of the chaperone machinery of the cell is probably not a desirable effect because cancer cell death may be delayed or require higher drug doses (39). Another noteworthy result of the global data analysis concerns the down-regulation of protein synthesis effected by the down-regulation of proteins all the way from ribosome biogenesis, mRNA splicing, and translation initiation. Very recently, a conceptually similar report on the global proteomic response of HeLa cells to the HSP90 inhibitor 17-DMAG has appeared in the online version of Molecular and Cellular Proteomics (13). Despite the many differences in experimental detail (different drug and dose, different cell line etc.; supplemental Fig. S19) both studies identified ~500 commonly regulated proteins (supplemental Fig. S20) that represent the two major expected global effects of HSP90 inhibition on the cellular proteome (supplemental Fig. S21). Notably, the unfolded protein response is increasing and kinase associated processes are decreasing suggesting that the experimental strategy for the identification of the HSP90 regulated proteome is valid. There are further similarities e.g. the highlighted DNA damage response although this is more pronounced in HeLa cells, which are a commonly used model system to study DNA damage. The comparison of the four cell

Fig. 7. HSP90 interactome. A, Co-immunoprecipitation and Western blot analysis reveals that DDR1 is a physical interactor of the HSP90/CDC37 complex. B, Results of HSP90 co-immunoprecipitation experiments and quantitative MS analysis from SILAC labeled Cal27 and MDAMB231 cells. The analysis in biological triplicates allowed for the identification of ~50 known and novel high confidence HSP90 interactors. C, Graphical representation of the HSP90 interactome as obtained in this study. HSP90 isoforms are shown in the center, surrounded by cochaperones in the next layer, kinases in the next layer and other proteins of diverse functions in the outer layer.
lines investigated in this study also revealed numerous differences and, not surprisingly, the Sharma study adds further complementary data most likely representing the different biologies of the different cell lines.

The very deep coverage of the cancer kinome (almost 300) obtained in this study allowed us to identify more than 50 protein kinases as potential novel targets of HSP90 inhibition. Although 90% of all kinases are down-regulated in response to the drug, 19 kinases showed the opposite behavior. Among these are a significant number of kinases involved in the cell cycle (AURA, PBK, PKAb, NEK2, ERK3, TTK, PKR). One of the effects of HSP90 inhibition is arresting cells in the cell cycle (G2/M), which may either be mediated by these kinases or result in their accumulation during the drug treatment (40, 41).

Two of the kinases are involved in stress responses (OSR1, MST3), which may explain why they are up-regulated along with other components of the cellular stress response. We serendipitously discovered that some kinases were removed from cells much faster than others upon drug treatment, which led us to investigate this phenomenon in more detail. The proteomic approach employed in this study measures the global pool of a kinase (or other protein) in a cell. Hence, we cannot directly measure the pool of protein associated with HSP90 and the observed quantitative changes are a mixture of HSP90 inhibition induced degradation and other cellular activities such as ordinary protein turn-over, induction of protein expression etc. To address the influence of the former, we conducted pulsed SILAC experiments to determine the turnover of individual proteins (mainly kinases, Fig. 5). For many of these, GA response and turnover correlate, which is consistent with an interpretation in which HSP90 inhibition leads to the rapid degradation of newly synthesized protein and the cellular pool of such proteins is depleted by their normal turn-over. For proteins that do not show this behavior, more complex scenarios must be discussed. The case in which proteins that are removed from cells by GA treatment at a faster rate than their normal protein turn-over may suggest that much of the cellular pool of the proteins is in complex with HSP90 (possibly indicating slow kinase maturation). In the reverse scenario, the regulated protein may be a HSP90 client but its expression is also induced by the drug treatment, which would result in an apparent slower removal from cells.

Yet other proteins may simply accumulate in GA treated cells which would result in an apparent slower removal from cells. This may be the case for a number of proteins, a fact that we believe has not been observed before. Given that kinase down-regulation is a major consequence of HSP90 inhibition, these differences in pharmacokinetics on individual targets may have several therapeutically relevant implications. First, it is likely that blocking kinase signaling cascades is the first and possibly major contributor to drug efficacy. Second, tumors that are driven by kinase of rapid turn-over rates (say AKT EPHA2, or DDR1, Fig. 5A) are likely to respond more quickly to the drug than tumors driven by longer lived proteins (e.g. EGRF). Third, rapid (or prolonged) removal of certain kinases may be undesirable. For example, BTK, which is an indispensable kinase for B lymphocyte development, differentiation, and signaling, will be very rapidly removed upon HSP90 targeted therapy and may therefore lead to a loss of function of immune cells of the innate as well as adaptive immune system (42, 43).

