© American Society for Mass Spectrometry, 2016

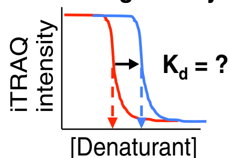
J. Am. Soc. Mass Spectrom. (2016) 27:1670–1676
DOI: 10.1007/s13361-016-1457-2

RESEARCH ARTICLE

Thermodynamic Analysis of the Geldanamycin–Hsp90 Interaction in a Whole Cell Lysate Using a Mass Spectrometry-Based Proteomics Approach

Yingrong Xu,[1,3] M. Ariel Geer Wallace,[2,3] Michael C. Fitzgerald[3]

[1]*Present Address:* Pfizer, Inc., Groton, CT 06340, USA
[2]*Present Address:* National Exposure Research Laboratory, U.S. Environmental Protection Agency, Research Triangle Park, NC 27709, USA
[3]Department of Chemistry, Duke University, Durham, NC 27708, USA



Abstract. Geldanamycin is a natural product with well-established and potent anti-cancer activities. Heat shock protein 90 (Hsp90) is the known target of geldanamycin, which directly binds to Hsp90's N-terminal ATP binding domain and inhibits Hsp90's ATPase activity. The affinity of geldanamycin for Hsp90 has been measured in multiple studies. However, there have been large discrepancies between the reported dissociation constants (i.e., K_d values), which have ranged from low nanomolar to micromolar. Here the stability of proteins from rates of oxidation (SPROX) technique was used in combination with an isobaric mass tagging strategy to measure the binding affinity of geldanamycin to unpurified Hsp90 in an MCF-7 cell lysate. The K_d values determined here were dependent on how long geldanamycin was equilibrated with the lysate prior to SPROX analysis. The K_d values determined using equilibration times of 0.5 and 24 h were 1 and 0.03 μM, respectively. These K_d values, which are similar to those previously reported in a geldanamycin–Hsp90 binding study that involved the use of a fluorescently labeled geldanamycin analogue, establish that the slow-tight binding behavior previously observed for the fluorescently labeled geldanamycin analogue is not an artifact of the fluorescent label, but rather an inherent property of the geldanamycin–Hsp90 binding interaction. The slow-tight binding property of this complex may be related to time-dependent conformational changes in Hsp90 and/or to time-dependent chemical changes in geldanamycin, both of which have been previously proposed to explain the slow-tight binding behavior of the geldanamycin–Hsp90 complex.

Keywords: Protein folding, Thermodynamic stability, Binding affinity, Heat shock protein 90, Chemical denaturation, Isobaric mass tags, Protein–ligand binding, Covalent labeling

Received: 5 May 2016/Revised: 14 July 2016/Accepted: 16 July 2016/Published Online: 16 August 2016

Introduction

Hsp90 is an important molecular chaperone protein involved in cellular signaling. Hsp90 binds to substrate proteins at a late stage of folding when proteins are nearly folded into their native conformations [1, 2], and many Hsp90 substrate proteins are involved in signal transduction. Hsp90 operates under stress conditions [2], and its function is regulated by many different protein–protein interactions involving co-chaperones such as Hsp70, Hsp40, Hop/Sti1, Aha1, p50/Cdc37, Pih1, Tah1, and p23/Sba1 [2–6]. Hsp90 inhibitors are attractive drugs for cancer therapy because Hsp90 is involved in maturation of oncogenic signaling proteins [7–11].

Geldanamycin (Figure 1) is one of several natural product Hsp90 inhibitors that exhibit anti-cancer activity by disrupting the association of Hsp90 with client proteins [12, 13]. Geldanamycin, which binds to the N-terminal ATP-binding domain of Hsp90, inhibits the ATPase activity of Hsp90 and prevents binding of a co-chaperone, p23/Sba1, to Hsp90 [14–17]. The effects of geldanamycin and a number of geldanamycin derivatives against cancer and disease states have been well-studied over the years [9, 13, 18, 19]. One measurement that has been elusive in these studies is the

Electronic supplementary material The online version of this article (doi:10.1007/s13361-016-1457-2) contains supplementary material, which is available to authorized users.

Correspondence to: Michael C. Fitzgerald;
e-mail: michael.c.fitzgerald@duke.edu

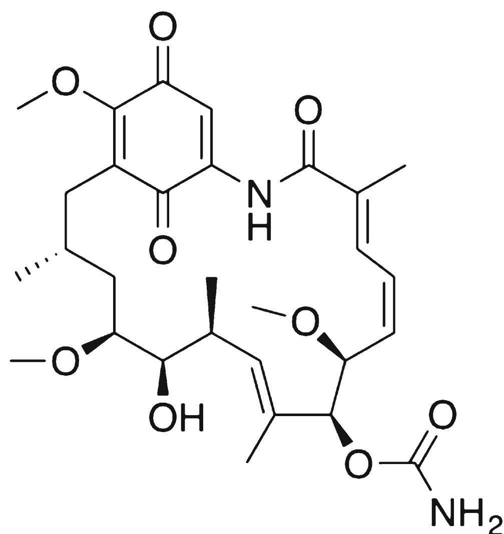


Figure 1. Chemical structure of geldanamycin

binding affinity of geldanamycin to Hsp90. Several studies on the purified protein have evaluated K_d values of the geldanamycin–Hsp90 complex in the low micromolar range [17, 20, 21]. However, IC_{50} values determined in cell-based assays have been in the low nanomolar range [21–23].

