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Distinguishing Allosteric effects from orthosteric binding in Protein-Ligand interactions --Manuscript Draft--

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Abstract:	A fundamental challenge in deciphering protein-ligand interactions is distinguishing binding changes at orthosteric sites from the associated allosteric changes at distal sites, as structural data does not always reveal allostery. Ligands mediate both orthosteric and allosteric effects on target proteins and hence in the context of screening low affinity fragments, it is important to describe fragment efficacy in terms of both direct binding and long-range allosteric responses. This presents a significant problem especially for low affinity ligands. Amide Hydrogen Deuterium Exchange mass spectrometry (HDXMS) is a robust method that can provide structural insights and information on conformational dynamics on both high affinity and transient protein-ligand interactions. Here, we describe the use of HDXMS on the ATPase domain of Hsp90, to parse orthosteric and allosteric effects mediated by two high affinity ligands and two low affinity fragment compounds. A comparison of deuterium exchange in ligand-bound-Hsp90 versus apo-Hsp90 was used to describe composite changes that combine both orthosteric effects and allosteric changes. Allostery can be discerned by correlating HDXMS results with structural information of orthosteric binding from crystallographic structures of protein-ligand interactions. Results from this approach indicate that fragments and ligands both mediate interactions at overlapping orthosteric sites but elicit distinct allosteric effects. However, orthosteric interactions of Hsp90 with fragments are inherently weaker due to faster dissociation rates (k _{off}). This finds important applications in fragment screening, ranking and lead compound design in fragment-based drug discovery.
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Question	Response
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below and explain in your cover letter.

Dr. Avital Braiman,
Director of Editorial,
Journal of Visualized Experiments

Dear Dr. Braiman,

Please find enclosed a manuscript titled '***Distinguishing Allosteric effects from orthosteric binding in Protein-Ligand***' to be considered for publication in *Journal of Visualized experiments*.

We thank the editors of Journal of Visualized experiments for inviting us to contribute this article on separating allosteric effects from orthosteric binding. Deciphering allosteric relays in protein complexes and distinguishing them from orthosteric effects is of great importance in drug discovery. HDXMS has proven to be a powerful tool to probe protein dynamics in response to various perturbations. Here, we have described the use of two different facets of HDXMS: to monitor orthosteric binding and effects, from differences in deuterium exchange, and to identify allosteric effects from kinetic analysis. Our results clearly demonstrate the use of this technique in identification of early orthosteric binding events and later allosteric events in the Heat shock protein, Hsp90, during ligand/fragment binding. This approach has a categorical implication in fragment based drug design and lead-compound optimization.

This manuscript is of great importance since it describes in detail an experimental HDXMS approach to distinguish between two distinct responses elicited by ligands/fragments: Orthosteric binding and Allosteric effects. These have broad significances in accelerating the understanding of allosteric effects and in fragment-based drug design. Importantly, this highlights the power of amide hydrogen/deuterium exchange mass spectrometry (HDXMS) in distinguishing between binding and allosteric events in protein-ligand interactions by deuterium uptake and kinetic analyses.

We hope you will find our manuscript of interest to readers of *Journal of Visualized experiments* and consider it for publication.

Thank you.
Sincerely

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Dr. Alisha DSouza,
Review Editor,
Journal of Visualized Experiments

Dear Dr. DSouza,

Thank you for giving us the opportunity to respond to the comments and suggestions provided by the reviewers. We thank the reviewers for their encouraging, excellent and constructive feedback. We have taken into consideration all of their feedback and have addressed their concerns.

These changes have greatly improved our manuscript and we thank the reviewers for their suggestions. We have also provided detailed responses to the reviewer's comments and have included this as a separate file with this resubmission. We have also made other suggested changes based on the editorial comments. All the changes made in the manuscript have also been tracked and highlighted.

We hope that these changes/additional data have satisfactorily addressed the reviewers' feedback and that this work will be of great interest to the readers of *Journal of Visualized experiments*.

Thank you.
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TITLE:

Distinguishing Allosteric Effects from Orthosteric Binding in Protein-Ligand Interactions

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KEYWORDS:

Protein-ligand interactions, fragment binding, HDXMS, fragment-based drug design, allostery, orthosteric binding, Hsp90.

SHORT ABSTRACT:

Application of amide hydrogen-deuterium exchange mass spectrometry to map interactions of low affinity fragment and ligands is demonstrated. This protocol describes a method for distinguishing orthosteric binding from allosteric changes accompanying high affinity ligand and low-affinity fragment binding to target protein, Hsp90, and finds important applications in fragment-based drug design.

LONG ABSTRACT:

A fundamental challenge in deciphering protein-ligand interactions is distinguishing binding changes at orthosteric sites from the associated allosteric changes at distal sites, as structural data does not always reveal allostery. Ligands mediate both orthosteric and allosteric effects on target proteins and hence, in the context of screening low affinity fragments, it is important to describe fragment efficacy in terms of both direct binding and long-range allosteric responses. This presents a significant problem especially for low affinity ligands. Amide Hydrogen Deuterium Exchange Mass Spectrometry (HDXMS) is a robust method that can provide structural insights and information on conformational dynamics for both high affinity and transient protein-ligand interactions. Here, we describe the use of HDXMS on the ATPase domain of Hsp90, to parse orthosteric and allosteric effects mediated by two high affinity ligands and two low affinity fragment compounds. A comparison of deuterium exchange in ligand-bound-Hsp90 versus apo-Hsp90 was used to describe composite changes that combine both orthosteric effects and allosteric changes. Allostery can be discerned by correlating HDXMS results with structural

information about orthosteric binding from crystallographic structures of protein-ligand interactions. Results from this approach indicated that fragments and ligands both mediate interactions at overlapping orthosteric sites but elicit distinct allosteric effects. However, orthosteric interactions of Hsp90 with fragments are inherently weaker due to faster dissociation rates (k_{off}). This approach finds important applications in fragment screening, ranking, and lead compound design in fragment-based drug discovery.

INTRODUCTION:

Drug development necessitates a complete understanding of the interaction of natural ligands with their target proteins, and utilizes this information to find alternate inhibitors or activators. Traditional drug development pipelines involve a high throughput screening (HTS) strategy to identify lead compounds¹. An alternative strategy is to use fragments as building blocks for lead compound generation. These have multiple advantages over traditional HTS strategies, including but not limited to, being intellectual property-free, optimizable, and modular². Fragments are defined as small chemical compounds (<300 Da) which mediate fewer than three H-bonding contacts with their target proteins³. Fragments are essentially the active moieties of drug molecules. Characterization of fragment-protein interactions poses unique challenges to current structural biology methods due to their low-binding affinities. Another important drawback of structural biology tools, such as X-ray crystallography and cryo-EM, is that they provide insights into kinetically constrained endpoint states which primarily provide information on orthosteric binding contacts between ligands and proteins. This is especially relevant in structures of protein-ligand interactions obtained by soaking ligands with protein crystals, where large-scale conformational movements in solution upon ligand binding, are likely to be undetected. X-ray crystallography also requires extensive optimization for crystallization and only provides a static structure of proteins. However, proteins in solution are dynamic molecules and this dynamics is important for their function⁴. In addition, monitoring proteins in solution offers the additional advantage of capturing transient intermediate changes. Hence, in order to comprehensively map binding effects of ligands to proteins, we need a dynamic overview in addition to the structural information available⁵. Nuclear magnetic resonance (NMR) spectroscopy can provide dynamic structural information but is limited by its analyte size and suffers from sensitivity issues. Additional techniques such as surface plasmon resonance (SPR)⁶ and bio-layer interferometry (BLI)⁷ can sensitively detect structural changes and capture the binding kinetics of protein-ligand interactions, but do not provide any local structural information. Consequently, capturing dynamic changes in both orthosteric binding sites and allosteric sites, with local structural information and binding kinetics, is critical to provide a systemic model for protein-ligand interactions⁸.

Hence, it is essential to work with a more holistic model of protein-ligand interactions, which includes both orthosteric and allosteric changes^{9,10}. The large body of available structural information on protein-ligand complexes is limited to details of binding interactions at orthosteric sites. This lack of information on changes at non-orthosteric regions upon ligand binding necessitates a complete description of the changes across the protein in solution. Protein dynamics has been shown to play an important role in distal allosteric communication and modulation, and hence capturing changes in conformational dynamics is crucial to develop a

systemic model for ligand binding^{11,12} that can be extended to fragment protein interactions. Amide hydrogen-deuterium exchange mass spectrometry (HDXMS) provides a map of the protein dynamics in solution at peptide-resolution, by measuring rates of deuterium uptake at peptide reporters across the protein. HDXMS measures changes in H-bonding and solvent accessibility in backbone amide hydrogens (H-bonding plays a major role in determining deuterium uptake rates) in protein-drug interactions¹³. Since H-bonds play an important role in protein-ligand interactions, HDXMS is uniquely poised to monitor ligand binding¹⁴ and has recently emerged as an important tool for biopharmaceutical discovery and development¹⁵⁻¹⁷. It offers significant advantages in studying protein-ligand complexes, which include no limitations on target protein size, ability to analyze proteins in physiological solution states without the need for concentrated protein samples, two advantages which eliminate artefacts due to aggregation and crowding.

