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Protein-Selective Capture to Analyze Electrophile Adduction of Hsp90 by 4-Hydroxynonenal

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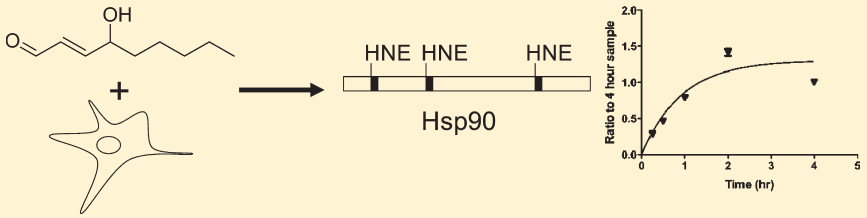
Supporting Information

ABSTRACT:



The analysis of protein modification by electrophiles is a challenging problem. Most reported protein−electrophile adducts have been characterized from in vitro reactions or through affinity capture of the adduct moiety, which enables global analyses but is poorly suited to targeted studies of specific proteins. We employed a targeted molecular probe approach to study modifications of the molecular chaperone heat shock protein 90 (Hsp90), which regulates diverse client proteins. Noncovalent affinity capture with a biotinyl−geldanamycin probe isolated both isoforms of the native protein (Hsp90α and Hsp90β) from human RKO colorectal cancer cells. Geldanamycin−biotin capture afforded higher purity Hsp90 than did immunoprecipitation and enabled detection of endogenously phosphorylated protein by liquid chromatography−tandem mass spectrometry (LC-MS/MS). We applied this approach to map and quantify adducts formed on Hsp90 by 4-hydroxynonenal (HNE) in RKO cells. LC-MS/MS analyses of tryptic digests by identified His^{450} and His^{490} of Hsp90α as having a 158 Da modification, corresponding to $NaBH_4$-reduced HNE adducts. Five histidine residues were also adducted on Hsp90β: His^{171}, His^{442}, His^{458}, His^{625}, and His^{632}. The rates of adduction at these sites were determined with Hsp90 protein in vitro and with Hsp90 in HNE-treated cells with a LC-MS/MS-based, label-free relative quantitation method. During in vitro and cell treatment with HNE, residues on Hsp90α and Hsp90β displayed adduction rates ranging from 3.0×10^{-5} h^{-1} to 1.08 ± 0.17 h^{-1}. Within the middle client-binding domain of Hsp90α, residue His^{450} demonstrated the most rapid adduction with k_{obs} of 1.08 ± 0.17 h^{-1} in HNE-treated cells. The homologous residue on Hsp90β, His^{442}, was adducted more rapidly than the N-terminal residue, His^{171}, despite very similar predicted pK_a values of both residues. The Hsp90 middle client-binding domain thus may play a signicant role in HNE-mediated disruption of Hsp90−client protein interactions. The results illustrate the utility of a protein-selective affinity capture approach for targeted analysis of electrophile adducts and their biological effects.

INTRODUCTION

Oxidative stress leads to the formation of reactive lipid-derived electrophiles. Species such as 4-hydroxy-2-nonenal (HNE) and malondialdehyde are generated from either radical or enzyme-mediated oxidation of polyunsaturated fatty acids such as arachidonic acid and linoleic acid. These species can covalently modify proteins and DNA, thus inducing mutation and changes in cellular signaling.[1−3] HNE adduction, in particular, is known to affect the activity of modified proteins.[4−6] Furthermore, HNE-adducted proteins have been identified in the plasma of children with autoimmune disease and in hepatocytes from rats fed a fat- and ethanol-rich diet, suggesting the involvement of such modifications in inflammatory disease and cancer.[4,5,7]

Recently, HNE has been shown to induce activation of heat shock transcription factor 1 and its nuclear localization in RKO cells after treatment at concentrations above 30 μM.[8] Activation of heat shock transcription factor 1 (Hsf1) occurs upon its dissociation from the heat shock chaperone complex, consisting of heat shock protein 90 (Hsp90), heat shock protein 70 (Hsp70), and Hsf1.[9] Hsp90 is a molecular chaperone with four isoforms: Hsp90α, Hsp90β, TRAP1, and Grp94. Of the two cytosolic forms, Hsp90α is inducible, whereas Hsp90β is constitutive; Grp94 is found in the endoplasmic reticulum and TRAP1 is the mitochondrial isoform. Hsp90 is an important therapeutic target for treatment of a variety of cancers dependent on chaperone-mediated stabilization of oncogenic proteins. Several classes of Hsp90 inhibitors have been discovered and developed for binding to the N-terminal ATPase domain.[10] The client proteins of Hsp90 span many classes of protein function

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and the chaperone protein can act as a repressor of proteins through inhibition by binding, as well as by stabilizing clients against degradation by the proteosome.[11,12] Hsp90 functions as a homodimer in the cytoplasm of cells, making up 1−2% of the total protein content of normal cells and up to 6% of the protein content in cancer cells.

Both Hsp90 and Hsp70 have been identified previously as targets of electrophilic modification in studies of rats fed with ethanol-rich diets and in proteomic inventories of HNE-adducted proteins in cell models.[4,5,13,14] In a previous study by Carbone et al., a single site of HNE modification of Hsp90 at Cys^{572} was identified after in vitro HNE treatment of purified protein and subsequent analysis by liquid chromatography−tandem mass spectrometry (LC-MS/MS). Modification of Hsp90 with HNE was shown to reduce the chaperone activity of the protein.[4]

The literature thus indicates that Hsp90 is a HNE target, but the only available data on site-specific adduction is from an in vitro study with purified protein. Mapping and quantitative comparison of site-specific adduction reactions on individual proteins remains a daunting analytical challenge. We and others have employed affinity-tagged electrophiles for this purpose,[13−16] but this strategy requires appropriate analogues and capture chemistries and captures all modified proteins rather than just the protein of interest. Others have used affinity-based probes that capture protein subclasses, such as kinases[17,18] or serine hydrolases,[19] but these approaches also do not enable selective targeting of individual proteins.