Several different hypotheses have been proposed to explain the discrepancy between the apparent binding affinity of geldanamycin to Hsp90 and its IC50 value. It has been proposed that the tumor cell environment alters the covalent structure of geldanamycin and generates a tight binding dihydroquinone analogue [24]. It has also been proposed that increased binding affinity observed in vivo is due to the presence of Hsp90 co-chaperones, such as p23/Sba1 and Hop, which are elevated in cancer cells [7]. Other researchers have proposed that the highly potent anti-cancer activity observed in cell line studies is an artifact of the intracellular accumulation of drug from the cell culture media [22]. The presence of two binding modes, one involving a fast, weak-binding event and one involving a slow, tight-binding event, has also been used to rationalize the anti-cancer effects of geldanamycin and the range of binding affinities that have been measured for the interaction of ansamycins like geldanamycin with Hsp90 [25]. Complicating interpretation of the quantitative kinetic and thermodynamic measurements, reported to date, is that they have involved the use of purified and unpurified Hsp90 samples and the use of geldanamycin and chemically modified geldanamycin analogues. One missing measurement is the geldanamycin–Hsp90 binding affinity determined using only the naturally occurring geldanamycin ligand and an unpurified Hsp90 sample.

Here we report on the thermodynamic properties of the geldanamycin–Hsp90 complex using the stability of proteins from rates of oxidation (SPROX) technique [26–29]. The SPROX technique is a mass spectrometry-based approach for measuring the thermodynamic stability of proteins and protein–ligand complexes [26, 27, 29, 30]. The technique has been previously validated using a series of different model systems, which have included both purified and unpurified proteins and protein–ligand complexes [27, 29, 30]. Of particular significance to the present study is that the SPROX technique does not require any special labeling of the ligand and that the technique is amenable to the thermodynamic analysis of protein–ligand binding reaction in whole cell lysates.

As part of this work, SPROX is used to measure the binding affinity of geldanamycin for Hsp90 in the context of an MCF-7 cell lysate. The work provides the first thermodynamic measurement of the geldanamycin–Hsp90 binding affinity without the use of a chemically modified geldanamycin ligand. The results reveal that wild-type geldanamycin has the same two binding modes previously observed with a fluorescently labeled geldanamycin derivative. The results of this work also help establish the generality of the SPROX technique for protein–ligand binding analyses.

Experimental

Materials

The MCF-7 cell line was obtained from the Duke University Cell Culture Facility (Durham, NC, USA). The following reagents were from Sigma-Aldrich (St. Louis, MO, USA): trypsin from porcine pancreas (proteomics grade, dimethylated), L-methionine, L-tryptophan, dimethyl (2-hydroxy-5-nitrobenzyl)sulfonium bromide (HNSB) (≥95 wt%), guanidinium hydrochloride (GdmCl), methyl methanethiosulfonate (MMTS), triethylammonium bicarbonate (TEAB) buffer (1.0 M, pH 8.5), sodium dodecyl sulfate (SDS), H_2O_2 (30%, w/w), ammonium acetate, 1,1,3,3-tetramethoxypropane (TMP), pyrrolidine, trichloroacetic acid (TCA), and trifluoroacetic acid (TFA). The following materials were from Thermo Scientific (Waltham, MA, USA): *tris*(2-carboxyethyl)phosphine hydrochloride (TCEP•HCl), UltraLink hydrazide resin, and Coomassie Plus Bradford Assay reagent. Methanol and isopropyl alcohol were from VWR International (West Chester, PA, USA). MacroSpin columns (Silica C18) and Pi^3 methionine reagent kits were from the Nest Group (Southborough, MA, USA). Ethanol (completely denatured) was from Avantor Performance Materials, Inc. (Phillipsburg, NJ, USA). Sodium phosphate dibasic was from EM Science (Gibbstown, NJ, USA). Sodium phosphate monobasic was from Mallinckrodt Backer, Inc. (Paris, KY, USA). Geldanamycin (≥98 wt%) was from Chem-Impex International, Inc. (Wood Dale, IL, USA). Dimethyl sulfoxide (DMSO) was from Acros (Morris, NJ, USA). Zirconia/silica beads (1 mm) were from Biospec Products (Bartlesville, OK, USA). The iTRAQ 8-plex reagents were purchased from AB Sciex (Framingham, MA, USA).

