Author's response to reviews

Title: The pan-HDAC inhibitor panobinostat acts as a sensitizer for erlotinib activity in EGFR-mutated and -wildtype non-small cell lung cancer cells

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Author's response to reviews: see over
Letter to the editor

Dear Dr. Solera,

thank you for providing us with many helpful and constructive comments regarding the manuscript by Greve et al. Below please find a point-by-point reply to the different criticisms in which we tried to address as much as possible within the timeframe allotted. Specifically, we are now providing Western blot experiments suggested by reviewer #1 and describe extensive additional cell line experimentation which we would prefer not to include in the manuscript because we think the data is still too preliminary (see extensive description in reply to comment 2 of referee #2 ).

Since Dr. Julia Schüler helped us conducting several important additional experiments we wish to include her in the list of authors.

We hope that after the additional experimentation performed, and revision of Results and Discussion, the manuscript may now be found acceptable for publication in the journal.

On behalf of the authors,

I remain

sincerely yours

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The first referee (#1 Jhanelle Gray) provided stimulating comments with her broad knowledge and expertise in this field. We could address all her points:

1. "Lines 90-96 appear to belong in the results section not the background section. If these are actually results from a prior trial then Please reference."

We apologize if these lines were misleading. We changed the text and hope that we made it comprehensible that we wanted to give a short preview of our study.

2. "Line 110 should state no instead of not"

We corrected the text accordingly.

3. "The authors comment that their studies demonstrated some possible effects of combination treatment with panobinostat and erlotinib even in the EGFR WT population based upon cell line data. While the authors make note of the phase 1 clinical trial combining these compounds, it is important to note the pertinent conclusions from the trial as they interpret their results. This is particularly true for the conclusions: where they recommend an phase II trial evaluating the combination in lung adenoca patients. A notation of EGFR mutation positivity is an additional likely criterion that would key to eligibility is needed so as not to mislead the reader."

We thank the referee for this helpful comment. It is indeed very interesting that the study could show that especially TKI-naïve EGFR-mutated patients might potentially benefit from combination therapy. This is very much in line with our own experimental data and we have therefore adapted the Discussion accordingly. We hope that we can provide new insights into the mechanism of the effects already seen in the clinic.

4. "Further they make no notation of the predictability of the CHK1 data also noted in the already published clinical trial. While pre-clinical work can support translation of projects to the clinic, Pre-clinical work that is performed on a combination that has already undergone clinical testing (ref 13) should add to the mechanistic understanding of the agents and further the exploration of biomakers. The authors should revamp not only their conclusions but also the results as presented, which I am confident they can do, to demonstrate how their data is additive to what has already been published and to further interrogate CHK1 as they did for e-cadherin. Examining Next steps for this combination overall can be better delineated and would significantly add to the impact of the manuscript."

The referee was absolutely right to suggest interrogation of CHK1 levels in our model. We have added the Western blot data of CHK1 and phosphorylated CHK1 to Figure 3 and have included the findings
in the manuscript. The data shows that panobinostat indeed is able to downregulate CHK1 most likely by corresponding upregulation of p21 and p53. In addition, up-regulation of phosphorylated CHK1 (in line with the immunoblot-signals of phospho-AKT) indicates a further inhibition of CHK1-function and might therefore explain the observed HDACi-induced growth inhibition.

5. "would also recommend outlining where these findings fit in this era of the third-generation EGFR TKIs such as AZD9291 and CO-1686."

We added these third-generation drugs in our outlook.

The other referee (#2 Hiromasa Yamamoto) also provided very helpful comments:

1. "Resolution of the figures is too low. I cannot understand the contents of Figures 1 and 3 (Major Compulsory Revisions)."

We apologize for the low resolution figures and have re-submitted all figures with higher resolutions.

2. "The number of cell lines used in this study is small. The authors chose only one cell line (A549) as an EGFR-wild type adenocarcinoma cell line. They should use more EGFR-wild type cell lines (KRAS-mutant or KRAS-wild type) to show the sensitizing effect of panobinostat to the effect of erlotinib. Other EGFR-mutant cell lines should be also used (Major Compulsory Revisions)."

We agree that the number of cell lines was limited. Therefore we chose 3 additional cell line models with different phenotypes and genotypes.

Two EGFR wt cell lines were selected: A427 (KRAS-mutant) and H1299 (KRAS wt), representing an adenocarcinoma and large-cell carcinoma, respectively. In addition, we chose an EGFR-mutant cell line, H1975 (also adeno carcinoma, KRAs wt), which carries the T790M mutation in addition to the missense L858R mutation, and is known to be highly TKI resistant. We designed the dose-finding experiments as follows: 1. we kept the 2 µM erlotinib concentration as in all the experiments in the manuscript, 2. we used a dose-range of 10, 20 and 30 nM, based on the literature, and our positive experience with these concentrations (see manuscript). Conducting these treatments in triplicates and with 3 treatment time points (24, 48 and 72 hours), we found that even after 72 hours the IC_{50} concentration was only reached in H1975 cells, whereas in A427 and H1299 proliferation was only reduced to 60% and 80% (compared to the DMSO control), respectively.

Therefore we conducted a next series of experiments using the same erlotinib concentration but increasing the panobinostat dose to 30, 40 and 50 nM in H1299 and A427, and to 40, 60 and 80 nM in the H1975 cell line.
Indeed at the highest panobinostat concentration we saw a 50% growth inhibition in H1299 and H1975, still, only 30% growth inhibition was observed in cell line A427. However, in the combination treatments unfortunately we could not detect a robust (i.e. more than 1 time point) sensitizing effect in any of the cell lines. This may be due to for instance to the p53-mutant genotype in cell lines H1299 and H1975. For the A427 we do not have a good explanation (possibly high passage number).

We are grateful to the reviewer for suggesting these experiments and, although the results are not clear and robust enough to warrant inclusion in a revised manuscript, we will continue the study of the EGFR L858R/T790M double mutant p53 mutant cell line H1975 with higher concentrations of both erlotinib and panobinostat, since this is an excellent model to prove that treatment with an HDAC inhibitor and erlotinib can overcome TKI resistance.

3. “The alignment of the protein bands in Figure 3 is difficult to understand. The protein bands should be arranged based on each cell line (Major Compulsory Revisions).”

The referee was right about the confusing arrangement of western blot bands. We have changed the order of bands according to the suggestion based on each cell line.

4. “Results can be simplified. It is redundant (Major Compulsory Revisions).”

We have removed the redundancies of the “Results”.