We addressed this problem by employing an affinity-tagged inhibitor of Hsp90 to capture the protein from cell lysates for LC-MS/MS analysis. The inhibitor is a derivative of geldanamycin, a natural product that selectively targets Hsp90 and binds in the N-terminal ATPase domain.[20] Geldanamycin induces a conformational change that results in the release of the Hsp90 client proteins and cochaperones. We demonstrate here that the commercially available reagent,[21] geldanamycin−PEG−biotin, will isolate both cytosolic forms of Hsp90. We used geldanamycin−biotin capture and LC-MS/MS to characterize sites of HNE modification of Hsp90 in RKO cells treated with exogenous HNE. We compared geldanamycin−biotin capture to Hsp90 immunoprecipitation and demonstrated detection of endogenous Hsp90 phosphorylations on protein isolated from RKO cells. We characterized adduction sites on Hsp90 captured from cells and treated in vitro with HNE. We also identified additional adduction sites on both Hsp90α and Hsp90β isolated from HNE-treated RKO cells. Reaction rates for HNE adduction at several sites on both isoforms of Hsp90 both in vitro and in intact cells were characterized by combining geldanamycin−biotin capture with targeted, label-free LC-MS/MS quantitation. Our results demonstrate the utility of protein-selective affinity capture for targeted analysis of electrophile adducts and their biological effects.

MATERIALS AND METHODS

Reagents. Geldanamycin and geldanamycin−biotin were purchased from Enzo Life Sciences (Plymouth Meeting, PA). High-capacity Neutravidin agarose resin was acquired from Thermo Scientific (Rockford, IL). All media were purchased from Invitrogen (Carlsbad, CA). Mouse monoclonal anti-Hsp90 used for Western blotting was purchased from BD Biosciences (San Jose, CA). Rabbit polyclonal anti-Hsp90 (Santa Cruz Biochemicals, Santa Cruz, CA) and protein G agarose (Roche Applied Science, Indianapolis, IN) were used for immunoprecipitation of Hsp90. Modified sequence grade porcine trypsin was purchased from Promega (Madison, WI). HNE was purchased from Cayman Chemicals (Ann Arbor, MI) or synthesized as described.[14] Unless otherwise stated, chemicals were purchased from Sigma and used as received.

Cell Culture. The human colon carcinoma cell line, RKO, was obtained from ATCC and maintained in McCoy's 5a medium supplemented with 10% fetal bovine serum. Cell treatments for less than 6 h were performed at 70−80% confluence in McCoy's 5a medium lacking serum. Treatments for longer than 6 h were performed in medium containing 5% fetal bovine serum. Cells were harvested in medium, pelleted, and then washed with 1× PBS before storage at −80C.

Isolation of Hsp90 with Geldanamycin−Biotin. Cell pellets were resuspended in lysis buffer (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.5% Igepal, 0.5 mM 4-(2-aminoethyl) benzenesulfonyl fluoride hydrochloride, 0.5 μM aprotinin, 10 μM leupeptin, 1.4 μM E-64, 20 μM betastatin, 1.5 μM pepstatin A, 1 mM NaF, and 10 mM β-glycerophosphate), sonicated, and clarified by centrifugation. Bicinchoninic acid (Thermo Fisher Scientific, Rockford, IL) was used to determine the protein concentration. Geldanamycin−biotin (40 μg in DMSO, 20 μg/μL stock) was added to 250 μL of 1 mg/mL diluted lysate and incubated at least 3 h at 4 °C. Before application to the resin, the lysate was further diluted to 0.25 mg/mL protein concentration in a volume of 1 mL. Neutravidin−agarose resin (375 μL slurry volume) was washed twice with lysis buffer before the addition of diluted lysates containing the affinity capture agent. The lysate and resin were incubated together at 4 °C overnight, after which the resin was washed four times with 1 mL of lysis buffer before elution in 150 μL of either SDS-PAGE loading buffer with 100 mM DTT or 2 mM geldanamycin−biotin in lysis buffer. The eluted proteins were purified by gel electrophoresis on an 8 cm × 8 cm, 10% polyacrylamide gel (Invitrogen) in the MOPS SDS running buffer system. The protein was analyzed by either immunoblot assay or LC-MS/MS analysis. For LC-MS/MS, the gel was stained with SafeStain Blue (Invitrogen) and the desired band was excised, reduced with 15 mM dithiothreitol for 15 min at 50 °C, alkylated with 10 mM iodoacetamide, and finally digested in-gel with trypsin (0.001 mg/mL).

Immunoprecipitation of Hsp90. Cell pellets were resuspended in lysis buffer (same as above), sonicated, and then clarified by centrifugation for 15 min at 15000*g*. Immunoprecipitations were performed with lysates diluted to 500 μg/mL of protein. Polyclonal anti-Hsp90 (20 μg) was added to 1 mL of dilute lysate and incubated with rotation overnight at 4 °C. Protein G-agarose was added to the lysate the following day and incubated at 4 °C for at least 3 h. The resin was washed four times with 1 mL of lysis buffer before elution of Hsp90 from the resin in SDS-PAGE loading buffer by heating at 95 °C for 10 min. The immunoprecipitated Hsp90 was separated from the antibody by SDS-PAGE, and either the protein band was excised and subjected to in-gel digestion and analysis by LC-MS/MS or the immunoprecipitate was analyzed by immunoblot assay.