SPROX Analyses

The SPROX analyses in this work were performed according to previously described protocols [28, 31]. A total of three SPROX experiments were conducted including

two in which the standard SPROX protocol [28] was used and one in which the hybrid protocol [31] was used. In each experiment, 180 μL portions of a ~5 μg/μL MCF-7 cell lysate, prepared as described in the Supplemental Information, were used to create the (–) control and (+) drug samples that were subjected to SPROX analysis. A 20 μL aliquot of DMSO was added to the (–) control samples, and 20 μL of 500 μM geldanamycin was added to the (+) drug samples. The (–) control and (+) drug samples were incubated at RT for either 30 min (for the SPROX-Short and Hybrid-Short experiments), or 24 h (for the SPROX-Long experiments), before 20 μL aliquots of each sample were diluted into 25 μL of 20 mM phosphate buffers (pH 7.4) containing different [GdmCl]. The final [GdmCl] in each sample was 0, 0.5, 1, 1.3, 1.5, 1.7, 2, 2.5 M for the SPROX-Short and Hybrid-Short eperiments, and 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4.1 M for the SPROX-Long experiment. The final geldanamycin concentration was 20 μM. The samples were equilibrated in the denaturant buffers for 1 h, before the chemical modification reactions used in each protocol were initiated. The oxidation reactions in the standard SPROX protocol were initiated with 5 μL of 30% H_2O_2 and allowed to proceed for 3 min before 1 mL of a 300 mM L-methionine solution was added to quench the oxidation reaction. In the Hybrid protocol, 5 μL H_2O_2 was first added, and after 2 min, 2.5 μL of a 46.2 mM aqueous HNSB solution was added. At 3 min, 1 mL of a solution containing 300 mM L-methionine and 4.9 mM L-tryptophan was used to quench the reactions. The proteins in each denaturant-containing buffer were precipitated with TCA and prepared for quantitative bottom-up proteomics analysis and iTRAQ 8-plex labeling as previously reported [28].

The (–) and (+) 0, 0.5, 1, 1.3, 1.5, 1.7, 2, 2.5 M (or 0.5, 1, 1.5, 2, 2.5, 3, 3.5, 4.1 M) GdmCl samples were labeled with the 113, 114, 115, 116, 117, 118, 119, and 121 iTRAQ tags, respectively. The non-enriched samples were generated by combining 10 μL aliquots of the eight different iTRAQ labeled samples within a set, (–) or (+), diluting the combined samples with 1.4 mL of 0.1% TFA, and desalting each of the combined samples using C18 resin ( ). The methionine/tryptophan-containing peptide enriched samples were generated by combining 30 μL aliquots of the eight different iTRAQ labeled samples within a set, (–) or (+). The methionine-containing peptide enrichment was performed using the Pi^3 Methionine Reagent kit according to the manufacturer's protocol, and the tryptophan-containing peptide enrichment was performed as described in reference [31]. The methionine-containing peptide enrichment was performed in the SPROX-Short and SPROX-Long experiment; both the methionine- and tryptophan-containing peptide enrichments were performed in the Hybrid-Short experiment.

LC-MS/MS Analysis

LC-MS/MS analyses were performed on one of two mass spectrometer systems, either a Q-Exactive Plus high resolution mass spectrometer with a nanoAcquity UPLC system (Thermo Scientific) or an Orbitrap Elite ETD mass spectrometer equipped with an Easy-nLC 1000 system. Samples generated in the SPROX-Short experiment were analyzed on the Q-Exactive Plus instrument, and the Orbitrap Elite instrument was used to analyze samples generated in the Hybrid-Short and SPROX-Long experiments. Detailed information about the LC conditions and MS and MS/MS data acquisition on the different instruments is provided in the Supplemental Information.

Peak lists were extracted from the raw LC-MS/MS data files and were subjected to database search. The LC-MS/MS data from the Q-Exactive Plus instrument were searched on Spectrum Mill Workbench Software B04 Rev. B against the SwissProt *Homo Sapiens* database and the data from the Orbitrap Elite instrument were searched using Proteome Discoverer. The enzyme was set as Trypsin, and up to two missed cleavages were allowed. The following modifications were used: fixed modifications of MMTS on cysteine residues, fixed modification of iTRAQ 8-Plex on N-terminus and lysine residues, and variable modification of oxidation on methionine residues. The mass tolerances for precursor and fragment ions were set to 10 ppm and 0.8 Da (respectively) for the Orbitrap Elite, and to 15 ppm and 0.02 Da (respectively) for the Q-Exactive Plus. Only peptides with high quality quantitative data (i.e., iTRAQ reporter ion intensities at *m*/*z* 113–121 that summed to >1000), with isolation purity >70%, and with false discovery rates (FDR) <5%, were used in subsequent analyses.

SPROX Data Analysis

The SPROX data analysis was performed as previously described [28]. Briefly, iTRAQ reporter ion intensities were normalized and used to generate chemical denaturation data sets for methionine/tryptophan-containing peptides from the (–) and (+) geldanamycin SPROX experiments. Hit peptides were identified as those with significant transition midpoint shifts in the (–) and (+) geldanamycin samples. Transition midpoints were assigned using a set of criteria (see Supplemental Information) that we have previously established for the analysis of SPROX data [28, 29]. Significant transition midpoint shifts were taken to be those resulting from iTRAQ reporter ion differences in the (–) and (+) geldanamycin samples at or between the transition regions of the two chemical denaturation curves obtained with and without ligand. Significant iTRAQ reporter ion differences were defined based on a global analysis of the data in each experiment, where differences less than the 15th percentile or greater than the 85th percentile were considered significant (see Supplementary Figure S1). This requirement for hits has two consecutive iTRAQ reporter ion differences less than the 15th percentile or greater than the 85th percentile produced hit peptides with an estimated *P*-value less than 0.03. Information about how K_d values were determined from $C_{1/2}$ value shifts can be found in the Supplemental Information.

