Author's response to reviews

Title: Comparative analysis of novel and conventional Hsp90 inhibitors on HIF activity and angiogenic potential in clear cell renal cell carcinoma: Implications for clinical evaluation

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Author's response to reviews: see over
Dear Editor,

Thank you for reviewing our manuscript ‘Comparative analysis of novel and conventional Hsp90 inhibitors on HIF activity and angiogenic potential in clear cell renal cell carcinoma: Implications for clinical evaluation’ by Bohonowych et al. We have addressed the reviewers’ comments herein and, where applicable, within the manuscript, as indicated.

Reviewer #1:

1) Constitutive HIF-1 can obviously be reduced (Fig.), however it would be valuable to see effects of inhibitors under hypoxic conditions as well (1%O2 or CoCL2)

Use of VHL deficient cells was to approximate the cellular context of hypoxia with respect to constitutive HIF activity. However, it is also known that hypoxia can further activate HIF through inhibition of the repressive FIH [1]. Although we have previously demonstrated that GA can suppress HIF activity and VEGF transcription under hypoxia, to account for the possibility of a hypoxia specific effect, we have taken the reviewer’s suggestion and examined the effects of EC154 and LBH589 with respect to VEGF and uPa secretion under hypoxia. These results are now presented in Fig. S4, and text has accordingly been added to the results section.

2) The authors should further clarify that an LBH589 mediated increase in HIF-2 does obviously not result in an increased HIF-2 activity, as determined by down-stream genes.

As per the reviewers suggestion, we have clarified this point through the addition of text in both the results and discussion sections. Our data clearly demonstrate that the increase in HIF-2 expression mediated by LBH-589 did not elicit a comparable increase in HIF-2 activity. This was shown by expression of HIF transcripts, including Oct4, which is regulated by HIF-2. Throughout the manuscript, there was no indication that LBH-589 elicited any increase in HIF-2 activity.

3) The figures are quite colorful and authors should consider a more legible format.

Due to the complexity of the figures and the corresponding key, we felt that the best way to depict the data would be to utilize distinct colors, rather than shading and patterns, which can be quite busy and detract from the main points. Use of a range of colors, rather than shades of grey, allow for clearer distinctions. If specifics need to be altered, we can certainly accommodate the request. We recognize that some of the figures are a bit busy, but we contend that this is more a reflection of the complexity inherent in the design, rather than the representational style.

Reviewer #2:

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1) Authors analyzed the effects of the drugs on expression of HIF1α and HIF2α, interestingly they found a decrease of expression but in some condition an increase... nevertheless the transcriptional activity is still drastically decrease...the authors referred to other papers to explain that the HIFα expression is not directly correlated with his activity, but lack to give other explanation... What about the regulatory subunit expression in grugs treated condition: HIFα expression? or ARNT expression? We certainly agree with the reviewer that the molecular basis for the uncoupling of HIF expression and activity we observed is an interesting and valid question. However, one of the central themes of this manuscript is to demonstrate the discrepancy between HIF levels and activity for several functional endpoints and the need for relevant and reliable surrogates/biomarkers in the evaluation of new Hsp90 inhibitors. Although it would be interesting to further examine the mechanistic basis for this, it is not within the scope of this paper, as innumerable possible regulatory subunits could be involved including ARNT, RACK, NF-kB, p300, and others, all of which are offered as possible explanations in the discussion. Given that HIF activity is regulated by acetylation, it is very possible that the increase in HIF acetylation by LBH-589 is inhibitory. Further, increased acetylation has also been shown to inhibit Hsp90 chaperone activity, which may further suppress HIF-2 activity. These points have been further clarified in the discussion. These possibilities have now been further clarified in the discussion.

2) Figure 3. In 786-O, drugs repress HIF1α specific targets (CAIX and GLUT1), while those cells do not express HIF1α... Do HIF2α compensate the HIF1α lack of expression? As mentioned in the results section, our data suggest a potential contributory role for HIF-2α in the regulation of CAIX and LOX-1. A further dissection of the specific transcriptional events controlled by HIF-1 vs HIF-2 is beyond the scope of this paper.

3) Mobility is inhibited as low concentration as 10 pM. At this dose there is a weak or no effect on ERK, FAK and Src phosphorylation, this pathway has to be exclude as an explanation... the other pathway suggested is uPA, but as described in figure 5, while LBH589 decrease uPA secretion, 17-AAG increase it, however both drugs reduce mobility! VEGF expression is not affected neither... how to explain this massive mobility reduction? Given that HIF has been reported to promote cell motility, and that cell motility is often correlated with metastatic potential, we included this metric within our analysis. Although it is an interesting question, defining the precise mechanistic basis of drug mediated suppression of cell motility is beyond the scope of this work. As reiterated by the reviewer, we did rule out ERK, FAK, and Src as participants of this effect. It is not possible to rule out uPA as a causative based solely on ELISA data, as uPA activity can be regulated by additional modifiers, such as PAI-1. Other possibilities include changes to the acetylation status of tubulin following inhibition of either Hsp90 or HDACs. These points have been further clarified in the discussion.

