Reviewer 1

Comments and Suggestions for Authors

Pediatric patients with PTEN Hamartoma Tumor Syndrome (PHTS) frequently develop lipomas. In this study, Kirstein AS et al. examined whether PI3K inhibitor alpelisib has growth-restrictive effects and induces apoptosis in lipoma cells, since mTORC1 inhibitor rapamycin has shown no significant effect on reversing lipoma growth. The authors used PTEN-haploinsufficient lipoma cells from three patients and treated them with alpelisib alone or in combination with rapamycin, and showed that 1-100 μM alpelisib alone or in combination with 10 nM rapamycin reduced the cell proliferation in a concentration- and time-dependent manner. The phosphorylation levels of AKT, mTOR and ribosomal protein S6 were reduced in alpelisib-treated cells. Rapamycin treatment alone led to increased AKT phosphorylation, while alpelisib could reduce rapamycin-induced AKT phosphorylation levels, the viability and proliferation of lipoma cells. Since alpelisib has been well tolerated in first clinical trials, this drug may exhibit a potential to be a therapeutic compound for lipoma cells. However, there are several issues needed to be clarified.

We thank the reviewer for this concise summary of our work.

1. In Figure 1, the authors should calculate the IC50 values of Alpelisib on the lipoma cells from three different patients. Is there an additional or synergistic effect of Alpelisib in combination with Rapamycin treatment on lipoma cell viability? In addition to PTEN-haploinsufficient lipoma cells, what is the effect of Alpelisib on wild-type PTEN lipoma cells?

We calculated the IC50 values (viability and proliferation) for the lipoma cells after 72 h alpelisib treatment. The values were added to the results section (page 3, lines 82-86) and supplement Table S1. For calculation of synergy, we performed additional WST-1 assays with rapamycin concentrations of 1 - 10 nM and alpelisib concentrations of 1 - 50 μM. In all cells tested (LipPD1-3) rapamycin synergistically enhanced the activity of alpelisib as determined with the statistical approach of Chou and Talalay. The results were described on page 3, lines 94-98 and in supplementary Figure S2 and discussed on page 11, lines 283-284.

We do not have access to lipoma cells from non-syndromic lipomas, but provide WST-1 and apoptosis assay data for two lipoma cell cultures from PROS patients, who have mosaic activating PI3KCA mutations and are PTEN-wild-type (Lip3 and Lip4, Methods page 12, Table 1). The results obtained with these cells are similar to those of the PHTS patients, pointing to similar effectiveness of the drug. We added the results (page 3, lines 82-86, supplementary Figure S1 (WST-1) and page 5, Figure 5 a (apoptosis)) and discussed them (page 12, lines 342-343). We restructured Figure 5 and showed the apoptosis assay results for combination treatment in supplementary Figure S4. Additionally, we want to point out that
PHTS is caused by germline PTEN mutations and therefore no PTEN-wildtype cells are present in these patients.

2. In Figure 2 and 3, what are the IC50 values of Alpelisib on these three lipoma cell viability and proliferation? Is it possible that Alpelisib has an effect on inducing the apoptosis of these three lipoma cells? Since there is a big inhibitory effect of Alpelisib on the cell viability after the 24-hour treatment, is this big effect of Alpelisib on the cell viability due to growth suppression, apoptosis induction or both?

- We thank the reviewer for this question/comment which was also mentioned by reviewers 2 and 3 and which prompted us to perform additional experiments regarding cell death and cellular senescence. The IC50 values were added on page 3, lines 82-86 (WST-1), page 4, lines 116-117 (Hoechst) and supplementary Table S1. We found downregulation of the proliferation markers PCNA and Ki-67. We did not observe changes in the amount of dead cells at any tested time points, which was also confirmed by a negative Annexin V/PI-assay. To confirm these results we performed an additional LDH cytotoxicity assay which was found to be negative as well. We therefore conclude that the effect seen in the WST-1 assay (based on metabolic activity) is mainly caused by an inhibition of cellular proliferation. We restructured Figure 5, added the results (Figure 5 b and page 6, lines 146-148 and supplementary Figure S5) and discussed them (page 11, lines 280-281). We also performed additional experiments on cellular senescence and added a new section (pages 9-10, lines 237-262). The elevated senescence marker p16 and positive β-galactosidase staining also indicate growth arrest as a cause of reduced cell viability measures, which was discussed on page 12, lines 326-337.

3. In Figure 4, 48 h, 10 μM Alpelisib treatment can reduce the cell proliferation marker Ki-67 in lipoma cells. How about the dose-response or time-kinetic effect of Alpelisib on the cell proliferation of the other two lipoma cells?

- We performed an additional Ki-67 staining experiment on LipPD2 and 3 cells after 72 h treatment. We found reduced fractions of Ki-67 positive cells after alpelisib treatment indicating inhibition of proliferation. The results were added to supplementary Figure S3a and an indication to this was given on page 5, lines 127-129.

4. In Figure 5, please show the whole results from the examination of the effects of Alpelisib on the cell death and apoptosis of these three lipoma cells. What are the IC50 values of Alpelisib on these three lipoma cell death and apoptosis?

- We only tested apoptosis of LipPD1 cells with Annexin V/PI assay after 24h once and found no apoptosis. This is why we continued to measure apoptosis after 72h (results for reviewer’s benefit):
To support this finding, we performed LDH cytotoxicity assays and showed that there was no cell death, neither at 24 nor 72 h treatment, as mentioned above (Figure 5b); therefore we cannot provide IC50 values here. The solubility of alpelisib in cell culture medium is limited to 50-100 μM which is why we tested those as maximum doses.

5. In Figure 6, please explain why rapamycin can induce the phosphorylation levels of AKT in LipPD1 cells. Is this phenomenon also shown in LipPD2 and LipPD3 cells? The results should be further statistically calculated to see whether there is a statistical significance between groups.

- Rapamycin induces phosphorylation of AKT via disrupting the negative feedback loop regulated by mTORC1, which is discussed as a cause of rapamycin resistance. We included this explanation in our discussion (page 11, lines 296-297 and references 4 and 7).

Our standard test (one sample t-test on the log of the fold change) did not show significant differences here. We now additionally performed a paired t-test on the normalized values for pAKT/AKT and found that pAKT is significantly upregulated after 10 nM rapamycin treatment (p=0.0448). A paired t-test between 10 nM rapamycin treatment alone or in combination with 10 μM alpelisib revealed a significant reduction after combination (p=0.0479). We performed additional Western blots for LipPD2 and LipPD3 cells and found the same phenomenon. We mention this in the results section (page 6, lines 171-174) and added the results to the supplementary Figures S6, S7, S9, S10 and supplementary Table S3.

6. In Figure 7, whether the phenomenon of Alpelisib-suppressed pS6 positive cells is also observed in the other two lipoma cells?

- We performed an additional pS6 staining experiment on LipPD2 and 3 cells after 72 h treatment. We found a dose dependent reduction in the fractions of pS6 positive cells after alpelisib. The results were added to supplementary Figure S3a and an indication to this was given on page 7, lines 183-184.