Results and Discussion

Experimental Workflow

Three SPROX experiments were performed using the experimental workflow outlined in Figure 2 to assay proteins in the MCF-7 cell lysate for geldanamycin-induced thermodynamic stability changes. The three experiments involved the use of two slightly different SPROX protocols, including one that involved the chemical modification of methionine residues in proteins with H_2O_2 (referred to hereafter as the SPROX protocol) and one that involved the chemical modification of methionine and tryptophan residues in proteins with H_2O_2 and HNSB, respectively (referred to hereafter as the Hybrid protocol). By using both methionine- and tryptophan-containing peptide probes to readout the thermodynamic stability of proteins in proteome-wide SPROX experiments, the Hybrid protocol results in a ~50% increase in proteomic coverage compared with using only methionine-containing peptide probes [31]. The three experiments also involved the use of different equilibration times. Experiments were performed using short and long equilibration times, where the MCF-7 cell lysate was equilibrated with geldanamycin for 0.5 or 24 h (respectively), prior to incubation with the chemical denaturant-containing SPROX buffers.

The proteomic coverages obtained in the three different proteome-wide experiments performed here are summarized in Table 1. As expected, the proteomic coverage achieved using the Hybrid protocol was ~50% larger than that achieved using the SPROX protocol. Between the different experiments, a total of 1188 proteins were assayed for thermodynamic stability changes induced by geldanamycin using 2756 peptide probes. The numbers of peptide and protein hits identified in each iTRAQ-SPROX experiment are listed in Table 1. The average peptide hit rate observed across the three experiments is ~1.5%, which is very close to the ~1% false-positive rate of protein target discovery previously established for iTRAQ-SPROX experiments [32]. In total, 48 unique peptides from 39 unique proteins were identified as hits in the three experiments (Supplementary Table S3). None of the hit peptides identified in the three SPROX Experiments, with the exception of those from Hsp90, displayed consistent hit behavior across the different experiments (e.g., the peptide probe was a hit in one experiment and not the other) (see Supplementary Table S3). Thus, all the peptide hits, with the exception of those from Hsp90, were deemed false-positives. In total, one tryptophan-containing and five methionine-containing Hsp90 peptide probes were assayed in these experiments. Shown in Figure 3 are iTRAQ-SPROX data sets generated for three of the peptide probes from Hsp90. The methionine and tryptophan residues in the 6 Hsp90 peptide probes assayed here are highlighted in Figure 4. The Hsp90 peptide probes derived from the N-terminal domain were consistently identified as hits (see Figure 3a, b, and c), whereas Hsp90 peptide probes from other domains of the protein were not (Figure 3d).

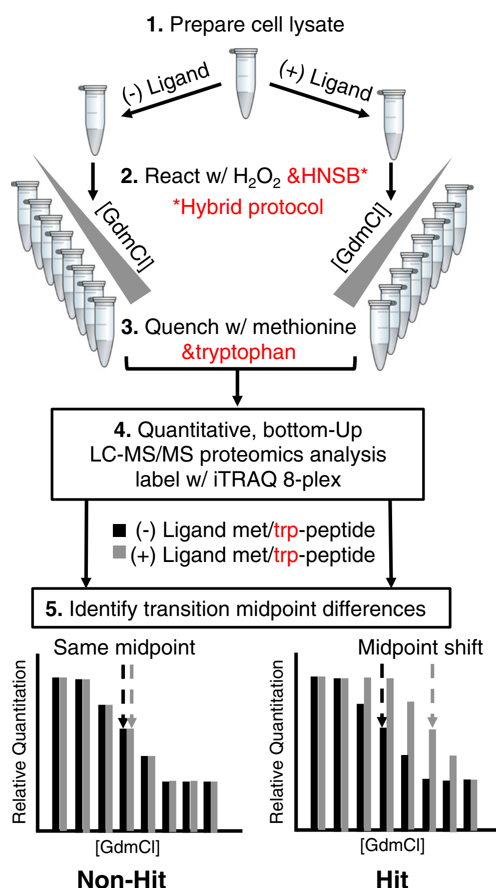


Figure 2. Schematic representation of the experimental workflow used in this work

Domain-Specific Hsp90 Stability Changes Upon Geldanamycin Binding

The location of the Hsp90 peptides assayed in the three experiments described here reveals information about the stability changes in the protein domains of Hsp90 upon binding of geldanamycin. Human Hsp90 has four domains, including an N-terminal ATP-binding domain (residues 1–224), a large middle domain (residues 293–469), a small middle domain (residues 470–547), and a C-terminal dimerization domain (residues 548–732) [33]. There is also as a disordered linker region (residues 225–292) [33]. Two of the detected methionine-containing peptide probes from Hsp90, TDTGEPM(180)GR and TLTIVDTGIGM(98)TK, mapped to the N-terminal ATP-binding domain (Figure 4b) that is