Treatment of Isolated Hsp90 with HNE. Hsp90 was isolated from untreated RKO cells grown to a confluency of 70−80%, as described above. After washing the Neutravidin resin containing the captured Hsp90 with NETN buffer, a final wash with 1× PBS at pH 7.5 was performed. HNE was diluted in 100 μL of PBS to a concentration of 1.0 mM and added to the Hsp90 protein bound to the Neutravidin resin, which gave a final volume of 400 μL and a final HNE concentration of 250 μM. The resin was incubated for various times, with rotation at room temperature. The reaction mixture was quenched with the addition of 2 M $NaBH_4$ to 10 mM and then frozen for subsequent analysis. The Hsp90 was released from the resin and gel purified as described above. The bands corresponding to Hsp90 were excised and the protein was alkylated and digested for LC-MS/MS analysis as described above.

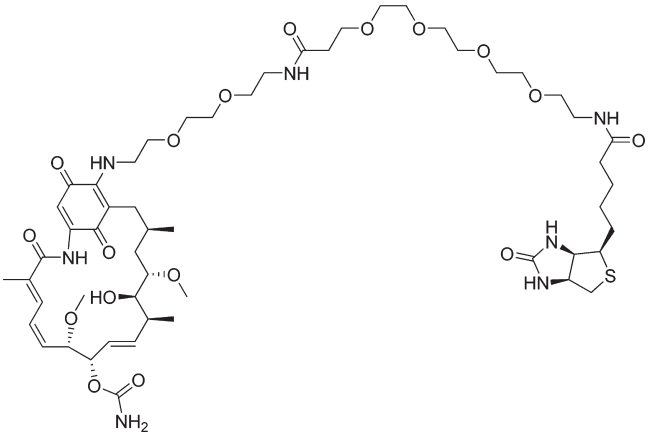
Treatment of Cells with HNE and Isolation of Hsp90. RKO cells were grown to 80−90% confluence in McCoy's 5a medium containing 10% FBS. The medium was aspirated and fresh medium with HNE was added to the cells. The cells were incubated with the electrophile for 1 h (unless otherwise noted) at 37 °C until being harvested in the culture medium. The harvested cells were washed once with 1× PBS before freezing. Cell lysates were prepared as described above. After rapid geldanamycin−biotin isolation (incubation time with geldanamycin−biotin was reduced to 1 h and incubation with neutravidin resin reduced to 1.5 h at 4 °C), neutravidin resin was washed four times with 1.0 mL of lysis buffer. The final wash was removed and 1/10 resin volume (50 μL for 500 μL bed volume resin) of 10 mM $NaBH_4$ was added to the resin. This slurry was incubated with rotation for 30 min at room temperature. After incubation, 1/5 bed volume of SDS loading buffer was added to the resin and incubated at 95 °C for 10 min to elute Hsp90. This sample was then analyzed by gel electrophoresis and the Hsp90 band was excised for digestion and analysis by LC-MS/MS as described above.

LC-MS/MS Analysis. Initial LC-MS/MS analyses of the geldanamycin capture and immunoprecipitation of Hsp90 were performed with a Thermo LTQ instrument (ThermoFisher, San Jose, CA) equipped with an Eksigent solvent delivery system, autosampler, and a microelectrospray source. Peptides were resolved on a 100 μm × 11 cm fused silica capillary column (Polymicro Technologies, LLC, Phoenix, AZ) packed with 5 μm, 300 Å Jupiter C18 (Phenomenex, Torrance, CA). Liquid chromatography was carried out at ambient temperature at a flow rate of 0.6 μL/min using a gradient mixture of 0.1% (v/v) formic acid in water (solvent A) and 0.1% (v/v) formic acid in acetonitrile (solvent B). Peptides eluting from the capillary tip were introduced into the LTQ source with a capillary voltage of approximately 2.4 kV. The heated capillary was operated at 150 °C and 40 V. MS/MS spectra were acquired in the data-dependent scanning mode, consisting of a full scan obtained for eluting peptides in the range of 350−2000 m/z, followed by four data-dependent MS/MS scans. MS/MS spectra were recorded using dynamic exclusion of previously analyzed precursors for 30 s with a repeat duration of 2 min. MS/MS data were analyzed using the SEQUEST algorithm for database searching and IDPicker for protein group assembly.[22,23]

To provide accurate mass characterization of HNE adducts on Hsp90, a Thermo LTQ-Orbitrap instrument with an Eksigent Nano 1D Plus pump and autosampler (Eksigent Technologies, Dublin, CA) was used to analyze Hsp90 samples from treated cells and from on-resin HNE treatments. Liquid chromatography was performed as described above. The data-dependent inclusion list screening was carried out with the following parameters, derived from previously described methods.[24] An Orbitrap MS scan from m/z 300−2000 at 60000 resolution was followed by 10 LTQ ion trap MS/MS scans. If eight or more ions on the inclusion list were present, then the eight most intense ions were selected for tandem MS analysis. If less than eight ions on the inclusion list were present, then those ions plus the most intense ions in the initial scan up to 8 were targeted. Dynamic exclusion was enabled, with a repeat count of 3 and a repeat duration of 10 s. The exclusion list size was 50, and the exclusion duration was 20 s. Threshold intensity for triggering peak detection was set at 100 with a collision energy of 28% for the entire list. The data were analyzed using MonsterMod, an algorithm similar to PMod,[25] to identify MS/MS spectra corresponding to Hsp90 peptides with mass shifts greater than 1 Da. Mass shifts of 158 Da corresponded to the reduced Michael adducts of HNE. Spectra of the adducted precursor and fragment ions were verified manually, with a requirement of less than 10 ppm error for peptide adduct precursor m/z measurements.

