oc-2022-00013z.R1

Name: Peer Review Information for "Development of a First-in-Class Small Molecule Inhibitor of the C-terminal Hsp90 Dimerization"

First Round of Reviewer Comments

Reviewer: 1

Comments to the Author

The article by Bhatia and coworkers entitled "Development of a first-in-class small molecule inhibitor of the C-terminal hsp90 dimerization" is a very interesting and However,, the manuscript is not written in good scientific form, but rather colloquial, which takes the readers attention off of the science-This is a major concern. In addition. there are several minor issues that should be addressed as well and include:

1) Hsp90 represents 4 isoforms, but is written as a single protein in the background and introduction.

2) RTA901 acts to stimulate the chaperone machinery and is not an inhibitor-furthermore, it also induces a robust HSR in contrast to many other c-terminal targeting agents.

3) The C-terminal binding site does not have a higher affinity for ATP as suggested by the authors who state it is an ATP binding site. This should be changed to a nucleotide binding site as research has shown a preference for guanine containing nucleosides.

4) how to reconcile that fact that 5b inhibits 40% dimerizatoin at 50 uM and has an ic50 of 1.3 uM against K562, but 5d has a 55% inhibition and only produces a 98 uM IC50. please explain.

5) Figure 5E appears to suggest that 5b can selectively disrupt Hsp90a dimerizaton in lieu of Hsp90b dimers. please provide insight or alternative explanations.

6) Why were western blots conducted after 48h? Most other Hsp90 inhibitors work withing 12-24 hours...

Reviewer: 2

Comments to the Author

This is a nice paper describing a new class of potential Hsp90 c-terminal inhibitors. The discovery of the compounds is largely based on previous studies of the Gohlke group on the determinants of PPI organisation in the C-terminal dimer of the chaperone.

The authors combine a number of different biophysical and biochemical techniques to validate interactions, and in cell experiments to prove the activity of their lead compound in tutor cell models.

Overall, the paper is nice and complete: I would suggest adding more discussion on the difference between the authors' compounds and other allosteric ligands designed previously (some based on rational computational approaches, acting as activators or inhibitors). In particular, the relevance of the M-C domain interface may be further discussed, in light of the possible use of combination of different molecular interventions on the chaperone

Reviewer: 3

Comments to the Author

The manuscript by Bathia et al. describes the development of a first-in class small molecule inhibitor of the C-terminal Hsp90 dimerization interface. In these years, Hsp90 has become a promising therapeutic target for cancer due to its key role in many cellular processes including cell cycle control, cell survival, as well as for its functional link with many signaling pathways involved in malignant transformation and progression of several tumor types. In this regard, considerable efforts are being under way to develop new Hsp90 inhibitors, mainly directed to the C-terminal domain in order to avoid the induction of heat shock response. In the present manuscript, the authors reported the identification of compound (5b) targeting the Hsp90 CTD dimerization interface, based on a tripyrimidonamide scaffold through a multidisciplinary approach, applying a rational design, chemical synthesis, assessment of the biochemical affinity and, finally, efficacy against therapy-resistant leukemia cells.Additionally, the identified inhibitor 5b reduces xenotransplantation of leukemia cells in zebrafish models, and induces apoptosis in TKI-resistant BCR-ABL1 mutant cells.

The manuscript is detailed and well written, and the obtained results are supported by the experimental data. Then, I believe it could be interesting for the readers of ACS Central Science. .

Some point should be ri-examined by the authors:

- A discussion about 5b and 5d with regard to the different values of KD observed.

-Some references on the identification of Hsp90 inhibitors (especially C-terminal) should be added, for example:

Molecular Cancer (2020) 19:161; Chem. Commun., 2015,51, 3850-3853.