Table 1. Peptide (protein) Coverages and Hits Obtained in the three SPROX Experiments Performed Here

Experiment	Assayed[a]	Hits
SPROX-Short	719 (414)	27 (23)
Hybrid-Short	1005 (508) Met[b]	11 (10) Met[b]
	706 (446) Trp[c]	4 (4) Trp[c]
SPROX-Long	1311 (612)	8 (8)

[a] Unique peptides (proteins) identified in both the (–) and (+) geldanamycin samples.
[b] Methionine-containing peptide probe data.
[c] Tryptophan-containing peptide probe data.

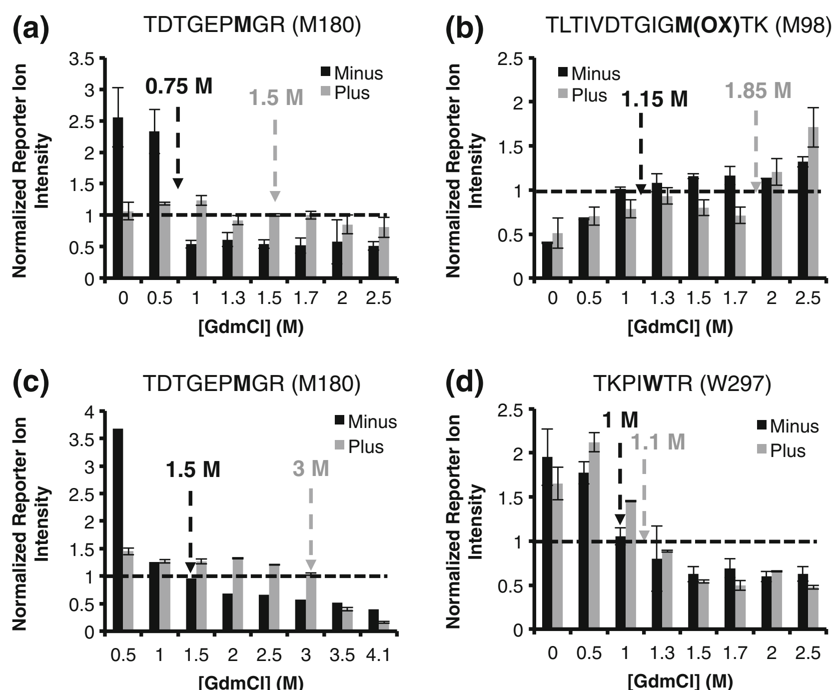


Figure 3. Chemical denaturation data recorded for selected assayed Hsp90 peptides. Shown in (**a**)–(**c**) are data for hit peptides, whereas (**d**) is for a non-hit peptide. The data in (**a**) is from the experiment using the SPROX protocol with the short equilibration time. The data in (**b**) and (**d**) are from the experiment using the Hybrid protocol with the short equilibration time, and the data in (**c**) is from the experiment using the SPROX experiment with the Long equilibration time. In cases where multiple product ion mass spectra were collected, the averaged data are shown with error bars representing ±1 standard deviation. The dotted arrows point to the $C_{1/2}$ values determined for each data set, and the horizontal dotted lines represent the normalized reporter ion intensity that best separates the pre- and post-transition baselines based on a global analysis of the data

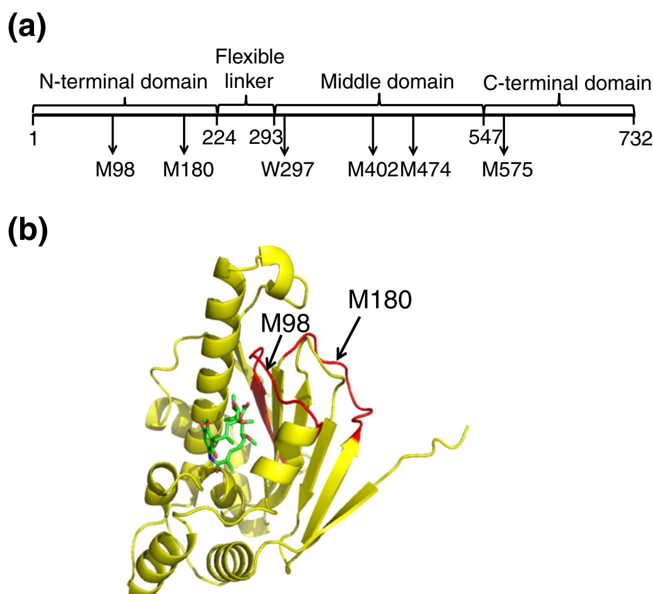


Figure 4. Schematic representation of the Hsp90 structure. (**a**) Schematic representation of the Hsp90 domain structure with the location of methionine (M) and tryptophan (W) residues in the assayed methionine- and tryptophan-containing peptides highlighted. (**b**) Crystal structure of the Hsp90 geldanamycin-binding domain (residues 9–236) with geldanamycin bound (PDB ID: 1YET [16]). The tryptic peptides containing M98 and M180 from the N-terminal domain are highlighted red