4) Figure 5. While the authors mention a possible increase of expression of uPA at low doses in discussion, nothing is described in the results section. We have now repeated this experiment and the results demonstrate that this transient early increase is not consistent. Our newly performed experiment is shown in Fig. 5 (top right panel). We have confidence in these results, and the data support our findings that these agents potently inhibit secretion of VEGF and uPA.
5) To verify the specificity of drugs effect on HIF\(\alpha\), it’s important to verify it, using HIF1\(\alpha\)-/-/HIF2\(\alpha\)-/- cells, and/or a model where HIF is constitutively active. Could the authors observe any expression modification of non-HIF target genes but HSP90 targets. We are unclear regarding the reviewer’s suggestion as the CCRCC cells (UMRC2 and 786-O) used for this study are indeed models that represent constitutive HIF activity, whereas the VHL-replaced derivatives of these cell lines reflect HIF-deficient counterparts. Results for the VHL-replaced cells are presented in Figures 2 and 4, as well as in Supplemental Figures 1 and 2, and demonstrate that the effects of drug treatment are consistent with a loss of HIF function. It is acknowledged that these drugs affect a number of other cellular proteins, and that they are not solely specific for HIF. However, our data convincingly show specific drug dependent effects upon HIF activity.

6) What about the dramatic drop of impedance with 17-AAG and EC154 at 1-2 hour? Could the drugs charged explain this change of impedance? Do the drugs modify ions efflux of the cells and not the permeability? To confirm these results it can be interesting to perform a quick dye diffusion experiment at different time point. The main take home message with Fig. 7 is that over time, both 17-AAG and EC154 improve barrier function. There is an initial transient decrease in impedance with these drugs that is not observed with LBH-589. We interpret this to mean that 17-AAG and EC154 normalize the vasculature, but in doing so, there may be an interim remodeling phase prior to this restoration of equilibrium. This notion is supported by the lack of a drop and a corresponding lack of an increase with LBH-589. Furthermore, as demonstrated in Supplemental Fig. 5, addition of 17-AAG alone to HUVEC monolayers did not induce a drop in impedance, therefore we do not think the charge of the drug is implicated in this change of impedance. Although a similar experiment can be performed with a dye diffusion approach, use of impedance with the ECIS model is much more sensitive than a dye experiment, and provides continuous data in real time. It is certainly possible that ion efflux is transiently affected, or perhaps gap junctions. Although interesting questions, the precise mechanism of the mechanistic basis of impedance variability is beyond the scope of this work.

7) LBH589 does not restore endothelial barrier function, one explanation will be a non-HSP90 effect of the drug... to verify non-HSP90 dependent effects of the 3 drugs, HSP90 deficient (HSP90-/-, siRNA)cells have to be used... could that explain some inconsistency between drugs? As addressed in point 6 above, this is beyond the scope of the work. It is certainly possible that this, and other effects are due to non-Hsp90 mediated effects (such as protein acetylation). In a complex cellular system, it is not possible to precisely elucidate this point, nor is this the main point of the data in Fig. 7. We raise some possible explanations as well as highlight data from previous reports regarding the effects of LBH589 on barrier function in the discussion. Finally, the type of analysis proposed is prohibited by the lethality of knocking out Hsp90 in eukaryotic cells.

8) While authors claim to analyze the “implication for clinical evaluation”, no in vivo experiment has been done!!!! While we agree with the reviewer that in vivo results would strengthen the manuscript, Biogen Idec has undergone a major re-organization and has stopped oncology work as well as drug synthesis of EC154. As a result, it is not possible to undertake an in vivo study at this time. However, there is still great merit in the drug comparisons presented herein with regards to elucidating appropriate ways to evaluate the efficacy and potency of novel Hsp90 inhibitors with new structures and improved toxicity profiles.
Discretionary Revisions:

1) p18. “via” and not “via”
   We are uncertain regarding the meaning of this comment.

2) p22. “via” and not “via”
   We are uncertain regarding the meaning of this comment.

3) Figure 1B. Standard deviation of the western Blot replicates.
   We have repeated the experiment multiple times and the blot shown is a representative result. The quantitation is performed from biological duplicates. Given that we obtained the same results twice, we contend that nothing will be gained by another duplication for the sake of providing a standard deviation.

4) Impedence or impedance? Have to be verified...
   We thank the reviewer for bringing this discrepancy to our attention. The correct word is “impedance” and this has been corrected throughout the manuscript.

Sincerely,

Jennifer Isaacs, Ph.D.