Author's Response to Peer Review Comments:

Journal: ACS Central Science
Manuscript ID: oc-2022-00013z
Original Submission Date: 05-Jan-2022
Title: "Development of a First-in-Class Small Molecule Inhibitor of the C-terminal Hsp90 Dimerization"
Author(s): Bhatia, Sanil; Spanier, Lukas; Bickel, David; Dienstbier, Niklas; Woloschin, Vitalij; Vogt, Melina; Pols, Henrik; Lungerich, Beate; Reiners, Jens; Aghaallaei, Narges; Diedrich, Daniela; Frieg, Benedikt; Schliehe-Diecks, Julian; Boop, Bertan; Lang, Franziska; Gopalswamy, Mohanraj; Loschwitz, Jennifer; Bajohgli, Baubak; Skokowa, Julia; Borkhardt, Arndt; Hauer, Julia; Hansen, Finn; Smits, Sander; Jose, Joachim; Gohlke, Holger; Kurz, Thomas

We are flexible in these unprecedented times affecting the global research community. If you need more time to complete authoring or reviewing tasks, please contact the editorial office and request an extension.

Dear Dr. Kurz:

Thank you for your recent submission to ACS Central Science. We have now received the reviews of your manuscript.

In its current form, your manuscript is not yet suitable for publication in ACS Central Science. The reviewers have raised points that require significant consideration and major revision that may include additional experiments/data and discussion. However, with adequate revisions, your manuscript may become acceptable for publication.

We would like to receive your revision no later than 11-Mar-2022. The revision should address the reviewers' comments and include a point-by-point response. Your manuscript may be subject to further peer review and likely sent back to one or more of the original referees.

In addition to a clean copy of the revised manuscript, please also submit a tracked version of the original submission that shows the actual changes (deletions and additions) made to the manuscript. You may highlight, color font, or underline the changes.

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Prior to submitting your revision, please also be sure to address the formatting issues listed above the reviewer comments. Further information regarding press, hero images, etc. is included in the attached 'Author Checklist' document. Please note that certain non-scientific needs are required prior to acceptance.

Funding Sources: Authors are required to report ALL funding sources and grant/award numbers relevant to this manuscript. Enter all sources of funding for ALL authors relevant to this manuscript in BOTH the Open Funder Registry tool in ACS Paragon Plus and in the manuscript to meet this requirement. See http://pubs.acs.org/page/4authors/funder_options.html for complete instructions.

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Best wishes,

Prof. Editor
Senior Editor ACS Central Science

Formatting Needs:

ABSTRACT: Please shorten the abstract to 200 words or less. It is currently 219.

Reply: We have shortened the abstract to 200 words.

SI: Please combine into one file.

SI PG#S: The supporting information pages must be numbered consecutively, starting with page S1.

Reply: We have combined the supplementary information into one file and numbered them from page S1-S44.

SI PARAGRAPH: If the manuscript is accompanied by any supporting information for publication, a brief description of the supplementary material is required in the manuscript. The appropriate format is: Supporting Information. Brief statement in non-sentence format listing the contents of the material supplied as Supporting Information.

Reply: We have added a SI paragraph on page 40, describing the provided supplementary materials:

Supporting Information:

- Chemical synthesis (general method, compound characterization and spectral data) (PDF)
- Supplementary Figures and Tables (PDF)

- Determination of aqueous solubility of 5b (PDF)
- Assessment of metabolic stability of 5b (PDF)

SYNOPSIS: The synopsis should be no more than 200 characters (including spaces) and should reasonably correlate with the TOC graphic. The synopsis is intended to explain the importance of the article to a broader readership across the sciences. Please place your synopsis in the manuscript file after the TOC graphic.

Reply: We have added the synopsis on Page 49 next TOC graphic, it reads:

"The tripyrimidonamide 5b is the first-in-class small molecule inhibitor targeting the Hsp90 CTD dimerization interface that shows antileukemic activity without inducing heat shock response (HSR)."

SAFETY STATEMENT: Authors must emphasize any unexpected, new, and/or significant hazards or risks associated with the reported work. This information should be in the Experimental Section of a full article and included in the main text of a letter. Statement examples can be found in the <Safety Statement Style Sheet> and additional information on communicating safety information from the ACS Guide to Scholarly Communication is freely available here.

Reply: 'No unusual or unexpected safety related hazards were encountered'

This safety statement is now inserted in the supplementary information file at Page S24.

Comment: REFS 10+ AU: References with more than 10 authors should list the first 10 authors, then be followed by "et al."

Reply: We have changed the reference style to 'ACS' and restricted the output style to 10 authors, then followed by et al.

FUNDING PLACEMENT: please move Funding Sources to the Acknowledgment section

Reply: We have moved the Funding Sources to the Acknowledgment section.