known to be the geldanamycin-binding domain [16, 34, 35]. Additional Met- and Trp-containing peptides from Hsp90 were assayed including, TKPIW(297)TR and EM(402)LQQSK from the large middle domain, YYTSASGDEM(474)VSLK from the small middle domain, as well as IM(575)KDILEK from the C-terminal domain (Figure 4a). These peptides from the large and small middle domains of Hsp90 did not show significant shifts in their $C_{1/2}$ values in the presence of geldanamycin, (see e.g., Figure 3d).

Calculation of Hsp90-Geldanamycin Binding Affinity

The SPROX data for the hit methionine-containing peptides from Hsp90 can be used to calculate a K_d value for the binding of geldanamycin to Hsp90. The chemical denaturation data collected on the Hsp90 hit peptides was used to determine $\Delta C_{1/2}$ values in

Table 2. Summary of Transition Midpoint Shifts (i.e., $\Delta C_{1/2}$ values) and K_d Values Calculated for the Hit Peptide Probes from Hsp90

Experiment	Sequence	$\Delta C_{1/2}$ (M)[a]	K_d (μM)
SPROX-Short	TDTGEPMGR (M180)	+0.75	0.8
Hybrid-Short	TLTIVDTGIGM(OX)TK (M98)	+0.7	1
SPROX-Long	TDTGEPMGR (M180)	+1.5	0.03

[a] Positive values indicate a shift to higher denaturant concentration in the plus geldanamycin samples.

each of the experiments; these $\Delta C_{1/2}$ values were used to calculate K_d values (see Table 2). The K_d values measured in the two experiments utilizing the short incubation time, 0.8 μM and 1 μM, were similar. The values were also within experimental error of a previously established K_d value of 1.2 μM for the geldanamycin binding to a purified Hsp90 sample using isothermal titration calorimetry [17].

It has been reported that two binding modes are involved in the interaction between geldanamycin and Hsp90, including a fast, weak binding event and a slow, tight binding event [25]. The short 30 min equilibration time used in the SPROX experiments described here enabled analysis of the thermodynamic properties associated with the fast, weak binding event. In order to probe the thermodynamic properties of the slow, tight binding event, a long (24 h) equilibrium time was used. Because the slow, tight binding event was expected to shift the $C_{1/2}$ value of the iTRAQ-SPROX data to a relatively high value, the SPROX experiments performed using the 24 h equilibration time utilized an extended [GdmCl] range (i.e., 0.5–4.1 M). The slow, tight binding event was detected in these experiments, and a K_d value of 0.03 μM (Table 2) was calculated using the iTRAQ-SPROX data collected using the 24 h equilibration time. The K_d value determined using the long equilibration time was ~30 times tighter than the K_d determined for the short equilibration time. The K_d value measured using the long equilibration time was also similar in magnitude to the 0.009 μM K_d value previously reported for the geldanamycin–Hsp90 complex in a mammalian cell lysate using a fluorescently-labeled geldanamycin analogue and a similarly long equilibration time in the binding assay [25].

Conclusion

The iTRAQ-SPROX methodology was used here to quantify the binding affinity of geldanamycin for Hsp90 in the context of a cell lysate derived from MCF-7 cells. The measured K_d values varied depending on the length of time geldanamycin was equilibrated with the MCF-7 cell lysate sample, prior to execution of the iTRAQ-SPROX protocol. The K_d value of 0.03 μM determined using a 24 h equilibration time was significantly lower than the K_d value of 1 μM determined using a 0.5 h equilibration time. The results are consistent with the presence of two binding modes between geldanamycin and Hsp90, including one involving a fast, weak binding interaction and one involving a slow, tight binding interaction. The present work represents the first geldanamycin–Hsp90 binding affinity measurement in a complex cell lysate that did not involve the use of a chemically modified geldanamycin analogue. The K_d values associated with the two geldanamycin binding modes observed here are consistent with those previously observed in binding studies conducted using a fluorescently labeled geldanamycin analogue and Hsp90 in a mammalian cell lysate [25]. These results establish that the slow, tight-binding behavior previously observed for the fluorescently labeled geldanamycin analogue is not an artifact of the fluorescent label, but rather an inherent property of the geldanamycin–Hsp90 binding interaction. The presence of time-dependent conformational changes in Hsp90 and time-dependent chemical changes in geldanamycin have both been proposed to explain the slow, tight binding behavior of the geldanamycin–Hsp90 complex [25, 36]. Either one or both of these explanations is consistent with the iTRAQ-SPROX results reported here.

Acknowledgment

The authors thank the Proteomics Facility at the Fred Hutchinson Cancer Research Center for collecting the LC-MS/MS data in Hybrid-Short and SRPOX-Long experiments. They also thank the Duke Proteomics Facility for collecting the LC-MS/MS data in the SPROX-Short experiment. This work was supported by a grant from the National Science Foundation (CHE-1308093) to M.C.F.