Kinetic Analysis of Hsp90 Adduction. Kinetic analysis of modification sites was performed using a label-free quantitation approach we described previously.[26] This approach measures signals for adducted peptides by LC-MS/MS using a Thermo LTQ instrument. Each peptide adduct was monitored by targeting the m/z of the doubly or triply charged precursor for MS/MS. Two unmodified peptides from each protein were also targeted in the same manner (see Supporting Information Table 2). Specific product ions generated by MS/MS fragmentation of the targeted peptide adducts and reference peptides were extracted with Thermo Xcalibur software, and peak areas were integrated. Three product ion signals were monitored for each peptide or peptide adduct and the peak area for each MS/MS transition were summed to generate a peak area for each peptide. MS/MS data for both doubly and triply charged precursor ions were acquired in some cases and the product ions yielding the greatest signal were used for subsequent analysis. The peak area of each peptide adduct was normalized to the average signal of the two unmodified reference peptides at each sample time point. The peak area reflecting HNE adduction after 24 h treatment was used as the end point for reactions with isolated Hsp90 in order to determine an observed rate of reaction. For analyses of adduction by HNE treatment in intact cells, a 4 h time point was used as an end point. Values of k_{obs} were calculated from plots of the ratio of normalized peak areas for adducted peptides at each time point to the average normalized peak area for the adducted peptide at the specified end point. Data were fitted to a single exponential association with Prism (GraphPad Software, La Jolla, CA).

Scheme 1. Chemical Structure of Hsp90 Capture Agent, Geldanamycin−Biotin



RESULTS

Geldanamycin−Biotin Isolates Both Isoforms of Hsp90 from Cell Lysates. To identify sites of HNE modification of Hsp90, a biotin-tagged analogue of the Hsp90 inhibitor, geldanamycin, was used to capture endogenous protein from the lysates of treated and untreated cells. This compound, geldanamycin−biotin (Scheme 1), binds tightly to all isoforms of Hsp90 and displaces proteins bound to Hsp90 by inducing conformational changes around the ATP-binding site.[21] As shown in Figure 1A, capture with geldanamycin−biotin was more efficient than immunoprecipitation of the native Hsp90. The captured target protein was eluted either with 2 mM geldanamycin or by boiling in SDS-PAGE loading buffer. Very little protein bound to the neutravidin resin alone, as observed in the negative control lanes shown in Figure 1A. LC-MS/MS shotgun analysis of the immunoprecipitation eluent identified Hsp90 and 79 other proteins, whereas analysis of the geldanamycin−biotin capture eluent identified only five proteins other than Hsp90. The identity of the protein band at 90 kDa was confirmed as Hsp90

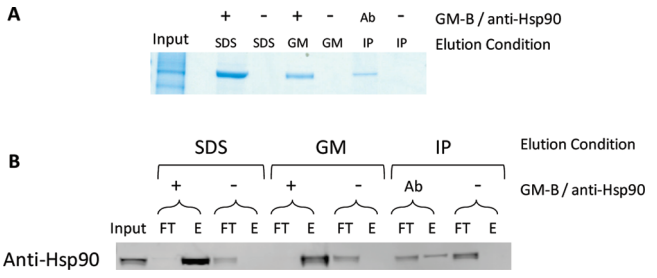


Figure 1. Geldanamycin−biotin efficiently captures Hsp90 from cell lysates. (A) SDS-PAGE analysis with Coomassie staining of Hsp90 captured with geldanamycin−biotin (GM-B) or Hsp90 antibody. The input lane contains 5 μg of cell lysate. Lanes are labeled as negative control (−), with GM-B (+), or with anti-Hsp90 (Ab). GM-B captured protein was eluted either by boiling in SDS gel loading buffer (SDS) or with 2 mM geldanamycin (GM). Immunoprecipitated Hsp90 was eluted by boiling in SDS gel loading buffer. (B) Western blot analysis with anti-Hsp90 antibody of the flow-through and elution from GM-B capture and immunoprecipitation of Hsp90. Lanes are labeled as flow-through (FT) or eluant (E), with elution and capture conditions noted as in (A). Flow-through lanes contain 2.5 μg total protein.

by immunoblotting with an anti-Hsp90 antibody (Figure 1B). (This antibody does not distinguish Hsp90α and Hsp90β, which migrate together on the gel.) The eluted Hsp90 was analyzed by LC-MS/MS to detect post-translational modifications. Sequence coverage of Hsp90 from analysis of three biological replicate samples was above 75% for both Hsp90α and Hsp90β (see Supporting Information Table 1 for representative peptide list). Hsp90 is known to be phosphorylated at two sites, Ser$^{226\beta/231\alpha}$ and Ser$^{255\beta/263\alpha}$, although the responsible kinase has not yet been identified.[27] These phosphorylation sites were routinely observed in Hsp90 isolated from untreated RKO cells (see Supporting Information Table 1 and Figures 1−2.)

Identification of Modification Sites Using in Vitro Treatment of Captured Hsp90. We identified potential sites of modification on Hsp90 using in vitro treatment of Hsp90 captured from RKO cells. Hsp90 was captured from untreated RKO cells in culture using geldanamycin−biotin and retained on Neutravidin resin after washing. HNE treatment was performed in PBS buffer on Hsp90 bound to the Neutravidin resin. Treatment for one hour with 500 μM HNE was followed by quenching with 10 mM $NaBH_4$. The modified Hsp90 was then eluted from the resin by boiling in SDS-PAGE loading buffer, purified by gel electrophoresis, and subjected to high mass accuracy LC-MS/MS analysis on a Thermo LTQ-Orbitrap. Peptide adduct ions were sampled by accurate inclusion mass screening and data-dependent MS/MS [24,28] Whereas the former method enables targeted acquisition of MS/MS spectra for selected peptides, the latter enables automated selection of peptide precursor ions based on MS spectral intensities. Ideally, the combination of these approaches enhances sampling of the most likely adducted peptide sequences while enabling discovery of unanticipated adducts. Predicted peptide adducts included potential cysteine adducts and the previously reported adduction at Cys$^{572\alpha}$. Although none of the predicted adducts were identified, we discovered five new sites of adduction on Hsp90β (His^{171}, His^{442}, His^{458}, His^{625}, and His^{632}) as shown in Table 1. MS/MS spectra of the peptide adducts are shown in Supporting Information Figures 3−7.