Reviewer(s)' Comments to Author:

Reviewer: 1

Recommendation: Reconsider after major revisions noted.

Comments:
The article by Bhatia and coworkers entitled "Development of a first-in-class small molecule inhibitor of the C-terminal hsp90 dimerization" is a very interesting and However,, the manuscript is not written in good scientific form, but rather colloquial, which takes the readers attention off of the science-This is a major concern. In addition. there are several minor issues that should be addressed as well and include:

Reply: We thank the reviewer for his/her comments. We took several steps to shape the manuscript in a good scientific form:

1) Removed general statements from the manuscript or backed up with specific examples. Below, new added section is underlined:

   a) On page 4, line 7: In cancer cells, Hsp90 is overexpressed, involved in uncontrolled proliferation and anti-apoptotic effects, and, that way, essential for the malignant transformation and progression of several cancer types, including in acute and chronic myeloid leukemia (AML and CML).
   b) On page 4, line 8-9: Multiple signal transduction-promoting oncoproteins are client proteins of Hsp90, including BCR-ABL1 fusion kinase, which is a molecular hallmark of CML.
   c) One page 4, line 11-14: Several Hsp90 inhibitors (Hsp90i) have been developed so far, for instance targeting Hsp90 N- or C-terminal domain (NTD or CTD) or with isoform selectivity, whereas, most of the inhibitors studied in clinical trials target the Hsp90 NTD ATP binding site and with a pan-inhibitory profile.

2) Added reviews summarizing the recent developments in the field of Hsp90 inhibitors development.

New citation (number 18): Banerjee, M., Hatial, I., Keegan, B. M. & Blagg, B. S. J. Assay design and development strategies for finding Hsp90 inhibitors and their role in human diseases. Pharmacol Ther 221, 107747, doi:10.1016/j.pharmthera.2020.107747 (2021).

New citation (number 19): Koren, J., 3rd; Blagg, B. S. J., The Right Tool for the Job: An Overview of Hsp90 Inhibitors. Adv Exp Med Biol 2020, 1243, 135-146.

New citation (number 20): Li, L.; Wang, L.; You, Q. D.; Xu, X. L., Heat Shock Protein 90 Inhibitors: An Update on Achievements, Challenges, and Future Directions. Journal of medicinal chemistry 2020, 63 (5), 1798-1822.

3) Removed non-scientific expressions, for instance:
a) On page 4, line 21, removed ‘drastic’ and changed it to ‘significant’.
b) On page 4, line 15, removed ‘pitfall’ and changed it to ‘clinical challenge’, updated sentence reads: ‘Another clinical challenge with the use of Hsp90 NTD targeting inhibitors is the induction of the pro-survival heat shock response (HSR).’

Comment #1) Hsp90 represents 4 isoforms, but is written as a single protein in the background and introduction.

Reply: We thank the reviewer for drawing our attention. We have now changed this in the background and in the introduction.

a) One page 3, first line of the abstract: Heat shock proteins 90 (Hsp90) are promising therapeutic targets due to their involvement in stabilizing several aberrantly expressed oncoproteins.
b) On page 4, first line of the introduction: The heat shock proteins of 90kDa (Hsp90) are abundant, molecular chaperones that modulate the folding, stabilization, and maturation of over 400 client proteins in eukaryotes, which are involved in essential processes such as signal transduction, cell cycle progression, and transcription regulation.

Comment #2) RTA901 acts to stimulate the chaperone machinery and is not an inhibitor-furthermore, it also induces a robust HSR in contrast to many other c-terminal targeting agents.

Reply: We agree with the reviewer's comment and hence we have removed RTA901 from the introduction as an example, and thus removed the actual number of Hsp90i that have entered in the clinical trials. The updated sentence reads:

On page 3, line 11-14: 'Several Hsp90 inhibitors (Hsp90i) have been developed so far, for instance targeting Hsp90 N- or C-terminal domain (NTD or CTD) or with isoform selectivity, whereas, most of the inhibitors studied in clinical trials target the Hsp90 NTD ATP binding site and with a pan-inhibitory profile.'

Comment #3) The C-terminal binding site does not have a higher affinity for ATP as suggested by the authors who state it is an ATP binding site. This should be changed to a nucleotide binding site as research has shown a preference for guanine containing nucleosides.