References

1. Pearl, L.H., Prodromou, C.: Structure, function, and mechanism of the Hsp90 molecular chaperone. Adv. Protein Chem. **59**, 157–186 (2002)
2. Zhao, R., Davey, M., Hsu, Y.C., Kaplanek, P., Tong, A., Parsons, A.B.: Navigating the chaperone network: an integrative map of physical and genetic interactions mediated by the hsp90 chaperone. Cell **120**, 715–727 (2005)
3. Millson, S.H., Vaughan, C.K., Zhai, C., Ali, M.M., Panaretou, B., Piper, P.W.: Chaperone ligand-discrimination by the TPR-domain protein Tah1. Biochem. J. **413**, 261–268 (2008)
4. Millson, S.H., Truman, A.W., Wolfram, F., King, V., Panaretou, B., Prodromou, C.: Investigating the protein-protein interactions of the yeast Hsp90 chaperone system by two-hybrid analysis: potential uses and limitations of this approach. Cell Stress Chaperones **9**, 359–368 (2004)
5. Eckert, K., Saliou, J.M., Monlezun, L., Vigouroux, A., Atmane, N., Caillat, C.: The Pih1-Tah1 cochaperone complex inhibits Hsp90 molecular chaperone ATPase activity. J. Biol. Chem. **285**, 31304–31312 (2010)
6. Johnson, J.L., Halas, A., Flom, G.: Nucleotide-dependent interaction of *Saccharomyces cerevisiae* Hsp90 with the cochaperone proteins Sti1, Cpr6, and Sba1. Mol. Cell. Biol. **27**, 768–776 (2007)
7. Kamal, A., Thao, L., Sensintaffar, J., Zhang, L., Boehm, M.F., Fritz, L.C.: A high-affinity conformation of Hsp90 confers tumour selectivity on Hsp90 inhibitors. Nature **425**, 407–410 (2003)
8. Fukuyo, Y., Hunt, C.R., Horikoshi, N.: Geldanamycin and its anti-cancer activities. Cancer Lett. **290**, 24–35 (2010)
9. Onuoha, S.C., Mukund, S.R., Coulstock, E.T., Sengerova, B., Shaw, J., McLaughlin, S.H.: Mechanistic studies on Hsp90 inhibition by ansamycin derivatives. J. Mol. Biol. **372**, 287–297 (2007)
10. Cardenas, M.E., Cruz, M.C., Del Poeta, M., Chung, N., Perfect, J.R., Heitman, J.: Antifungal activities of antineoplastic agents: *Saccharomyces cerevisiae* as a model system to study drug action. Clin. Microbiol. Rev. **12**, 583–611 (1999)
11. Cazal, C.M., Choosang, K., Severino, V.G.P., Soares, M.S., Sarria, A.L.F., Fernandes, J.B.: Evaluation of effect of triterpenes and limonoids on cell growth, cell cycle and apoptosis in human tumor cell lines. Anti Cancer Agent Med. Chem. **10**, 769–776 (2010)
12. Whitesell, L., Mimnaugh, E.G., De Costa, B., Myers, C.E., Neckers, L.M.: Inhibition of heat shock protein HSP90-pp60v-src heteroprotein complex formation by benzoquinone ansamycins: essential role for stress proteins in oncogenic transformation. Proc. Natl. Acad. Sci. U. S. A. **91**, 8324–8328 (1994)
13. Franke, J., Eichner, S., Zeilinger, C., Kirschning, A.: Targeting heat-shock-protein 90 (Hsp90) by natural products: geldanamycin, a show case in cancer therapy. Nat. Prod. Rep. **30**, 1299–1323 (2013)
14. Ali, M.M., Roe, S.M., Vaughan, C.K., Meyer, P., Panaretou, B., Piper, P.W.: Crystal structure of an Hsp90-nucleotide-p23/Sba1 closed chaperone complex. Nature **440**, 1013–1017 (2006)