Although Hsp90α was present in these experiments, we did not identify adducts on this protein in the in vitro experiment.

Table 1. Hsp90 Peptides Adducted by HNE

peptide[a]	in vitro[b]	cell treatment[c]
LGIH*EDSQNR (His$^{450\alpha}$)		*
H*IYYITGETK (His$^{490\alpha}$)		*
ADH*GEPIGR (His$^{171\beta}$)	*	*
LGIH*EDSTNR (His$^{442\beta}$)	*	*
YH*TSQSGDEMTSLSEYVSR (His$^{458\beta}$)	*	
KH*LEINPDHPIVETLR (His$^{625\beta}$)	*	
HLEINPDH*PIVETLR (His$^{632\beta}$)	*	

[a] Modified residue denoted by *. [b] In vitro treatment: 500 mM HNE for 1 h in 1× PBS. [c] Cell treatment: 250 mM HNE for 1 h in serum-free medium.

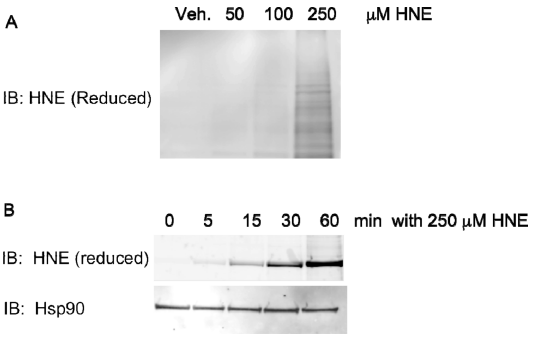


Figure 2. Analysis of HNE adduction in RKO cells. (A) Immunoblot analysis of lysates from cells treated with DMSO, 50, 100, and 250 μM HNE and detected with an antibody to reduced HNE adducts. (B) Immunoblot analysis of Hsp90 isolated from RKO cells treated with 250 μM HNE for 0, 5, 15, 30, and 60 min and detected with an antibody to reduced HNE adducts. Loading control for total Hsp90 detected with anti-Hsp90 is shown below.

Unmodified Hsp90α peptides were present in the data-dependent experiments, confirming capture of both isoforms. However, it appears that the relative amount of Hsp90α was lower than that of Hsp90β, which would reflect the relative abundance of these proteins in untreated cells. If formed, Hsp90α adducts may have been below our limit of detection. All HNE adducted peptides were identified by a +158 Da mass shift, which corresponds to reduced Michael adducts. Measurements of the precursor ions for each adducted peptide were within 10 ppm of predicted values.

Analysis of Hsp90 Modification by HNE in RKO Cells. The isolation of Hsp90 using geldanamycin−biotin allowed monitoring of the adduction of cellular Hsp90 by exogenous HNE. RKO cells were treated with HNE at concentrations of 0, 50, 100, and 250 μM HNE for one hour. The accumulation of adducted proteins was visualized by the immunoblot analysis of treated cell lysates with an anti-HNE antibody (Figure 2A). The Michael adducts of HNE in the cell lysates were reduced with 2 mM $NaBH_4$ before capture with geldanamycin−biotin. HNE adduction of Hsp90 captured from treated cells was visualized though immunoblot analysis with an antibody to reduced HNE adducts, as shown in Figure 2B. Adduction of Hsp90 by HNE was identified after only 5 min of treatment with 250 μM HNE, and the signal increased with treatment length. After isolation of Hsp90 from HNE-treated RKO cells, the protein was gel-purified for LC-MS/MS analysis. The protein band was excised, reduced, alkylated, and digested with trypsin before analysis by LC-MS/MS

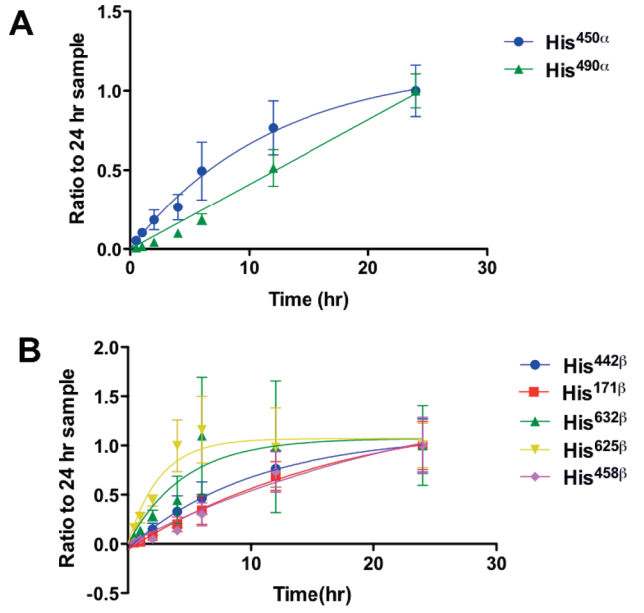


Figure 3. Kinetic analysis of HNE adduction sites during on-bead treatment as analyzed by label-free relative quantitation using LC-MS/MS. (A) Reaction rates for adduction sites on Hsp90α. Each time point represents 3−6 replicate analyses. (B) Reaction rates for adduction sites on Hsp90β. Each time point represents 3−6 replicate analyses. Nonlinear single exponential regression was used to fit the curves.