A comparative analysis of deuterium exchange across multiple peptide reporters in the presence and absence of a ligand provides a protein-wide map of the changes in solution dynamics due to ligand binding^{18,19}. This offers a read-out of protein dynamics from seconds to longer timescales, determined by the deuterium labelling time^{20,21}. HDXMS of protein-ligand complexes reports on both orthosteric changes at the binding site and long-range conformational changes at allosteric sites, in response to ligand binding^{22,23}. Overlaying information on protein dynamics with structural data from orthosteric sites enables us to describe long-range conformational changes distal from binding sites. A complete description of both these changes has important applications in describing the interactions of low-affinity fragments with proteins. An approach to map these composite changes involves an initial dynamic description of the natural inhibitors or tight-binding ligands, which provides a standard reference to compare the binding effects of fragments. This initial interaction map of natural ligands acts as the reference fingerprint to compare different fragments to test their binding interactions. The reference fingerprint includes information on the peptide-reporters and magnitude of deuterium uptake changes and kinetics of these changes.

Here, we apply HDXMS to describe orthosteric and allosteric changes in response to the binding of fragments and high-affinity ligands to the N-terminal ATPase domain of Hsp90²⁴⁻²⁸. The protocol focuses initially on Hsp90 and its interactions with two of its high-affinity ligands purified from natural sources: Radicicol²⁹ and 7-N-Allylamino-17-demethoxygeldanamycin (17-AAG)³⁰. The composite changes are differentiated into orthosteric and allosteric changes based on crystallographic structures to identify the regions and HDXMS-specific peptide-reporters that correspond to these respective changes. This information can then be expanded to map the effects of two low affinity fragments, the phenolic compounds, Methyl 3,5-Dihydroxyphenylacetate (Fragment 1) and 2,4 Dihydroxypropiophenone (Fragment 2)^{24,31} with dissociation constants of ~500 μ M. Further, a workflow is described for the application of this approach to fragment screening for generating a ranking system to sort fragments based on the magnitude of changes in protein dynamics at various loci.

The main advantage of this approach is its wide-applicability to any protein or multi-protein complex. HDXMS studies of proteins have been carried out in various environments, for instance membrane proteins can be characterized in membrane-mimetic nano-discs, detergents, and as

assembled macromolecular complexes such as viruses. These highlight the robustness of the approach in describing the dynamics of a wide range of protein targets. HDXMS analyses of the binding of peptide-inhibitors to their target protein offer additional insights into complementary interface residues on the inhibitor end. Since HDXMS involves no disruptive labels, dyes, or specific osmolyte conditions, protein-ligand interactions can be monitored in solution at physiologically similar conditions. These also offer the possibility of studying these interactions with different physical perturbants such as temperature, osmolyte, pH, and other perturbants such as lipids, nucleic acids, and other proteins.

PROTOCOL:

1. Preparing D₂O buffer, Quench and Hsp90 Protein Solutions

1.1. Prepare 100 μ M Hsp90 protein solution (expressed in *E. coli*²²) in Hsp90 aqueous buffer (20 mM HEPES (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid), 300 mM NaCl, 10% (v/v) glycerol, 0.5 mM TCEP (tris(2-carboxyethyl)phosphine), pH 7.5).

1.2. Prepare Hsp90 D₂O buffer by vacuum evaporation of the Hsp90 aqueous buffer. Dry the Hsp90 aqueous buffer by vacuum evaporation to remove H₂O. Subsequently, reconstitute the dried buffer constituents with an equivalent volume of D₂O to make the Hsp90 D₂O buffer.

1.2.1. pH stability is critical. Ensure that the pH of buffers is stable and measured accurately. Control pH and pH_{read} carefully at each step of the process.

1.2.2. Prepare sufficient aliquots of Hsp90 D₂O buffer for the entire set of experiments from a single buffer solution batch to minimize variability from factors such as pH, temperature, concentrations, and buffer constituents.

1.2.3. Optimize and determine specific temperature, concentrations, and buffer constituents for each protein-ligand system. Refer to^{32,33} for in-depth methodological support.

1.3. Determine deuterium exchange reaction ratios (Ratio of Hsp90 protein solution: ligands in dimethyl-sulfoxide (DMSO): Hsp90 D₂O buffer: Hsp90 quench solution) to achieve the highest deuterium concentration by maximizing the ratio of D₂O buffer.

1.3.1. For Hsp90 deuterium exchange reactions, mix 1 μ L of 100 μ M Hsp90 protein solution (from step 1.1) with 2 μ L of ligand in DMSO/water (for ligand-bound experiments) or 2 μ L of DMSO/water-blank (for ligand-free condition) and 27 μ L of Hsp90 D₂O buffer (from step 1.2) to achieve a final D₂O concentration of 90% and a final Hsp90 protein concentration of 3.3 μ M in a 30 μ L deuterium exchange reaction volume.

1.3.2. If needed, concentrate protein solutions prior to initiating the deuterium exchange reaction. For lower concentrations of proteins, use 2 – 4 μ L of protein and adjust the deuterium exchange reaction volumes accordingly to obtain the highest D₂O concentration achievable; e.g.,

increase total reaction volume to 100 - 250 μ L.

1.4. Prepare ligand solutions in water (for high-affinity inhibitors) or DMSO (for low-affinity fragments).

Note: The concentrations of ligand solutions are determined to ensure that the ligand concentration saturates the binding site under the final deuterium exchange reaction conditions (step 1.3.1).

1.4.1. Subsequently, for mapping high-affinity inhibitor binding of radicicol and 17-AAG, prepare 300 μ M radicicol and 17-AAG inhibitor solutions in DMSO to maintain final deuterium exchange reaction concentrations (step 1.3.1) of radicicol (K_D = 19 nM) and 17-AAG (K_D = 33 nM)³³ at 20 μ M with a final Hsp90 protein concentration of 3.3 μ M (6:1 ligand to target protein ratio) to saturate the Hsp90 binding site with inhibitors.

1.4.2. For the low affinity fragments, prepare 75 mM solutions of Fragment 1 and Fragment 2 in DMSO to maintain final deuterium exchange reaction concentrations of Fragment 1 (K_D = 490 μ M) and Fragment 2 (K_D = 570 μ M) at 5 mM with a Hsp90 protein concentration of 3.3 μ M (~ 1500:1 ligand to target protein ratio) to saturate the Hsp90 binding site with fragments.

1.5. Prepare the Hsp90 quench solution by adding 2% trifluoroacetic acid (TFA) to water such that addition of 20 μ L of Hsp90 quench solution to 30 μ L of deuterium exchange reaction (step 1.3.1) reduces the pH of the final quenched exchange reaction to 2.5.

1.6. Prepare fresh Hsp90 quench solutions before each set of experiments and test their ability to reduce the pH of the final deuterium exchange reaction to 2.5 before every experimental set.

1.7. Alternatively, use a phosphate buffered quench of pH 2.5 to reduce the pH of the final quenched deuterium exchange reaction to pH 2.5. Ensure that addition of high molarity phosphate buffers does not cause salting out in capillaries.

Note: TFA is preferred as a quench solution for its relative inertness. Estimated time required for preparation of D₂O buffers and Hsp90 quench solution is 3 - 4 hours. Prepared buffers can be stored at 4 °C.

2. Setting Up Deuterium Exchange-Liquid Chromatography Coupled to Mass Spectrometry System (LC/MS)

2.1. Prepare the trapping stage of the LC/MS by adding an online pepsin column and a C18-trap column in a commercial HDX specific module. Maintain the immobilized pepsin at 12 °C and trap column at 4 °C to reduce deuterium back-exchange with solvent.

Note: A simple setup in the absence of a commercial HDX-manager module involves maintaining the trap and C18 columns at 0 to 4 °C (ice-bath or a refrigerator). The quenched sample is then

221 automatically eluted through the pepsin column under high pressure with solvent water at pH
222 2.5 (by adding formic acid) and the resultant peptides are trapped in a trap column. Another
223 alternative to an inline pepsin column is use of immobilized pepsin-beads for proteolytic cleavage
224 before injection into the trap column.

225
226 2.2. Prepare the analytical stage of the LC/MS by attaching a reverse phase C18 LC column
227 downstream of the trap column such that the trapped peptides are eluted onto the reverse-
228 phase C18 column by a gradient of water:acetonitrile (pH 2.5 by addition of formic acid).

229
230 2.3. Set an LC gradient with three distinct steps: an initial 92:8 water:acetonitrile ratio to remove
231 non-specific peptides, a linear gradient from 92:8 to 15:85 water:acetonitrile ratio, and 15:85
232 water:acetonitrile ratio to remove any residual peptides or un-cleaved products.

233
234 2.4. Connect the LC outlet to the source of the mass spectrometer.

235
236 2.5. Calibrate the mass spectrometer with reference compounds before data collection (*e.g.*, Glu-
237 fibrinogen peptide or leucine-enkephalin solutions). Add the reference compound solutions to
238 the mass spectrometer and select 'continuous calibration' mode during data collection. Collect
239 mass spectrometry data in MS/MS mode for data independent analysis (DIA).

240
241 Note: Data collection automatically collects precursor ion and fragment ion spectra along with
242 the retention time of individual precursor ions.

243
244 2.6. Modify trapping time for pepsin proteolysis, LC gradient, and elution time to improve
245 sequence coverage, if required.

246
247 Note: The experimental setup, peptide identification, and deuterium exchange have also been
248 previously described^{22,32}. This representative LC/MS setup described for Hsp90 can be directly
249 applicable for non-aggregating homogenous protein samples. The estimated time required for
250 preparation of solutions, columns, and the calibration of the mass spectrometer (MS) is around
251 2 - 3 hours.