Reply: On page 5, line 9 and on page 26, line 15: We have changed ATP binding site to nucleotide binding site.

Comment #4) how to reconcile that fact that 5b inhibits 40% dimerizatoin at 50 uM and has an ic50 of 1.3 uM against K562, but 5d has a 55% inhibition and only produces a 98 uM IC50. please explain.

Reply: This can be explained by three-fold differences in the apparent K_D between 5b (3.4 ± 1.0µM) and 5d (11.7 ± 1.0µM) in microscale thermophoresis (MST) measurements (Figure 2e). Moreover, the different orders of K_D (Figure 2c) and IC50 (Figure 2d) values of 5b and 5d with respect to the percentages of inhibition (Figure 2b) might be due to uncertainties in the autodisplay dimerization assay. The autodisplay assay is a FACS assay, which was applied as an initial screening method with one-point measurements (at 50 µM). Hence, it can give an indication on the inhibitory profile of the compounds but is not sufficient to predict quantitative values like IC50 values reliably.

5d is less potent than 5b likely because of the larger 4-benzyloxyphenylethyl substituent compared to 4-methoxybenzyl (Figure 1e), which sterically interferes when binding to the H4/H5 interface. In contrast to the 4-methoxy-benzyl side chain of 5b, the 4-benzyloxyphenylethyl substituent of 5d cannot mimic the Y689' hot spot and the 4-methoxy-benzyl side chain of 5b does it and should also act as a (weak) hydrogen bond acceptor for S673' and T669'.

We have inserted this text in the discussion on Page 26, Line 27, which reads: The three-fold difference in the K_D between 5b and 5d was reported, which is likely because of the larger 4-benzyloxyphenylethyl substituent compared to 4-methoxybenzyl (Figure 1e) that sterically interferes when binding to the H4/H5 interface. In contrast to the 4-methoxy-benzyl side chain of 5b, the 4-benzyloxyphenylethyl substituent of 5d cannot mimic the Y689' hot spot and the 4-methoxy-benzyl side chain of 5b does it and should also act as a (weak) hydrogen bond acceptor for S673' and T669'.

Comment #5) Figure 5E appears to suggest that 5b can selectively disrupt Hsp90a dimerizaton in lieu of Hsp90b dimers. please provide insight or alternative explanations.

Reply: We thank the reviewer for this comment. As it can be seen in Figure 5E, we have also incubated K562 cells with novobiocin (NB) and AUY922 HSP90i as controls, and the extent of Hsp90α or β complex/monomer/dimer disruption by 5b was comparable to the controls. Moreover, the expression of detected Hsp90α monomeric/dimeric species was prominently lower than the Hsp90β monomeric/dimeric species in the blue native PAGE analysis, which makes it difficult to conclude whether 5b had any intracellular isoform selectivity, especially in disrupting Hsp90 dimerization. Additionally to determine whether 5b exhibit any Hsp90 isoform specificity, we performed MD simulations with Hsp90α and β proteins, however we did not determine any isoform specificity with 5b (Figure 4a and SI Figure 24). Furthermore, in TR-FRET assay by taking Hsp90α and β CTD recombinant proteins (Figure 3e, SI Table 1), 5b did not display any Hsp90 isoform selectivity in blocking the binding of a CTD interacting chaperone (PPID).

We have inserted this explanation in the result section on Page 18, Line 5: The extent of Hsp90α or β complex/monomer/dimer disruption by 5b was comparable to the controls (NB and AUY922). Moreover, the expression of detected Hsp90α monomeric/dimeric species was prominently lower than the Hsp90β monomeric/dimeric species in the blue native PAGE analysis, which makes it difficult to conclude whether 5b had any intracellular isoform selectivity, especially in disrupting Hsp90 dimerization.

We have also added this sentence in the discussion after MD simulation results on Page 27, line 8: Furthermore, in TR-FRET assay by taking Hsp90α and β CTD recombinant proteins, 5b did not display any Hsp90 isoform selectivity in blocking the binding of a CTD interacting chaperone (PPID).

Comment 6) Why were western blots conducted after 48h? Most other Hsp90 inhibitors work withing 12-24 hours...