on a Thermo LTQ-Orbitrap. The Orbitrap was set with a threshold of 100 for the data-dependent scans, which included an accurate mass inclusion list of potential adducted peptide masses. As shown in Table 1, four sites of adduction were identified on Hsp90 isolated from HNE-treated RKO cells using this data-dependent method, including two new sites that were not identified during the in vitro treatment. The MS/MS spectra of these two adducts are shown in Supporting Information Figures 8 and 9. In replicate analyses, the sites most often identified were on the homologous histidine residues, His^{450} of Hsp90α and His^{442} of Hsp90β, which fall in the middle domain of the protein and are required for binding to client proteins.[29,30]

Kinetic Analysis of Hsp90 Adduction Sites during in Vitro Treatment of Isolated Protein. The adducts identified both in RKO cells and in isolated Hsp90 treated with HNE were used to create targeted methods for quantifying HNE adducts of both Hsp90α and Hsp90β. These methods were used to measure the observed reaction rates for each adduction site by analyzing the extent of modification with time. The peptides for each isoform were analyzed in separate LC-MS/MS analyses on the LTQ. The LTQ targeted the identified peptide adduct precursor ions for MS/MS, as well as precursors for two normalization peptides, which were chosen for their reproducible detection and lack of modification by HNE or by oxidation. To determine the reaction rate for each adduction site, the ratio of the adducted peptide signal to the averaged signal from the normalization peptides was calculated at each time point. This normalized signal was then plotted as a fraction of the normalized signal for the same adducted peptide at the chosen end point (24 h), as shown in Figure 3A,B. These plots were then fit using a single exponential nonlinear association to determine the k_{obs}. The measured reaction rates are presented in Table 2.

Table 2. k_{obs} Values for the Reaction of HNE with Hsp90 Peptides after On-Bead and Cell Treatments

peptide[a]	on-bead k_{obs} (h^{-1})	cell treatment k_{obs} (h^{-1})	pK_a[b]
LGIH*EDSQNR ($His^{450\alpha}$)	0.09 ± 0.01	1.08 ± 0.17	6.78 (s)
H*IYYITGETK ($His^{490\alpha}$)	3.0×10^{-5}	ND	4.44
ADH*GEPIGR ($His^{171\beta}$)	0.04 ± 0.02	0.51 ± 0.13	6.5 (s)
LGIH*EDSTNR ($His^{442\beta}$)	0.08 ± 0.02	0.54 ± 1.3	6.78 (s)
YH*TSQSGDEMT SLSEYVSR ($His^{458\beta}$)	0.04 ± 0.02	ND	4.55 (b)
KH*LEINPDHPIVETLR ($His^{625\beta}$)	0.39 ± 0.08	ND	4.59 (b)
HLEINPDH*PIVETLR ($His^{632\beta}$)	0.22 ± 0.10	ND	6.79 (s)

[a] Modified residue denoted by *. [b] Values for pK_a determined using PROPKA online pK_a calculator. (S) refers to surface residue, and (b) refers to buried residues

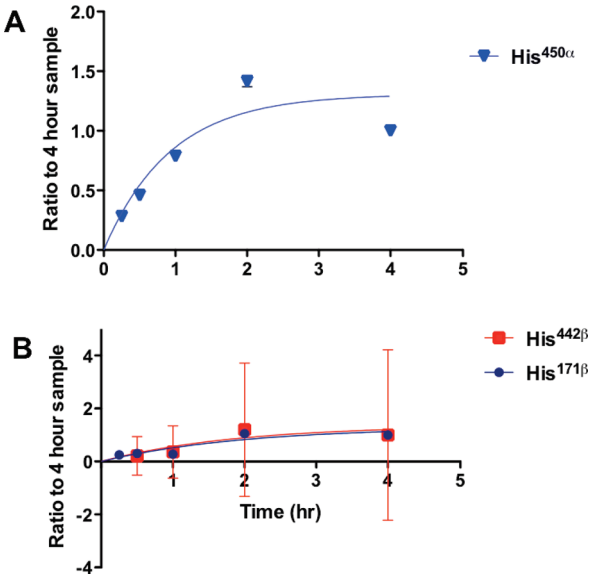


Figure 4. Determination of the relative reaction rates of HNE-modified Hsp90 peptides after treatment of RKO cells with 250 μM HNE. (A) Reaction kinetics of $His^{450\alpha}$ with HNE in Hsp90α. (B) Reaction kinetics of $His^{442\beta}$ and $His^{171\beta}$ with HNE in Hsp90β. Replicate analyses of 4−8 samples were used for each time point. Nonlinear single exponential regression was used to fit the curves.

Kinetic Analysis of HSP90 Adduction during HNE Treatment of RKO Cells in Culture. The rates of reaction for adduction after treatment of cells with HNE are shown in Figure 4. The measurement method was as described above, except that 4 h was chosen as the end point. The 4 h end point was chosen in order to maximize the potential observed adduction and to avoid complication of the analysis by biological degradation of adducted protein. The measured reaction rates for three adduction sites ($His^{442\beta}$, $His^{171\beta}$, and $His^{450\beta}$) during cell treatments with 250 μM HNE are given in Table 2, as well as the calculated pK_a for each adducted residue. Relative rates of adduction were measured using the targeted LC-MS/MS method described above. Only three adduction sites on Hsp90 were regularly observed during these experiments: $His^{450\alpha}$, $His^{442\beta}$, and $His^{171\beta}$. Reaction rate plots for these sites are shown in Figure 4A,B, and the reaction rates are given in Table 2. $His^{450\alpha}$

displayed the highest rate for both cell and in vitro treatments; however, the rate of reaction at that residue during cell treatment was found to be an order of magnitude higher than the rate determined from in vitro experiments. All of the rates of adduction observed in the cell culture experiments were higher than those observed in the in vitro treatments and ranged from 0.5 to 1 h^{-1}.