252 253 **3. Determining Peptide-List from Undeuterated Hsp90 LC/MS Experiments**

254
255 3.1. Prepare undeuterated reactions of ligand-free Hsp90 by mixing 1 μ L of Hsp90 protein
256 solution (step 1.1) + 2 μ L DMSO-blank + 27 μ L of Hsp90 aqueous buffer. Add 20 μ L of Hsp90
257 quench solution (step 1.5) to reduce the pH of the solution to 2.5.

258
259 3.2. Inject this sample into the HDX-manager fitted with pepsin, trap, and C18 columns with
260 outlets to a mass spectrometer (steps 2.1 to 2.5). Press the 'start' button in the HDX-manager to
261 start pepsin-proteolysis followed by mass spectrometry data collection during LC gradient.

262
263 3.3. Obtain the peptide-database (theoretical list of all possible peptides) using the protein
264 primary sequence and the proteolytic enzyme used for cleavage (pepsin)^{34,35} using the vendor

provided^{34,35} or other integrated analysis software^{37,38}.

3.4. Search for peptides identifiable in the sample against the peptide-database using the MS/MS mass spectrometry data from the undeuterated Hsp90 protein experiments (step 3.1).

3.5. Identify and collect a list of peptides along with their LC retention times from precursor and fragmentation profiles obtained simultaneously throughout the LC gradient³⁶.

3.6. Filter the peptide-list to remove peptides with low intensity, poor fragmentation profiles, and high error. Typically use cut-offs, in validated or vendor-provided software³⁷⁻³⁹, to select peptides with minimum peak intensity of 2,000 Arbitrary Units (AU), maximum MH⁺ error of 10 ppm and a minimum of one fragment.

3.7. Filter the peptide list to ensure that peptides that were eluted only during the gradient (step 2.3) are selected, to maintain reproducibility in obtaining a final peptide list.

4. Additional Optimization

4.1. Add additional post-quench reagents such as denaturants in quench solution (urea and guanidine hydrochloride) and repeat steps 3.1 to 3.7 to increase the number of well-resolved peptides. Modify the chromatographic gradient and time to improve separation and resolution of pepsin fragment peptides.

4.2. Add reducing agents such as tris(2-carboxyethyl)phosphine (TCEP) or dithiothreitol (DTT) in quench solutions for better proteolytic cleavage of proteins that have disulphide bonds.

4.3. Incubate quenched samples with denaturants such as urea/guanidinium-HCl (which helps to unfold the protein for optimal proteolytic cleavage) to improve primary sequence coverage.

4.4. Improve data resolution by optimizing the protocol to generate multiple overlapping and nested peptides.

Note: Estimated time required for a single LC/MS experiment is 20 min and is dependent on the LC gradient time. Four deuterium labelling time points and an undeuterated sample experiment are together estimated to take 3 – 4 h per individual experimental replicate, per ligand, or fragment-bound or ligand-free condition. LC/MS data collection time for three individual replicates for each fragment or ligand binding condition is expected to be 9 – 12 h.

5. Deuterium Exchange Reaction of High-Affinity Ligand-Protein Interaction to Identify Peptide-Reporters

5.1. Prepare deuterium exchange reactions of ligand-free Hsp90 by addition of 1 µL of 100 µM Hsp90 protein solution (step 1.1) + 2 µL DMSO-blank + 27 µL of Hsp90 D₂O buffer (step 1.2) resulting in a final labelling D₂O concentration of 90% and Hsp90 concentration of 3.3 µM.

5.2. Prepare similar deuterium exchange reactions of high-affinity inhibitor-bound Hsp90 protein by adding 1 μ L of Hsp90 protein solution (step 1.1) + 2 μ L high-affinity inhibitor solution (radicol and 17-AAG from step 1.4.1) + 27 μ L of Hsp90 D₂O buffer (step 1.2) resulting in a final labelling D₂O concentration of 90% and Hsp90 concentration of 3.3 μ M.

5.3. Perform deuterium exchange reactions by incubating deuterium exchange reactions (steps 5.1 and 5.2) for specific deuterium labelling time-points (0.5 min, 1 min, 2 min, 5 min and 10 min).

5.4. Include additional deuterium exchange time-points such as 100 min and 24 h, if needed. Include millisecond deuterium exchange reactions with a stopped-flow instrument, if required.

Note: A deuterium exchange reaction for Hsp90 was carried out for the following deuterium labelling times: $t = 0.5, 1, 2, 5$, and 10 min. Although, this time series represents a shorter labeling time series for deuterium exchange, this was the optimal labeling-time-window where the largest changes were observed.

5.5. Add the prepared Hsp90 quench solution (step 1.5) to quench the deuterium exchange reaction by reducing the pH to 2.5. Inject quenched samples to liquid chromatography coupled to a mass spectrometer (LC/MS) setup (step 3.2).

5.6. Analyze the peptides in the peptide-list (step 3.7) and determine deuterium uptake values for each peptide in all the experimental conditions: ligand-free Hsp90 (step 5.1) and high-affinity inhibitor-bound Hsp90 (step 5.2). Calculate deuterium uptake values for each peptide at each of the deuterium labelling time-points (step 5.3).

5.7. Compare deuterium uptake values for peptides and calculate differences in deuterium uptake for each peptide, between ligand-free Hsp90 and high-affinity-bound Hsp90.

5.8. Filter peptides that show significant differences in deuterium uptake above the significance threshold of 0.5 Da.

5.9. Identify Hsp90 residues involved in ligand binding by analyzing ligand-bound structures of Hsp90 from PDB.

5.9.1. Determine residues within 4 Å distance from the ligand using a structure visualization tool such as PyMOL and classify them as orthosteric residues. Load the protein-ligand complex structure in PyMOL using the PDB identifier 4EGK for the Hsp90-Radicicol structure. Click and select the ligand radicol (RDC) in the sequence and use the action menu to modify the selection to include residues within 4 Å distance. Classify these amino-acids as orthosteric residues.

5.9.2. Additionally, include residues that have been annotated as binding sites for the ligand (in either PDB or literature)²².

5.9.3. Determine the list of peptides in Hsp90 that show significant differences (>0.5 Da) in deuterium uptake between the ligand-free and ligand-bound states.

5.10. Classify peptides that show significant differences from step 5.9 and span one or more orthosteric residues as orthosteric reporter-peptides.

5.11. Classify peptides that show significant differences from step 5.9 but do not include any orthosteric residues as allosteric reporter-peptides.

Note: Changes at these peptides represent long-range allosteric changes in response to ligand binding at orthosteric sites.

6. Deuterium Exchange Reactions of Fragment-Protein Interactions to Determine Orthosteric and Allosteric Effects due to Fragment-Binding to Hsp90

6.1. Prepare deuterium exchange reactions of fragment-bound Hsp90 by addition of 1 μ L of 100 μ M Hsp90 protein solution (step 1.1) + 2 μ L of fragment solutions in DMSO (Fragment 1 and 2 from step 1.4.2) + 27 μ L of Hsp90 D₂O buffer (step 1.2) resulting in a final labelling D₂O concentration of 90% and Hsp90 concentration of 3.3 μ M and fragment concentration of 20 mM.

6.2. Select a small-set of suitable deuterium labelling time-points (30 s and 5 min) which show the highest changes in high-affinity-ligand-protein interactions.

6.3. Preferably include shorter deuterium labelling time-points (30 s), since differences in deuterium uptake upon fragment-binding are readily apparent at these short deuterium labelling time-points due to the weak binding affinities of fragment compounds.

6.4. Perform deuterium exchange reactions for fragment-Hsp90 interactions by incubating the deuterium exchange reactions (step 6.1) for the specific time points (30 s and 5 min), followed by addition of Hsp90 quench solution to reduce the pH to 2.5.

6.5. Determine deuterium uptake values (similar to step 5.7) for orthosteric and allosteric reporter-peptides identified in steps 5.10.3 and 5.11.

6.6. Analyze fragment-protein interaction data at these reporter-peptides to qualitatively determine the orthosteric and allosteric effects of fragment-binding based on the number of peptides or regions that show changes upon each fragment binding.

6.7. Quantitate these changes by measuring the differences in deuterium uptake at each of these reporter-peptides. The differences indicate the amount of protection at these reporter-peptides due to fragment-binding and indirectly report the strength of the interaction¹⁰.

6.8. Identify reporter-peptides that show significant differences in deuterium uptake above the significance threshold of 0.5 Da (similar to 5.10). Determine the number of orthosteric and

allosteric peptides that show significant differences upon fragment-binding for each fragment (Fragment 1 and 2, similar to 5.11 and 5.12).

7. Additional Interpretation

7.1. Analyze the deuterium uptake kinetics over time to predict the relative k_{off} rates for ligands (or fragments) with similar dissociation constants (K_D). Measure decreases in observed deuterium uptake differences (ligand-bound protein versus ligand-free protein) with increases in deuterium labelling time.

7.2. Compare dissociation rates (k_{off}) of ligands and rank them based on faster observed decreases in deuterium uptake differences with increases in deuterium labelling times at orthosteric reporter-peptides.

7.3. Identify ligands with faster dissociation rates by comparing those that show earlier decreases in deuterium uptake differences with increases in deuterium labelling time at orthosteric reporter-peptides.