Reply: We have treated the leukemic cells for 48h to determine the impact of 5b in several *ex vivo* functional assays, such as Caspase 3/7 dependent, annexin V/PI apoptosis assay (Figure 7b, 7e, 7g and SI Figure 28) or differentiation induction (SI Figure 29), and therefore to stay coherent with this time frame we have chosen 48h treatment for western blotting. Furthermore in our previous publication (Bhatia et al., Blood. 2018 Jul 19;132(3):307-320) we have also performed the western blotting after 48h incubation with predecessor compound (AX). Of note, this does not imply that the compound 5b acts only at a later time points, as 24h exposure of 5b to leukemic cell lines and primary patient samples was sufficient to see an anti-cancer effect in colony forming assay (Figure 7d, 7h and SI Figure 30).

Additional Questions:
Quality of experimental data, technical rigor: Top 5%

Significance to chemistry researchers in this and related fields: Top 1%

Broad interest to other researchers: Top 5%

Novelty: Top 5%

Is this research study suitable for media coverage or a First Reactions (a News & Views piece in the journal)?: No

Reviewer: 2

Recommendation: Publish in ACS Central Science after minor revisions noted.

Comments:
This is a nice paper describing a new class of potential Hsp90 c-terminal inhibitors. The discovery of the compounds is largely based on previous studies of the Gohlke group on the determinants of PPI organisation in the C-terminal dimer of the chaperone.
The authors combine a number of different biophysical and biochemical techniques to validate interactions, and in cell experiments to prove the activity of their lead compound in tutor cell models.
Overall, the paper is nice and complete: I would suggest adding more discussion on the difference between the authors' compounds and other allosteric ligands designed previously (some based on rational computational approaches, acting as activators or inhibitors). In particular, the relevance of the M-C domain interface may be further discussed, in light of the possible use of combination of different molecular interventions on the chaperone

Reply: We thank the reviewer 2 for reviewing our manuscript and for the encouraging comments. We have added new introduction and discussion section with the references:

1) On page 5, line 12: (5) modulators addressing an allosteric binding site between CTD and MD.

2) On page 26, line 12-15: The CTD of Hsp90 contains several binding areas: the C-terminal ATP binding site, the MEEVD motif at the end of the CTD, the region at the border between the MD and the CTD (located ~60 Å away from the NTD ATP binding site, which has been indicated to host a druggable allosteric binding site) and the primary dimerization interface of Hsp90.

New citation (number 80): D'Annessa, I., Raniolo, S., Limongelli, V., Di Marino, D. & Colombo, G. Ligand Binding, Unbinding, and Allosteric Effects: Deciphering Small-Molecule Modulation of HSP90. *J Chem Theory Comput* **15**, 6368-6381, doi:10.1021/acs.jctc.9b00319 (2019).

New citation (number 81): Sanchez-Martin, C., Serapian, S. A., Colombo, G. & Rasola, A. Dynamically Shaping Chaperones. Allosteric Modulators of HSP90 Family as Regulatory Tools of Cell Metabolism in Neoplastic Progression. Front Oncol 10, 1177, doi:10.3389/fonc.2020.01177 (2020).

3) On page 26, line 19-21: For the mitochondrial Hsp90 paralog TRAP1, small-molecule inhibitors were rationally found that target the allosteric site and found Hsp90 activators were indicated to also act via this site.

New citation (number 29): Sattin, S. *et al.* Activation of Hsp90 Enzymatic Activity and Conformational Dynamics through Rationally Designed Allosteric Ligands. *Chemistry* **21**, 13598-13608, doi:10.1002/chem.201502211 (2015).

New citation (number 30): Sanchez-Martin, C. *et al.* Rational Design of Allosteric and Selective Inhibitors of the Molecular Chaperone TRAP1. *Cell Rep* **31**, 107531, doi:10.1016/j.celrep.2020.107531 (2020).

Additional Questions:
Quality of experimental data, technical rigor: High

Significance to chemistry researchers in this and related fields: High

Broad interest to other researchers: High

Novelty: Moderate

Is this research study suitable for media coverage or a First Reactions (a News & Views piece in the journal)?: No

Reviewer: 3

Recommendation: Publish in ACS Central Science after minor revisions noted.