DISCUSSION

Efficient mapping of post-translational modifications on proteins often requires capture of the specific proteins of interest. Global affinity approaches based on capture of the adduct moiety do not enable selective enrichment of individual proteins. Immunoprecipitation of epitope tagged target proteins requires the development of expression constructs for each target protein and the tag itself may influence both modification of the protein and affect the level, interactions, and subcellular location of the target. Target proteins may also be captured by immunoprecipitation, but this approach is often limited by the specificity and affinity of antibodies. We demonstrate here that an affinity-tagged small-molecule inhibitor enables efficient capture and analysis of a target protein and its adducts.

This report describes the first identification of HNE adducts of Hsp90 generated within a cellular environment. Our method allowed us not only to identify these adducts but also to study rates of reaction of different target sites. To accomplish this, we have employed a high-affinity small-molecule inhibitor linked to biotin to isolate both isoforms of Hsp90 from cell lysates for analysis by immunoblotting and LC-MS/MS. In comparison with immunoprecipitation, small-molecule capture more efficiently isolates Hsp90. The small-molecule capture method does not require the introduction of epitope-tagged Hsp90 into cells, nor does it require the ectopic expression of elevated levels of protein. We were thus able to evaluate Hsp90 modifications in a native cellular context. We mapped two known phosphorylation sites on both Hsp90 isoforms isolated from untreated cultured RKO cells.

Our studies demonstrate the utility of a biotin-tagged, high-affinity ligand for targeted protein capture to map modifications generated on Hsp90 by reactive species. Using capture with geldanamycin−biotin and MS/MS analysis on a Thermo LTQ-Orbitrap, we identified two sites of adduction by HNE on Hsp90α and two sites of HNE adduction on Hsp90β from RKO cells treated with 250 μM HNE. Although this concentration is higher than endogenous levels of HNE (0.1−4 μM), it was required to obtain a detectable extent of modification.[31] It has been shown that the heat shock response, in the form of Hsf1 activation, is induced in RKO cells by concentrations of HNE greater than 50 μM.[8,32]

To characterize potential sites of modification on both isoforms of Hsp90, the protein was isolated using geldanamycin−biotin from untreated RKO cells and treated with HNE while it was retained on the Neutravidin resin after isolation. This treatment allowed the identification of four additional adduction sites on Hsp90β, with a data-dependent LC-MS/MS method supplemented with an accurate inclusion mass list for detection on a Thermo LTQ-Orbitrap. Although we regularly identified the peptide containing unmodified $Cys^{572\alpha/564\beta}$, which was previously reported as adducted by HNE,[4] we did not detect either cysteine adduct in our analyses. Had the adduct been present, it should have been detected, as our analysis workflow can detect HNE−cysteinyl Michael adducts.[13] In considering possible reasons for our failure to detect the cysteine adduct reported by Carbone et al.,[4] we note first that, according to the crystal structure of the carboxy-terminal domain of Hsp90 from *Leishmania major* (PDB: 3HJC), that residue is buried below the protein surface. Carbone et al. detected the adduct after treatment of purified protein in vitro with 0.5 mM HNE overnight, whereas our in vitro incubations were at 0.5 mM for only 1 h. It seems possible that some denaturation of the protein during the longer incubation made this cysteine available for adduction in the experiments described by Carbone et al. It is also possible that $Cys^{572\alpha/564\beta}$ adduction altered the binding of Hsp90 to the geldanamycin probe, although this seems unlikely given the ability of the probe to capture several different modified Hsp90 forms.

We also performed data-dependent analysis of HNE adducts on Hsp90 from HNE-treated RKO cells. From these experiments, two additional adducts, $His^{490\alpha}$ and $His^{450\alpha}$ and two Hsp90β adducts identified during in vitro treatments were observed. The difference in adduction sites observed between in vitro and intact cell experiments may be due to differences in the structures or composition of the Hsp90 complex in cells during HNE treatment. Furthermore, the α isoform of Hsp90 may be induced during cell treatment with HNE, thus increasing the concentration of the protein available for reaction with HNE. Two of the adduction sites we identified in Hsp90 from treated cells, $His^{450\alpha}$/$His^{442\beta}$, are homologous in Hsp90α and Hsp90β. The adduction site $His^{490\alpha}$ is found only in Hsp90α and is replaced by a serine in the β isoform. Conversely, $His^{171\beta}$ is adducted in the N-terminal region of Hsp90β, and is not present in the α isoform.

Using in vitro HNE treatment of isolated Hsp90 and targeted LC-MS/MS analysis, we were able to measure the kinetics of modification of specific residues on Hsp90 α and β. The precursor ions corresponding to the modified peptides were targeted for MS/MS. For quantification, signals for the expected fragment ions were extracted and then normalized to signals obtained from two unmodified peptides from each Hsp90 isoform. This normalization step corrected for differences in capture efficiency or recovery of Hsp90 protein and enabled data from multiple independent capture preparations to be compared. All seven adduction sites gave sufficient data for kinetic characterization. We did not track the potential doubly adducted peptide containing both $His^{632\beta}$ and $His^{625\beta}$ because we did not observe that bis-adduct in our mapping experiments. This study was only the second report, to our knowledge, to compare kinetics of protein adduction at different sites in a protein in intact cells. We previously employed a similar approach to compare kinetics of cysteine residue modifications in the sensor protein Keap1.[33]