Note: Estimated time for data analysis can be from 2 – 10 days and is dependent on the number of fragments or ligands analyzed.

REPRESENTATIVE RESULTS:

In order to identify the reporter peptides that represent changes in Hsp90 upon ligand binding, changes in deuterium uptake were quantified for Hsp90 in the presence and absence of the high affinity ligands. Differences in deuterium uptake were determined at pepsin-proteolyzed peptides between the high-affinity-ligand bound-Hsp90 and ligand-free-Hsp90 and reporter peptides that showed significant differences in deuterium uptake (≥ 0.5 Da) were identified. The error in a single deuterium exchange experiment has been shown to be 0.17 Da⁴⁰. This is a composite of variability in pH, temperature, samples, and instrumentation. The maximum standard error in a subtractive analysis is twice that – 0.34 Da. Hence, a threshold of 0.5 Da was set for any difference to be considered significant. Among all the reporter peptides that showed ligand-specific changes, orthosteric reporter peptides were determined using crystallographic structural information. Peptides that span residue(s) within a 4 Å H-bonding distance, or annotated to be part of the binding pocket by PDB, are considered as orthosteric reporter peptides and monitor changes at the binding site. For Hsp90, multiple residues were annotated in PDB to make contacts with the ligand in the orthosteric binding pocket. Peptides spanning residues L48, N51, D54, A55, and K58 were classified as orthosteric peptides in region O1 since these residues were shown to be involved in direct binding to the ligand. Similarly, orthosteric regions O2 spanned residues D93, I96, M98, D102, N106, L107 and K112, region O3 with residues G135, V136, G137 and F138 and region O4 with residues T184 and V186 that are known to make orthosteric contacts with the ligand. Peptides spanning these residues were classified as orthosteric reporters (**Figure 1**). Peptides that showed significant differences, but are not part of the orthosteric regions, are considered allosteric reporter peptides and monitor changes at the allosteric site. For instance, peptide spanning residues 62-76 showed differences in deuterium

uptake between the ligand-bound and ligand-free Hsp90 although the residues involved do not have orthosteric binding contacts with the ligand, hence the changes observed in this peptide locus were classified as representing allosteric changes that are distal to the binding site. High-affinity ligands are used to identify these orthosteric and allosteric reporter peptides (**Figure 1**). A protein-wide difference plot is used to determine the entire set of reporter peptides (orthosteric and allosteric) across Hsp90. These clearly show four distinct orthosteric and allosteric regions represented by multiple reporter peptides (**Figure 2**). Mapping these regions on the structure of Hsp90 show that the orthosteric regions (colored blue) surround the ligand-binding pocket, while allosteric regions are distal to the binding site (**Figure 2**). A few orthosteric reporter peptides are sensitive enough to capture differences between the binding kinetics of the two high-affinity ligands (Orthosteric reporter peptide I, **Figure 1**).

Kinetics of deuterium exchange indicate that the early time points of exchange show the highest magnitude differences in deuterium exchange and these differentially drop with increasing deuteration times, dependent on the ligand dissociation rates (k_{off}) (**Figure 2**). Hsp90-Fragment interactions showed the largest differences at labelling time-points up to 10 min, specifically at 30 s and 5 min. Some peptide reporters showed decreased differences at time-points after 10 min due to fast k_{off} rates of fragments. HDXMS data (**Figure 2**) showed that the faster decreases in difference in deuterium uptake for 17-AAG (bottom-panel, **Figure 2**) compared to radicicol (top-panel), over increased deuterium labelling time, suggesting that 17-AAG dissociates faster than radicicol despite their similar dissociation constants (K_D). A higher magnitude of difference in deuterium exchange observed in radicicol-Hsp90 complex compared to 17-AAG-Hsp90 complex also suggests the radicicol makes additional or stronger contacts at the orthosteric binding site (Orthosteric region O2, **Figure 2**, data from²²).

[Place Figure 1, 2 here]

Reporter peptides identified from high-affinity-ligand analysis (in both orthosteric and allosteric regions) are used to monitor changes across Hsp90 in response to fragment binding. Fragments mediate contacts across all the orthosteric contacts observed with high affinity ligands, but the magnitude of difference and time-dependent differences are considerably lower (**Figure 3**, data from ²²). Fragments can be compared and ranked in terms of their magnitude of differences, the number of orthosteric regions where changes are observed, and the time-dependent increases or decreases in deuterium uptake. Each of these can be represented as below: Both fragments 1 and 2 show significant changes in all four orthosteric sites and the four allosteric sites observed in high affinity ligands. However, fragment 1 shows changes at an additional allosteric peptide at region A5 (colored orange in **Figure 3**). Furthermore, fragment 1 shows higher magnitude changes in regions O1, O4, A1, A3 and A4, compared to fragment 2. Kinetics of deuterium uptake also show that changes due to fragment 1 at orthosteric site O2 are observable until the 10 min deuterium labelling time-point, compared to fragment 2, where there are no significant changes observable after 30 s of deuterium exchange. These results suggest that this method of monitoring low-affinity fragments and the changes (orthosteric and allosteric) using HDXMS allows the comparison and ranking of ligands and fragments based on their binding and allosteric effects in solution.

[Place Figure 3 here]

FIGURE LEGENDS:

Figure 1: Identification of reporter peptides for monitoring orthosteric binding and allosteric effects. Comparison of deuterium uptake values between the ligand-bound-Hsp90 and apo-Hsp90 shows changes at distinct regions in the protein. Representative pepsin-proteolyzed peptides and their differences in deuterium uptake are shown from four different regions of Hsp90 upon binding two high affinity ligands (Radicicol and 17-AAG). Orthosteric reporter peptides contain residues (side chains represented as sticks) in the binding sites that make orthosteric contacts (Orthosteric reporter peptide I and II). These orthosteric peptides show differences in deuterium uptake upon ligand binding. Comparing these orthosteric peptides, peptide I (boxed red and colored red on the structure of Hsp90) is able to differentiate between the two ligands, while changes in peptide II (boxed in blue and colored blue on the structure of Hsp90) are similar for both the ligands in the deuterium labelling time-points observed. A representative allosteric reporter peptide (boxed in cyan and colored cyan on the structure of Hsp90) shows changes in deuterium uptake upon ligand binding and does not contain any residue which makes orthosteric contacts with the ligand and is distal to the binding site. These changes in this allosteric reporter peptide are allosteric changes due to ligand binding. A control peptide (boxed in yellow and colored yellow on the structure of Hsp90) is shown, which neither involved in orthosteric binding nor shows any distal allosteric changes. Deuterium uptake plots for the highlighted reporter peptides are represented in boxes. The deuterium uptake values (Y-axis in Daltons) are plotted against their deuterium labelling times (logarithmic X-axis) for at least three independent HDXMS experiments in each set of conditions and colored according to key. The reporter peptides residues are mapped on to the structure of Hsp90 (grey). Radicicol bound at the ligand binding pocket is shown as green sticks (PDB ID: 4EGK). (Figure adapted from²²)

Figure 2: Dissecting orthosteric and allosteric changes in Hsp90. A plot of differences in deuterium exchange at reporter peptides upon ligand binding across Hsp90 shows orthosteric regions that show changes and distal sites that show allosteric effects. **(A)** The absolute difference in numbers of deuterons (Y-axis) between the free and ligand bound-Hsp90 is plotted for each pepsin digest fragment listed from the N to the C terminus (x-axis) for each deuterium exchange time point ($t = 0.5, 2, 5, 10$ min) in a 'difference plot'. Shifts in the positive scale represent decreases in deuterium exchange and shifts in the negative scale represent increases in deuterium exchange when compared to apo-Hsp90. Regions showing significant differences above a threshold of 0.5 Da (red dashed line) are compared with orthosteric sites (blue boxes) to predict allosteric regions. Peptides (highlighted in red) show regions with differences in distal allosteric regions, which are not involved in orthosteric binding. Peptides spanning these regions (marked in red boxes) are divided into four allosteric regions A1 to A4. Radicicol and 17-AAG showed differences in A1 and A2, while only radicicol showed changes in A3 and A4. Time points are colored according to key. **(B)** Predicted allosteric regions are mapped on to the structure of Hsp90 (red), together with the orthosteric regions, in blue. Radicicol bound at the ligand binding pocket is shown as sticks (PDB ID: 4EGK). (Figure adapted from²²)

Figure 3: Fragments 1 and 2 differ in the nature of their allosteric effects on Hsp90. (A) The absolute difference in numbers of deuterons (inferred from the difference in mass in Daltons (Da)) (Y-axis) between the free and ligand bound state is plotted for each pepsin digest fragment, listed from the N to the C terminus (X-axis) of Hsp90 for each deuterium exchange time point ($t = 0.5, 2, 5, 10$ min) in a 'difference plot'. Shifts in the positive scale represent decreases in deuterium exchange and shifts in the negative scale represent increases in deuterium exchange when compared to the apo-Hsp90. Regions showing significant differences above a threshold of 0.5 Da (red dashed line) are compared with orthosteric sites (blue boxes) to establish allosteric regions (red boxed). Fragment 2 does not show any changes in region A4, similar to 17-AAG, while fragment 1 shows differences, similar to Radicicol. In addition, fragment 1 shows an allosteric response at region A5 (residues 201–213 shown in orange box), which is not observed in the other three ligands. Time points are colored according to the key. **(B, C)** The identified orthosteric (blue) and allosteric regions (red) for fragments are mapped on to the structure of Hsp90 in blue. **(C)** The changes in allosteric site A5 in Hsp90 are observed only for fragment 2 (highlighted in orange). Radicicol bound at the ligand binding pocket is shown as sticks (PDB ID: 4EGK). (Figure adapted from²²)

DISCUSSION:

Critical steps in the protocol: It is essential that the pH of solutions, including protein buffers and LC-solutions, are all maintained at a pH of 2.5 to minimize loss of deuterium labelling. It is also critical that deuterium exchange experiments be carried out at saturating concentrations of ligands to maintain a homogenous population of ligand-bound protein. This can be estimated from the ligands' or fragments' dissociation constants and need to be consistent among all fragment-protein deuterium exchange experiments. Identification of reporter peptides should involve differences in deuterium uptake, together with a detailed structural characterization of residue-wise bonding contacts at the ligand-protein interface to identify peptides that contained at least one non-hydrogen atom proximal to the ligand and are thus involved in ligand binding. Spatially distal peptides showing changes in deuterium uptake need to be carefully examined to exclude regions with orthosteric ligand contacts.

