***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 dose 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 combine 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*](http://s.th/)*. from*[*s.th*](http://s.th/)*." (not "…and…"), "information about/on*[*s.th*](http://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 Kd.*

-The dissociation rate discussed in the manuscript is the rate koff and not the constant KD. We have added “(koff)” to clearly indicate the dissociation rate.

Since the dissociation constant KD is a ratio (KD = koff / kon), despite both the high-affinity ligands having similar KD values, their dissociation rates (koff) 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.