The observation that a large number of human proteins with diverse molecular functions appear to be affected by geldanamycin begs the question if different HSP90 inhibitors would have different target profiles possibly leading to different cellular outcomes. Our preliminary comparison of geldanamycin and the inhibitor PU-H71 show no drastic differences in their kinase profiles (Fig. 6). This may not be surprising as both drugs target the ATP binding site of HSP90 and thus their primary mechanism of cellular action should be similar. However, Moulick et al. recently showed by using immobilized HSP90 inhibitors (including PU-H71) that different inhibitors can favor distinct HSP90 client and co-chaperone complexes (44). This apparent discrepancy can be resolved by the fact that our kinobeads experiments purify kinases from cells (with or without interacting HSP90 and complex members) and thus monitor the outcome of the HSP90 inhibition by the drug rather than capturing the physical interaction. At least in principle, it should be possible to drug different parts of the HSP90 protein (2, 5). Once such molecules become more widely available, proteome-wide profiling experiments such as the ones described here should help to define their specificities. HSP90 inhibition is also being considered in the context of combination therapies in order to suppress resistance formation by “oncogene switching” (2, 45) or in order to extend the effective treatment time of targeted drugs such as kinase inhibitors (46). Our preliminary data using combinations of geldanamycin and the EGFR inhibitor lapatinib (Fig. 6) confirm earlier work that the drug combination is more effective than either drug alone but more work is required to establish if this concept can be generalized (47). A further pharmacologically interesting outcome of this proteomic study is the observation that certain signaling pathways (e.g. PI3K/mTOR) are more affected by HSP90 inhibition in some cancer cells than in others (Fig. 3). This may, on the one hand, merely reflect the overall activity of these pathways in the respective cell line but, on the other hand, may also highlight alternative therapeutic intervention points exemplified by the AKT independent killing of Cal27 cells by the dual PI3K/mTOR inhibitor PF-04691502 (Fig. 6). Alternatively, the discovery of highly active pathways in cancer cells using HSP90 drugs may also provide a means to sensitize cells for more targeted drugs.

It should be noted that the catalog of HSP90 regulated proteins reported here should not be mistaken for a list of
HSP90 clients. However, it is likely that HSP90 clients and physical interactors are significantly represented in the catalog. In order to identify novel interaction partners, we used HSP90 immunoprecipitation and pull-down experiments employing immobilized geldanamycin. Although classical experiments, they are somewhat complicated by the fact that the affinity of most clients to HSP90 is not very strong (12) and they may therefore be difficult to retrieve within the time frame of the biochemical experiment. Nevertheless, the HSP90 IPs purified more than 30 known HSP90 interaction partners including many of the cochaperones and client kinases (Fig. 7). In addition, the experiments highlight about 20 significant potential novel interactors. Again, these include a number of kinases (AXL, TRIO) but also GTPases and activators (ARHGAP29, SMAP1, XPOT), metabolite transporters (ASCT2, SLC3A2, GLUT1) as well as other (signaling) proteins such as the apoptosis related proteins BIRC6. It is beyond the scope of this manuscript to discuss in detail how these proteins relate to HSP90 and cancer, but a few interesting observations can be made. The protein BAG2 inhibits the chaperone activity of HSP70/HSC70 by promoting substrate release (48). Whether or not BAG2 also binds to HSP90 or is co-purified with HSC70 is currently not known. EDRF1 is a transcription factor involved in erythroid differentiation (49) but its relationship to HSP90 has not been described. FLII is a coactivator in transcriptional activation by hormone-activated nuclear receptors and is involved in estrogen hormone signaling, which is particularly relevant for breast cancer (50). ISG15 is a ubiquitin-like protein that is conjugated to several intracellular target proteins including EIF2AK2, which was found in the same IP experiment. ISG15 may thus be a ubiquitin donor in HSP90 mediated protein degradation. Finally, BIRC6 is a protein that contains a BIR (baculoviral inhibition of apoptosis protein repeat) domain and a UBCc (ubiquitin-conjugating enzyme E2, catalytic) domain and functions as an apoptosis inhibitor by promoting the degradation of apoptotic proteins by ubiquitination (51). Although many HSP90 cochaperones have been identified that often confer substrate specificity, the number of ubiquitin ligases that form part of HSP90 complexes has only recently begun to expand from the established E3-ligase CHIP (44). It is therefore tempting to speculate that BIRC6 may be part of the HSP90 ubiquitination system. Recent literature also suggests that BIRC6 is a potential therapeutic target in colon cancer stem cells, which warrants further research on this protein (52). Around 50 of the proteins that were identified in HSP90 complexes (purified by HSP90 IP or immobilized GA) are also on the list of regulated proteins of the global proteome profiling experiments. These proteins represent our currently best validated novel HSP90 clients and include several eukaryotic translation initiation factors, the ubiquitin-like protein ISG15, the protein kinases ARAF and AXL, the transcriptional coactivator FLII, and the HSP70 inhibitor BAG2.

Conclusions and Outlook—The results of this study show that the systematic identification of HSP90 regulated proteins from cancer cells by quantitative proteomics provides valid data that includes novel biological information and from which multiple starting points for further research in the area can be defined. On the technical level, one logical extension of this work would be to repeat the geldanamycin profiling experiment in the presence and absence of heat stress or proteasomal and translation inhibitors, which may provide a sharper distinction between direct effects of HSP90 inhibition and more distantly related cellular responses to drug treatment. Profiling of further cell lines from other tumor entities, profiling tumor models in rodents and comparing the effects of different HSP90 drugs on the proteome are further attractive lines of future inquiry as these may provide clinically valuable insights into pharmacokinetic details that may be used to stratify tumor entities for HSP90 targeted therapy or to predict the response of an individual’s tumor to such treatment.

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This article contains supplemental Figs. S1 to S21 and Tables S1 to S15.

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