Modifications and troubleshooting: The observed differences at reporter peptides represent composite changes due to ligand binding and include both orthosteric changes at the binding site and allosteric changes at the ligand-binding site. Any known structural information on additional co-factors and partner binding sites can be defined as allosteric regions which have been reported earlier to respond to ligand binding. Finding the optimal deuterium exchange and ligand concentrations can be a challenge. It is advisable to utilize deuterium uptake profiles of ligand-free-protein to optimize the deuterium labelling times and to preferentially pick the shortest labelling times, in case there is a lack of differences between them. In case of availability of structural information in homologous proteins or other ligands, these are also likely to provide important clues in terms of dissociation constants, multiple binding sites, and any observable conformational changes.

Limitations of the method: HDXMS is extremely sensitive to changes in protein conformation and offers a perturbation map for fragment and ligand binding to the target proteins. One limitation is that it provides this map at a peptide resolution. The authors wish to emphasize that HDXMS when combined with X-ray crystallography can provide important complementary insights into dynamics of protein-ligand interaction. Hence, this method is also most suited to study protein-ligand systems where structural information about orthosteric contacts is available. The availability of high-resolution structural information eases prediction and separation of allosteric effects from orthosteric contact points. Additionally, the resolution of this approach depends on the ability to obtain pepsin-proteolyzed reporter peptides that span the orthosteric and allosteric sites to monitor conformational changes. Changes observed in longer peptides (>15 residues in length) lack the resolution to accurately localize changes. However, shorter, overlapping and nested peptides (<10 residues in length) from commercially available high-pressure pepsin proteolysis can overcome these limitations.

Significance of the method with respect to existing alternative methods: Dynamics is a major consideration of allosteric processes in proteins and represents a challenge for biophysical and structural characterization of conformational changes. Additionally, fragments with weak dissociation constants mediate transient interactions and are difficult to capture due to their high k_{off} rates. Since deuterium exchange reactions are initiated by mixing a ligand and its target protein with deuterated buffer, it is possible to capture steady-state and equilibrium kinetics from a range of deuterium labelling times. The ability to distinguish between similar high-affinity ligands and fragments based on the magnitude and kinetics of deuterium exchange differences has been demonstrated in the results section. In addition, HDXMS has especially important implications in drug and fragment screening in proteins that are challenging to crystallize due to factors such as aggregation. This method is uniquely poised to provide a comprehensive description of protein-ligand complexes: structural localization of binding, allostery, and kinetic information on the relative k_{on} and k_{off} rates. These applications have enabled HDXMS to become an emerging tool in small-molecule and biopharmaceutical discovery¹⁵⁻¹⁷.

Future applications or directions of the method: An extension of monitoring changes in protein-fragment interactions is the ability for this approach to be utilized as an efficient fragment screening method. The magnitude of deuterium exchange difference and kinetics of difference observed directly reports on fragment efficacy. It is hence possible to rank-order fragments based on: 1) Number of loci or peptides showing differences in deuterium exchange. 2) Magnitude of difference at each of these loci. 3) Reduction in differences over deuterium labelling time. These offer a HDXMS framework for iterative fragment screening and optimization in the FBLD pipeline. Optimization of lead-compound by monitoring differences due to modification of multiple single moieties can be used to preferentially optimize some components of the lead-compound vs the others. Overall, the method demonstrated here is expected to accelerate fragment-based drug discovery.

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DISCLOSURES:

The authors declare that they have no competing or financial interests.