To understand the observed kinetic reaction rates of the various adduction sites on Hsp90, we calculated the pK_a of each histidine residue using the PROPKA online algorithm (http://propka.ki.ku.dk/). We had previously found that the pK_a of His residues in human albumin correlated with measured reaction rate constants.[6] For the adducted residues of Hsp90β, it was first necessary to create a homology model containing the residues of interest. Using the Swiss-model algorithm (www.swissmodel.expasy.org), we threaded the amino acid sequence (Uniprot: PO8238) onto the crystal structure of the c-terminus of Hsp90 from *Leishmania major* (PDB: 3HJC), which displayed the highest sequence identity to the human protein. This produced a homology model with a root-mean-square deviation from the

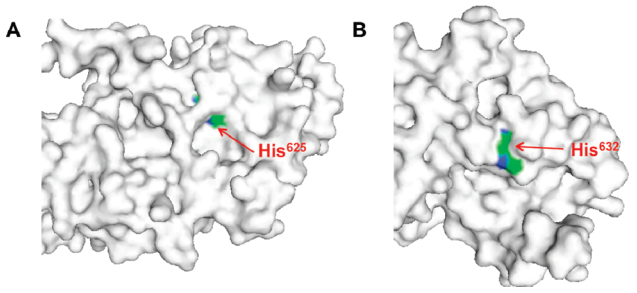


Figure 5. Comparative surface exposure for adduction sites on $His^{625\beta}$ and $His^{632\beta}$ on Hsp90β. Gray surfaces are the homology model created by threading the human Hsp90β sequence onto the structure of Hsp90 from *Leishmania major*. Adducted histidine residues are colored with green carbon atoms and blue nitrogen atoms.

original crystal structure of 2.38 Å. Using this homology model, the pK_a values of the adducted histidines were predicted (Table 2). PROPKA also predicts whether a residue is surface-exposed or buried. In particular, all the cysteines in the carboxyl-terminal region of Hsp90β except Cys^{632} are predicted to be buried. The HNE target residue, $Cys^{572\alpha/564\beta}$, previously observed by Carbone et al., was calculated to have a pK_a of 9.0 and to be buried within the structure.

From our preliminary kinetic analysis of HNE adduction during in vitro treatment of isolated Hsp90α and β, two residues, $His^{625\beta}$ and $His^{632\beta}$, displayed the highest overall reactivity, with k_{obs} rates of 0.39 and 0.22 h^{-1}, respectively. $His^{625\beta}$ reacts slightly faster than His^{632} despite the potential occlusion of its imidazole ring by a ridge on the Hsp90 protein, as shown in Figure 5A. This correlates with the predicted pK_a of $His^{625\beta}$ (4.59) being lower than the predicted pK_a of $His^{632\beta}$ (6.79), which resides directly on the surface of the protein (Figure 5B).

The homologous histidines, $His^{442\beta}$ and $His^{450\alpha}$, are characterized by similar reaction rates during the on-bead treatment. These residues are C-terminal to a region shown to be required for Hsp90 chaperone activity toward both the estrogen receptor and the progesterone receptor, although the region including $His^{442\beta}$ was not directly analyzed in the study.[29] Surprisingly, the observed reaction rates of $His^{450\alpha}$ and $His^{442\beta}$ during cellular treatment with HNE differ by a factor of 2 and both rates are almost an order of magnitude higher than the values measured for isolated Hsp90 in vitro. The same observation holds true for adduction of $His^{171\beta}$; the in vitro adduction exhibited a relatively low observed rate of 0.04 h^{-1}, whereas the rate obtained from cell treatment was 0.51 h^{-1}. This difference may be due to the altered structure of Hsp90 upon binding to geldanamycin during the on-bead reactions or to changes in the N-terminal domain with cochaperone or client protein binding in the cell.

CONCLUSION

We developed an inhibitor-based capture method for LC-MS/MS analysis of Hsp90 that affords good yield and sufficient coverage of the protein. Our analyses identified several new adduction sites on Hsp90 formed by in vitro HNE treatment of the captured proteins. We were also able to identify a smaller number of adduction sites on Hsp90 following HNE treatment of RKO cells followed by geldanamycin−biotin capture and LC-MS/MS analysis. The detection of a fewer adducts on Hsp90 in intact cells may reflect interactions with client proteins, which may obscure binding sites that are available in the isolated protein in vitro. The use of the geldanamycin−biotin probe could be adapted for studies of other reactive species thought to modify Hsp90. Moreover, the approach of using high-affinity, small-molecule probes linked to biotin offers a useful strategy for targeted analysis of protein modifications by endogenous and exogenous electrophiles.

ASSOCIATED CONTENT

Supporting Information. Representative list of Hsp90 α and β peptides; targeted peptides for relative quantitation of HNE adducts by LC-MS/MS; mass spectra identification of peptide including phosphorylation site, Hsp90β pS226: EKEIS-(79.9)DDEAEEEK; mass spectral identification of peptide including adduction site Hsp90β H171: ADH(158)GEPIGR; mass spectral identification of peptide including adduction site Hsp90β H442: LGIH(158)EDSTNR; mass spectral identification of peptide including adduction site Hsp90β H458 (YH-(158)TSQSGDEM(16)TSLSEYVSR; mass spectral identification of peptide including adduction site Hsp90β H625: KH(158)LEINPDHPIVETLR; mass spectral identification of peptide including adduction site Hsp90β H632: HLEINPDH-(158)PIVETLR; mass spectral identification of peptide including adduction site Hsp90α H450: LGIH(158)EDSQNR; mass spectral identification of peptide includinmg adduction site Hsp90α H490: H(158)IYYITGETK. This material is available free of charge via the Internet at http://pubs.acs.org.

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ABBREVIATIONS

HNE, 4-hydroxynonenal; Hsp90, heat shock protein 90; LC−MS/MS, liquid chromatography−tandem mass spectrometry; LTQ, linear ion trap quadrupole mass spectrometer; PBS, phosphate-buffered saline; RKO, human colorectal cell line

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