Comments:
The manuscript by Bathia et al. describes the development of a first-in class small molecule inhibitor of the C-terminal Hsp90 dimerization interface. In these years, Hsp90 has become a promising therapeutic target for cancer due to its key role in many cellular processes including cell cycle control, cell survival, as well as for its functional link with many signaling pathways involved in malignant transformation and progression of several tumor types. In this regard, considerable efforts are being under way to develop new Hsp90 inhibitors, mainly directed to the C-terminal domain in order to avoid the induction of heat shock response. In the present manuscript, the authors reported the identification of compound (5b) targeting the Hsp90 CTD dimerization interface, based on a tripyrimidonamide scaffold through a multidisciplinary approach, applying a rational design, chemical synthesis, assessment of the biochemical affinity and, finally, efficacy against therapy-resistant leukemia cells.Additionally, the identified inhibitor 5b reduces xenotransplantation of leukemia cells in zebrafish models, and induces apoptosis in TKI-resistant BCR-ABL1 mutant cells.
The manuscript is detailed and well written, and the obtained results are supported by the experimental data. Then, I believe it could be interesting for the readers of ACS Central Science. .
Some point should be ri-examined by the authors:

Reply: We thank the reviewer 3 for reviewing our manuscript and for the encouraging comments.

Comment #- A discussion about 5b and 5d with regard to the different values of KD observed.

Reply: 5d K_D (11.7 ± 1.0μM) is ~3-fold less potent than 5b (3.4 ± 1.0μM), likely because the larger 4-benzyloxyphenylethyl substituent compared to 4-methoxy-benzyl (Figure 1e) that sterically interferes when binding to the H4/H5 interface. In contrast to the 4-methoxy-benzyl side chain of 5b, the 4-benzyloxyphenylethyl substituent of 5d cannot mimic the Y689' hot spot and the 4-methoxy-benzyl side chain of 5b does it and should also act as a (weak) hydrogen bond acceptor for S673' and T669'.

We have inserted this text in the discussion on Page 26, Line 27, which reads: The three-fold difference in the K_D between 5b and 5d was reported, which is likely because of the larger 4-benzyloxyphenylethyl substituent compared to 4-methoxybenzyl (Figure 1e) that sterically interferes when binding to the H4/H5 interface. In contrast to the 4-methoxy-benzyl side chain of 5b, the 4-benzyloxyphenylethyl substituent of 5d cannot mimic the Y689' hot spot and the 4-methoxy-benzyl side chain of 5b does it and should also act as a (weak) hydrogen bond acceptor for S673' and T669'.

Comment #-Some references on the identification of Hsp90 inhibitors (especially C-terminal) should be added, for example:
Molecular Cancer (2020) 19:161; Chem. Commun., 2015,51, 3850-3853.

Reply: We have added the suggested reference and few other reviews summarizing the development of different HSP90 C-terminal inhibitors on page 5, line 13.

New citation (number 27): Strocchia, M. *et al.* Targeting the Hsp90 C-terminal domain by the chemically accessible dihydropyrimidinone scaffold. *Chemical communications* **51**, 3850-3853, doi:10.1039/c4cc10074c (2015).

New citation (number 29): Sattin, S. *et al.* Activation of Hsp90 Enzymatic Activity and Conformational Dynamics through Rationally Designed Allosteric Ligands. *Chemistry* **21**, 13598-13608, doi:10.1002/chem.201502211 (2015).

New citation (number 30): Sanchez-Martin, C. *et al.* Rational Design of Allosteric and Selective Inhibitors of the Molecular Chaperone TRAP1. *Cell Rep* **31**, 107531, doi:10.1016/j.celrep.2020.107531 (2020).

New citation (number 31): Mak, O. W.; Sharma, N.; Reynisson, J.; Leung, I. K. H., Discovery of novel Hsp90 C-terminal domain inhibitors that disrupt co-chaperone binding. Bioorg Med Chem Lett 2021, 38, 127857.

Additional Questions:
Quality of experimental data, technical rigor: High

Significance to chemistry researchers in this and related fields: High

Broad interest to other researchers: Moderate

Novelty: Moderate

Is this research study suitable for media coverage or a First Reactions (a News & Views piece in the

journal)?: No

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