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Figure 1

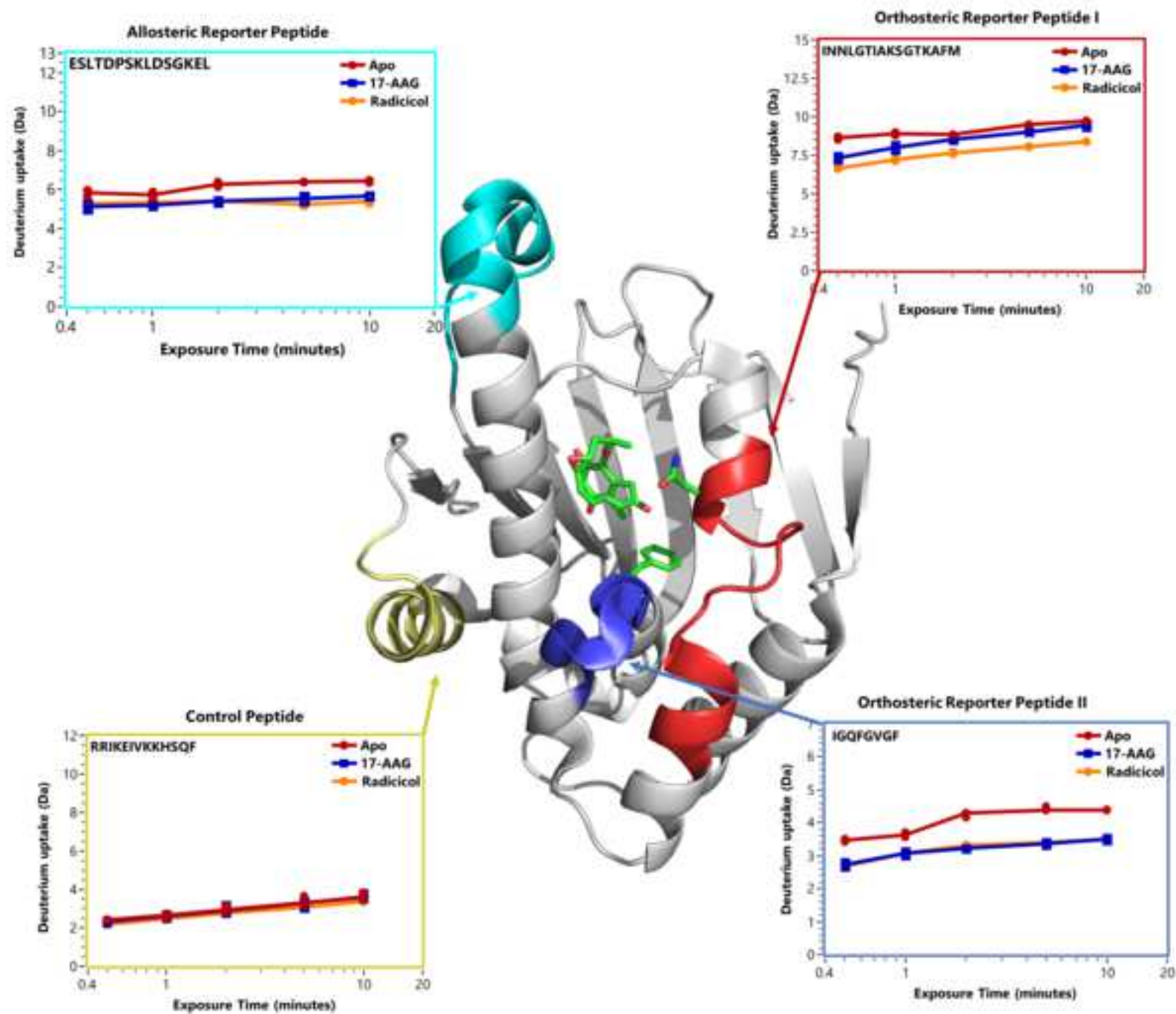


Figure 2

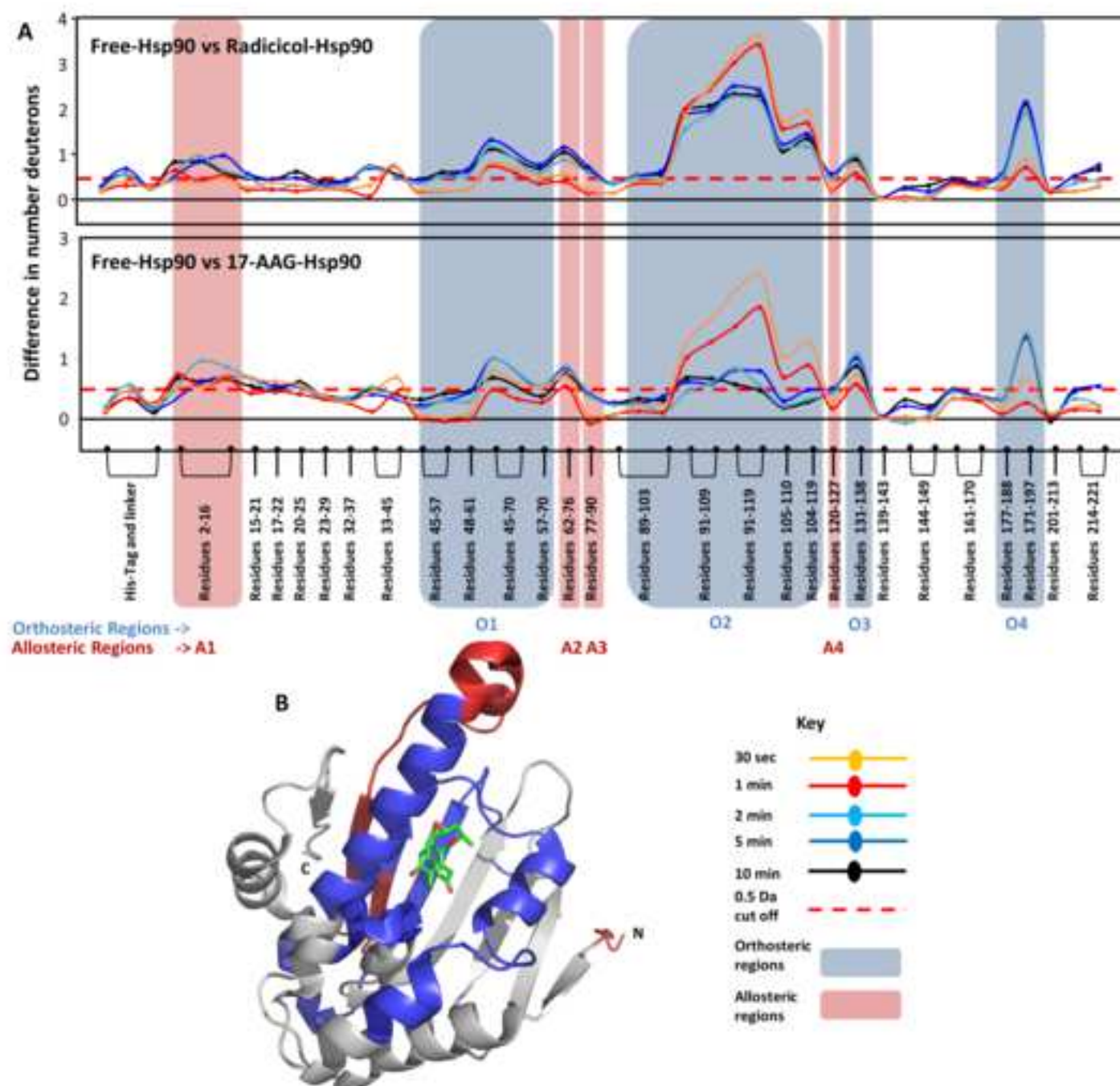
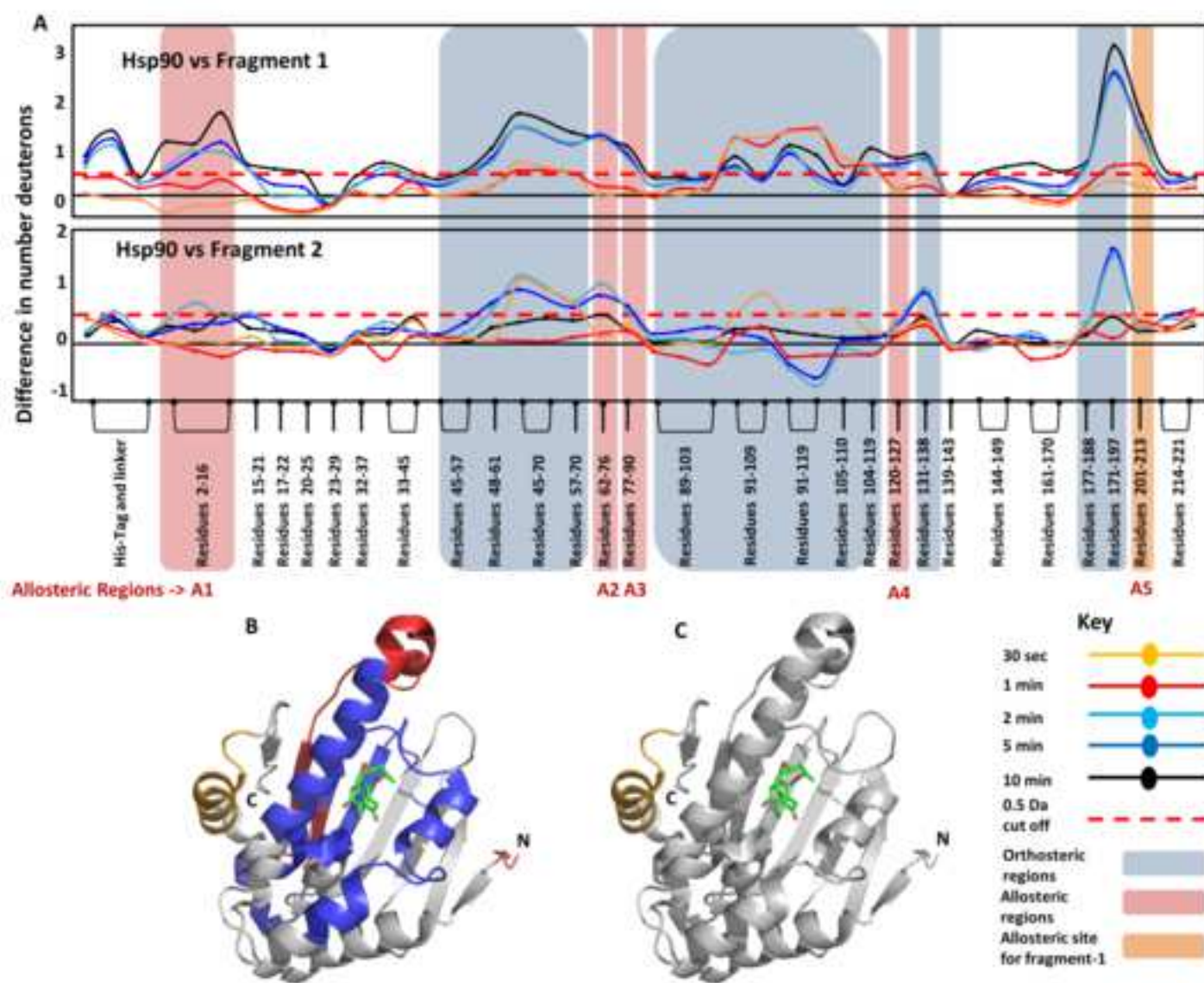
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Figure 3



Name of Material/ Equipment	Company	Catalog Number
Deuterium Oxide	Cambridge Isotope, Tewksbury, MA	DLM-6-1000
HEPES Buffer	Sigma Aldrich, St. Louis, MO	H3375 SIGMA
Glycerol	Sigma Aldrich, St. Louis, MO	G5516
NaCl	Sigma Aldrich, St. Louis, MO	S7653
TCEP	Sigma Aldrich, St. Louis, MO	C4706
DMSO	Sigma Aldrich, St. Louis, MO	D8418
TFA	Sigma Aldrich, St. Louis, MO	302031
FA	Fisher Scientific, Singapore	A117-50
Glu-fibrinogen	Sigma Aldrich, St. Louis, MO	F3261
Leucine-Enkaphalin	Waters, Milford, MA	186006013
ACN	Sigma Aldrich, St. Louis, MO	34851
pNIC28-Bsa4 vector	Addgene, Cambridge, MA	26103
BL21(DE3) E. coli strain	Merck-millipore Novagen, Singapore	69450
Terrific Broth medium	Sigma Aldrich, St. Louis, MO	T9179
Kanamycin	Sigma Aldrich, St. Louis, MO	60615
chloramphenicol	Sigma Aldrich, St. Louis, MO	C0378
isopropyl β -D-thiogalactopyranoside (IPTG)	Sigma Aldrich, St. Louis, MO	IPTG-RO ROCHE
imidazole	Sigma Aldrich, St. Louis, MO	I5513
Protease Inhibitor Mixture Set III, EDTA free	Merck-Millipore, Singapore	539134
Vibra-Cell processor	Sonics & Materials Inc., Newtown, CT	VC 505 / VC 750
nickel-nitrilotriacetic acid Superflow resin	Qiagen Inc., Valencia, CA	30410
HiLoad 16/60 Superdex-200 column	GE Healthcare, Waukesha, WI	28989335
Vivaspin 20 filter concentrators	GE Healthcare, Waukesha, WI	28932360
Poroszyme Immobilized Pepsin Cartridge, 2.1 mm x 30 mm	ThermoFischer, Singapore	2313100
ACQUITY UPLC BEH C18 Column	Waters, Milford, MA	186002350
ACQUITY UPLC BEH C18 VanGuard Pre-column	Waters, Milford, MA	186003975
nanoAcquity HDX sample manager	Waters, Milford, MA	
Synapt G1 ESI mass spectrometer	Waters, Milford, MA	
nanoAcquity Auxillary Solvent Manager	Waters, Milford, MA	
nanoAcquity Binary Solvent manager	Waters, Milford, MA	

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Signature: S. G. Arad Date: 28-02-2017

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Reviewer #1:*Manuscript Summary:*

The authors describe the use of amide hydrogen deuterium exchange spectrometry (HDXMS) for assessing structural changes and conformational dynamics of ligand-protein interactions. Using the highly conserved molecular chaperone protein hsp90, two clinically relevant hsp90 inhibitors (17-AAG and radicicol), and two fragment components as prototype molecules, HDXMS is shown to be a powerful tool for evaluating distinct orthosteric and allosteric changes on a target protein, which are not readily discernible using other more conventional biophysical approaches. When combined with structural data and kinetic studies, HDXMS can provide highly useful information for lead compound screening and fragment-based drug discovery.