15. Karagoz, G.E., Duarte, A.M., Ippel, H., Uetrecht, C., Sinnige, T., van Rosmalen, M.: N-terminal domain of human Hsp90 triggers binding to the cochaperone p23. Proc. Natl. Acad. Sci. U. S. A. **108**, 580–585 (2011)
16. Stebbins, C.E., Russo, A.A., Schneider, C., Rosen, N., Hartl, F.U., Pavletich, N.P.: Crystal structure of an Hsp90–geldanamycin complex: targeting of a protein chaperone by an antitumor agent. Cell **89**, 239–250 (1997)
17. Roe, S.M., Prodromou, C., O'Brien, R., Ladbury, J.E., Piper, P.W., Pearl, L.H.: Structural basis for inhibition of the Hsp90 molecular chaperone by the antitumor antibiotics radicicol and geldanamycin. J. Med. Chem. **42**, 260–266 (1999)
18. Patel, K., Piagentini, M., Rascher, A., Tian, Z.Q., Buchanan, G.O., Regentin, R.: Engineered biosynthesis of geldanamycin analogs for Hsp90 inhibition. Chem. Biol. **11**, 1625–1633 (2004)
19. Hitotsuyanagi, Y., Mitsui, K., Fukaya, H., Takeya, K.: Fluorinated and rearranged gedunin derivatives. Phytochem. Lett. **5**, 486–489 (2012)
20. Chiosis, G., Rosen, N., Sepp-Lorenzino, L.: LY294002-geldanamycin heterodimers as selective inhibitors of the PI3K and PI3K-related family. Bioorg. Med. Chem. Lett. **11**, 909–913 (2001)
21. Panaretou, B., Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W.: ATP binding and hydrolysis are essential to the function of the Hsp90 molecular chaperone in vivo. EMBO J. **17**, 4829–4836 (1998)
22. Chiosis, G., Huezo, H., Rosen, N., Mimnaugh, E., Whitesell, L., Neckers, L.: 17AAG: low target binding affinity and potent cell activity—finding an explanation. Mol. Cancer Ther. **2**, 123–129 (2003)
23. Neckers, L.: Heat shock protein 90 inhibition by 17-allylamino-17-demethoxygeldanamycin: a novel therapeutic approach for treating hormone-refractory prostate cancer—Commentary. Clin. Cancer Res. **8**, 962–966 (2002)
24. Maroney, A.C., Marugan, J.J., Mezzasalma, T.M., Barnakov, A.N., Garrabrant, T.A., Weaner, L.E.: Dihydroquinone ansamycins: toward resolving the conflict between low in vitro affinity and high cellular potency of geldanamycin derivatives. Biochemistry **45**, 5678–5685 (2006)
25. Gooljarsingh, L.T., Fernandes, C., Yan, K., Zhang, H., Grooms, M., Johanson, K.: A biochemical rationale for the anti-cancer effects of Hsp90 inhibitors: slow, tight binding inhibition by geldanamycin and its analogues. Proc. Natl. Acad. Sci. U. S. A. **103**, 7625–7630 (2006)
26. West, G.M., Tang, L., Fitzgerald, M.C.: Thermodynamic analysis of protein stability and ligand binding using a chemical modification- and mass spectrometry-based strategy. Anal. Chem. **80**, 4175–4185 (2008)
27. West, G.M., Tucker, C.L., Xu, T., Park, S.K., Han, X., Yates III, J.R.: Quantitative proteomics approach for identifying protein-drug interactions in complex mixtures using protein stability measurements. Proc. Natl. Acad. Sci. U. S. A. **107**, 9078–9082 (2010)
28. Strickland, E.C., Geer, M.A., Tran, D.T., Adhikari, J., West, G.M., DeArmond, P.D., Xu, Y., Fitzgerald, M.C.: Thermodynamic analysis of protein-ligand binding interactions in complex biological mixtures using the stability of proteins from rates of oxidation. Nat. Protoc. **8**, 148–161 (2013)
29. Geer, M.A., Fitzgerald, M.C.: Characterization of the *Saccharomyces cerevisiae* ATP-Interactome using the iTRAQ-SPROX technique. J. Am. Soc. Mass Spectrom. **27**, 233–243 (2016)
30. Dearmond, P.D., Xu, Y., Strickland, E.C., Daniels, K.G., Fitzgerald, M.C.: Thermodynamic analysis of protein–ligand interactions in complex biological mixtures using a shotgun proteomics approach. J. Proteome Res. **10**, 4948–4958 (2011)
31. Xu, Y., Strickland, E.C., Fitzgerald, M.C.: Thermodynamic analysis of protein folding and stability using a tryptophan modification protocol. Anal. Chem. **86**, 7041–7048 (2014)
32. Strickland, E.C., Geer, M.A., Hong, J., Fitzgerald, M.C.: False-positive rate determination of protein target discovery using a covalent modification- and mass spectrometry-based proteomics platform. J. Am. Soc. Mass Spectrom. **25**, 132–140 (2014)
33. Lee, C.C., Lin, T.W., Ko, T.P., Wang, A.H.: The hexameric structures of human heat shock protein 90. PLoS One **6**, e19961 (2011)
34. Prodromou, C., Roe, S.M., O'Brien, R., Ladbury, J.E., Piper, P.W., Pearl, L.H.: Identification and structural characterization of the ATP/ADP-binding site in the Hsp90 molecular chaperone. Cell **90**, 65–75 (1997)
35. Grenert, J.P., Sullivan, W.P., Fadden, P., Haystead, T.A., Clark, J., Mimnaugh, E.: The amino-terminal domain of heat shock protein 90 (hsp90) that binds geldanamycin is an ATP/ADP switch domain that regulates hsp90 conformation. J. Biol. Chem. **272**, 23843–23850 (1997)
36. Onuoha, S.C., Mukund, S.R., Coulstock, E.T., Sengerova, B., Shaw, J., McLaughlin, S.H., Jackson, S.E.: Mechanistic studies on Hsp90 inhibition by ansamycin derivatives. J. Mol. Biol. **372**, 287–297 (2007)