Major Concerns:

1. More technical detail describing the technique as it relates to hsp90 and assayed ligands should be provided in Protocol Steps 3-7. While the authors understandably intend for other investigators to adapt this technique to their particular proteins of interest, having the authors include more of the specifics of the protocol as it was used for completing this investigation on the hsp90 system would expedite the development/adaptation process and increase the protocol's overall utility. The addition of a few key sentences in these steps as notes or additions to individual sub-steps providing technical details (e.g., as done in Protocol Steps 1-2) would be highly beneficial to the reader.

-We thank the reviewer for these suggestions and we have added multiple sentences in the protocol (steps 2-7) to include details for the Hsp90 system to increase the protocol's utility. We have however added these sentences as notes since these are specific to hsp90 and may not always be applicable as a general protocol.) We have additionally included a citation to a new textbook 'Hydrogen deuterium exchange mass spectrometry of Protein: Fundamentals, Methods and Applications', Editor: D. Weiss, 2016, Wiley Press. This textbook offers in-depth methodology support for carrying out HDXMS on a wide range of proteins.

2. Inclusion of additional references to the current literature, particularly those presenting applications of HDXMS in drug discovery, would better allow the reader to place this protocol in context and would be greatly appreciated. These additional references could be added in both the Introduction and Discussion sections.

-We thank the reviewer for this suggestion and have included references to recent literature, which describe the application of HDXMS to drug and small molecule discovery.

3. Explaining how the data obtained using HDXMS compare to those data previously reported

for hsp90 and 17-AAG/radicicol using other kinetic and biophysical techniques would be useful. Adding this information (to the extent it is available) to the Representative Results section could enable the reader to better assess the power and validity of this technique. Please include reference citations here as well.

-We thank the reviewer for their suggestion. We have complemented HDXMS with other biophysical and kinetic techniques to provide additional insights into ligand binding. For instance, we have utilized dissociation constants from kinetic studies to additionally infer and distinguish between the dissociation rates of the two high-affinity ligands from our HDXMS experiments.

Minor Concerns:

1. Please avoid the use of "etc.", replacing it with more descriptive examples of what may be expected.

-We have removed “etc” and replaced it with additional examples.

Reviewer #2:

Manuscript Summary:

The MS describes a method to estimate and study structural changes in a protein away from its ligand/substrate binding site.

Major Concerns:

Though the article does not carry any novelty, it might still be useful to people who are studying solution structures of proteins.

Additional Comments to Authors:

the limitations are not discussed

-HDXMS offers a sensitive perturbation map for fragment and ligand binding to target proteins. One limitation is that it provides this map at a peptide resolution. The authors wish to emphasize that HDXMS when combined with X-ray crystallography can provide important complementary insights into dynamics of protein-ligand interaction. These have also been added to the manuscript.

Reviewer #3:

Manuscript Summary:

General comments and major concerns:

1. The method HDXMS is presented as an approach to identify and characterize fragments for lead structure discovery in drug research. This method is widely applicable to ligand binding proteins such as ATPase or GTPases and therefore of high interest to scientists in several fields. However, the authors should mention the key point of this research article: Hsp90 is an ATPase, hydrolyzes ATP and this research article focuses on the effects of competitive ATPase inhibitors on one isolated single N-terminal domain of Hsp90. Global structural dynamics and changes are hence neglected, they would have to include the complete, dimeric protein with each monomer consisting of 3 domains. Thus, the allosteric effects investigated in this article are all short-ranged (around 2 nm) within the single NTD. With respect to this, what is the argument for the choice of 4 Å as a cut off for considering peptides orthosteric?

-We thank the reviewer for their comments. We specifically chose the N-terminal domain of Hsp-90 as a test protein for our studies as application of fragment-based drug discovery (FBDD) on this particular domain has been extensively described in the public domain. This offered us a system previously characterized and for which X-ray crystal structures were available. Since this article is targeted towards investigators to adopt this approach and due to the broad applicability of this strategy to multiple protein-ligand systems, we have focused on the strategy rather than the protein of interest. We have included references to the study where the results with respect to Hsp90 are discussed in detail.

Allostery, in this study, is described as long-range conformational changes distal to the binding site. While there are multiple levels of allostery that operate at each level of protein structure, we focus on the allostery between distal regions in the protein. We have hence clarified in the manuscript that we capture long-range allosteric changes within the ATPase domain of the Hsp90 protein.

The 4 Å cut-off for orthosteric contacts chosen is based on the cut-offs used to determine hydrogen bonding contacts in structural studies such as X-ray crystallography and NMR. This was chosen to identify putative hydrogen bonding contacts in crystallographic structures reported in PDB. This cut-off also allows for capturing H-bond contacts (2.5 – 3.5 Å) and factors in any side-chain movements. The changes observed in HDXMS experiments within these regions can be expected to be due to direct binding of ligands at orthosteric sites. Consequently, long-range changes observed would operate at distances greater than 4 Å from the orthosteric site.

2. The authors neglect the available structural data on Hsp90 and the common name used to reference certain motifs within the domain, such as 'the ATP-lid' or 'the cross-monomer contact'. Using these identifiers could help the Hsp90 community to take this method into account for further research and increase the method's popularity. As the idea of drugs binding to the

nucleotide pocket of Hsp90 is their competition with ATP, a comparison of this method in the presence and absence of ATP and ADP would be of high interest to the Hsp90 community.

We thank the reviewer for the suggestion. The focus of this study is to describe an approach using HDXMS to compare fragment binding at both orthosteric and allosteric sites. And hence we have taken high-affinity ligands and fragments that have been previously studied and tested extensively. Hence, although it would be of great interest to the Hsp90 community if the ATP and ADP ligands were compared with fragments using HDXMS, we would prefer to use Hsp90 only as a representative protein to highlight the applicability of HDXMS to distinguish and monitor orthosteric and allosteric responses to ligand/fragment binding. The results of this study are already published and this detailed study has been referenced in the articles and will indeed be of interest to the Hsp90 community.

3. The main advantage of this method over the most common method in drug screening, i.e. x-ray crystallography, is the applicability to proteins that tend to aggregate, form amorphous or even no crystals at all. This should be mentioned in the text more clearly.

-We thank the reviewer for their comment and have described this advantage of HDXMS where we discuss the advantages of HDXMS over such structural tools. Further, in parsing orthosteric and allosteric changes in response to ligand binding, structural information greatly complements HDXMS. High-resolution structures offer a framework to overlay solution HDXMS data to more clearly identify allostery.

4. The authors mention the ability of their method to resolve protein dynamics. It would be very useful for the reader if the authors provide numbers on which timescales are accessible to this method and give appropriate references.

-HDXMS offers a read-out of protein dynamics at the seconds and longer timescales. These have been addressed in the revised manuscript.

5. The interaction of a protein with fragments that bind with a very high dissociation constant results in very low specificity. How do the authors exclude artifacts from binding of the fragments at sites distal to the native ligand binding site? The latter would result in different HD-exchange dynamics and would then be misinterpreted as allosteric effects.

-By carefully choosing fragment concentrations, we greatly reduce the possibility of the fragments binding at these distal sites. Additionally, structural studies with much higher concentrations of fragment used do not detect electron density at any other sites and hence we

do can expect that fragments concentrations used do not lead to artifacts due to binding at non-orthosteric sites. It should also be noted that not all of these allosteric regions are surface accessible and changes observed at these loci are allosteric changes due to binding at a distal site.

Would this method be applicable to the full-length, dimeric protein? What would be obstacles to such an experiment and how could they be overcome? Why is trypsin digestion not mentioned as alternative to create fragments?

-Yes, indeed this method is applicable to full-length dimeric and high oligomeric states of proteins. The only consideration is that the samples must be homogenous in terms of sample purity and oligomer state. HDX reactions are directly affected by pH and temperature. In order to trap deuterons exchanged on the protein of interest, the reaction quench conditions must be maintained at pH 2.5 and 4 °C. The only proteases that will cleave the deuterated protein are acid stable proteases of which pepsin is the most robust. Trypsin is inactive at pH 2.5 and therefore cannot be used for HDXMS analysis.

6. This method is meant to be used in drug screening. It would be very helpful and increase the articles impact if the authors provide numbers on the time consumption of the single steps and the general procedure.

-We thank the reviewer for the comment and have included details of time required for steps in the single steps and in the overall procedure.

7. How is the value 0.5 Da for a significant difference in deuterium uptake justified?

-The standard deviations in deuterium exchange measurements have been determined across multiple studies to be within 0.2. 0.5 Deuterons (2X standard deviation) is accepted as a threshold for significant difference (Houde et al, 2011 in the revised manuscript)

Minor comments:

-Writing word1/word2 in sentences decreases the readability of the manuscript (e.g. line75).

-Replaced '/' with suitable conjunctions

-Please check the language "distinguishing [s.th.](#) from [s.th.](#)" (not "...and..."), "information about/on [s.th.](#)" (not ... "of..."), last sentence in abstract includes the word fragment 3x in one line

-These have been modified, as suggested, to improve readability.

-General question: Which organism does this Hsp90 come from and how was it expressed and purified; what is the buffer composition incl. pH?

-Human Hsp90 expressed in E.coli. Buffer constituents and a citation to reference the purification of recombinant Hsp90 has been included.

-L28: Why should structural data not include allosteric information? This method's main advantage would be that it makes further structural data redundant?

-Structural information does not always capture allostery. Dynamics is a major consideration of allosteric processes in proteins and represents a challenge for biophysical and structural characterization of conformational changes. Consequently, capturing protein dynamics is essential to completely characterize allosteric changes within proteins. There are multiple examples of allosteric regulation through dynamic (entropic) mechanisms, which can only be captured in solution (Popovych *et al*, 2006, Nature structural and Molecular Biology). HDXMS is uniquely poised to capture such changes in protein dynamics. However HDXMS complements structural data and provides insights into dynamics and taken together, they comprehensively describe allosteric changes upon ligand binding in proteins.

-L44: The authors claim the dissociation rate of the fragment is increased- this is not shown directly in the data, the exact conclusion is an increase of the K_D .

-The dissociation rate discussed in the manuscript is the rate k_{off} and not the constant K_D . We have added “(k_{off})” to clearly indicate the dissociation rate.

Since the dissociation constant K_D is a ratio ($K_D = k_{off} / k_{on}$), despite both the high-affinity ligands having similar K_D values, their dissociation rates (k_{off}) can be different. The difference in deuterium uptake at orthosteric peptides are a direct consequence of the dissociation rates. Ligands which dissociate faster lose protection from deuterium exchange over time and can be inferred from faster decreases in deuterium uptake differences over time.

So, in a direct comparison between two ligands with similar dissociation constants, ligands with faster dissociation rates result in loss of deuterium protection in earlier time-points. HDXMS data (Fig 2) showed that the faster decreases in difference in deuterium uptake for 17-AAG (bottom-panel in Fig 2) compared to radicicol (top-panel in Fig 2), over increased deuterium labelling time, suggests that 17-AAG dissociates faster than radicicol despite their similar dissociation constants.

-L50: ATP/ADP are the natural ligands, not Radicicol or 17-AAG. Data on ADP or ATP would be very interesting.

-True. However, HDXMS of ATPase domain of Hsp90 interactions with ATP would not solely reflect binding since ATP-Hsp90 interaction is not a binding interaction but an enzymatic reaction, where ATP is continuously hydrolyzed. Changes observed would be a combination of dynamics involved in both binding and the enzymatic hydrolytic reaction. Hence, for an enzymatic study of Hsp90, it would be interesting to look at HDXMS data with ATP/ADP however for the focus of the paper, inhibitors like radicicol and 17-AAG are preferred. Also, it has been shown that radicicol and 17-AAG both bind to the same pocket as ATP.

-L60: Most structural data represents a global (or local and kinetically trapped) energy minimum. But why should the comparison of 'endpoints' not reveal allosteric effect? Wouldn't the advantage of the exchange method rather be that it is capable of revealing transient intermediate changes inaccessible to x-ray/cryo-EM?

-L63: Soaking crystals can initiate reactions and therefore lead to structural changes that can indeed be monitored. Thus, this claim is wrong.

-We concur with the reviewer and have modified the manuscript based on these suggestions.

-L71: Again, structural data indeed contains information of allosteric effects, as long as they are preserved in the structures that are captured.

-We do agree that information on allosteric effects can be captured, however those allosteric changes cannot be readily discerned except for large conformational change such as movement of a helix etc.

-L82: The natural ligands contain 2/3 phosphates and hence a large charge (same for e.g. GTPases). Therefore, I suppose the authors speak of drugs rather than ligands in general.

-We agree with the reviewer and have modified the manuscript, as suggested.

-L85: This sentence is unclearly verbalized, please rephrase.

-L107: This number is the dissociation constant, please rephrase.

-L116: This sentence is a repetition of sentence in L112.

-L117: This sentence is unclear, do the authors suggest the application of this method on peptide

inhibitors as well?

-L146: I suggest the authors to speak of binding site saturation rather than 100% binding.

-We thank the reviewer for the above comments and have modified these sentences to improved clarity.

-L168ff: I suggest the authors to give the names and exact specifications of the equipment they use: mass spectrometer, columns, beads.

-L185: Please specify the reference compound or give a literature reference for readers unaware of this procedure.

-We have adhered to JOVE's policy of not including specific vendor names etc. The Hsp90 specific results from this protocol have been previously published with detailed specifications etc. We are also willing to add these details to this current manuscript, however, we strongly feel that would make it a vendor specific protocol. The calibration compounds are also vendor specific and hence although, we have mentioned the calibration compound used in this study, it would likely differ based on the mass spectrometer used and hence cannot be generalized.

-L202: Please give the name and reference the databases that are used.

-We have also added a note to better describe the database of master-list which can be determined from protein-sequence alone.

-L316f: The authors give error numbers, which is highly useful. But where do these numbers come from? Please reference to the appropriate literature and/or show the data underlying these numbers.

-We have added references as suggested. These are similar to the 0.5 Da error threshold mentioned in Q7 (major comments)

-L325: This is not protein-wide, but domain-wide data.

-Sentence has been modified to exclude protein-wide.

-L346: I suggest the authors speak more general of faster vs. slower exchange kinetic, because this method is not able to discriminate on/off rates clearly.

-We do agree that it is not able to report on 'on' rates. However, increase in deuterium labelling over time has been shown to be used to calculate 'off' rates (Jorgenson *et al*, 2004, biochemistry and Zhang *et al*, 2015, Analytical Chemistry) since deuterium competes directly with the ligand at the orthosteric site. A ligand with a faster 'off' rate results in deuterium labelling at earlier time points compared to a ligand with a slower 'off' rate. Hence with multiple deuterium labelling time points, it is possible to correlate ligand dissociation with deuterium exchange. In this study, we only qualitatively compare between two ligands based on the loss of protection from deuterium exchange with increasing time.

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1) Editor re-wrote some of your protocol steps (in section 1) in the imperative tense.

We thank the editor for rewording some of our protocol steps. We have also carefully examined the rest of the protocol and converted the protocol steps to imperative tense.

2) There are numerous notes, please limit notes in number and their length.

3) Notes should be written out as steps wherever appropriate. As an example see the tracked changes to the Note following 1.2 (now step 1.3)

The notes have been either condensed, or written out as steps, as suggested.

4) Several steps need to be rewritten in the imperative tense: 2.5

The steps in section 2.5 have been modified accordingly, as suggested.

• **Protocol Detail:** Please note that your protocol will be used to generate the script for the video, and must contain everything that you would like shown in the video. **Please add more details to the following protocol steps.** There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol.

1) 1.2.1: What is the sample used? What is its source?

The source has been added and the steps modified to include references. We have also replaced 'sample' with 'deuterium reaction' for a more accurate description.

2) 1.4: When is the quench solution added to the sample?

Details of addition of quench have been included.

3) 2.2 Note: This should be converted into steps.

The note has been converted to protocol steps.

4) 2.5.3.1: Please mention what button is clicked on in the software to do this, or which menu items need to be selected.

These are vendor specific information and hence the interface and 'methods' that need to be set differ between different Mass Spectrometry vendors and hence it is difficult to indicate a specific button or menu item. However, continuous calibration is an option that is available during adding a 'Mass Spectrometry method' in most of the vendor-provided software. These steps have also been modified to appeal to a general mass spectrometry reader.

5) 3.2,3.4 : Please cite references for each step.

References have been added for these steps.

6) 4.1: Is quenching done as in Section 1?

Yes, the quenching is done as in section 1 and the section has been referenced in this step for ease of readability.

7) 4.3: Please mention what button is clicked on in the software to do this, or which menu items need to be selected.

These are again vendor specific details that are difficult to add since they have vastly difference interfaces. However, we have modified the steps to include 'MS/MS mode' option details that are common across different Mass spectrometer providers.

8) 4.4: *How is this done? Please add a reference.*

We have added references to a few such analysis soft wares and to studies where such analyses have been detailed.

9) 4.5: *What are the denaturants you use? Please provide examples. Please cite the referenced steps by the step number.*

We have provided examples and referenced the steps by step numbers.

10) 5.1: *What is the quantity of ligands, protein and buffers? At what time points?*

We have added the details of ligands, protein and buffers ratios used, to this step to aid in continuity and improved readability.

11) 5.2: *Incubate at what temperature? For what duration? At what time points?*

We have added details of incubation temperature, deuteration time points etc to this step.

12) 5.3: *How is the data analyzed? Perhaps cite a reference?*

The data analysis has been discussed in earlier steps, and references to those steps have been added.

13) 5.4: *How?*

Details of classification of orthosteric reporters have been added.

14) 5.5: *Needs a reference.*

This has been moved to 5.4 and a reference has been added.

15) 6.2: *Mention the steps by number.*

Step numbers have been added.

16) 6.3: *Needs a reference for the data analysis. What do you analyze for?*

These have been described in earlier steps and hence we have mentioned the relevant step numbers and added more details of what we analyze for.

17) 7.2,7.5: *Needs a reference.*

These have been moved to 5.4 and 7.3, respectively, and references have been added.





















