Reviewer 2

Dear Editor,

Followings are my review comments to the authors of “The novel phosphatidylinositol-3-kinase (PI3K) inhibitor alpelisib effectively inhibits growth of PTEN-haploinsufficient lipoma cells. (Cancers-537425)

In this manuscript, the authors presented that novel PI3K inhibitor, alpelisib alone or in combination with rapamycin reduced proliferation of PTEN-haploinsufficient lipoma cells. They also described that alpelisib treatment reduced phosphorylation of AKT, mTOR and ribosomal protein S6. Although these findings are intriguing and worth investigating, the manuscript demands sufficient evidence to support authors’ conclusions. Furthermore, there are following concerns about the study and the authors should pay attention to the interpretation of the results.

We thank the reviewer for this evaluation.

The review comments to the authors are as follows.

The proportion of apoptotic cells after 72 h treatment with alpelisib and rapamycin showed slight elevation, but the authors did not observe cell death after 72 h treatment. However, alpelisib alone or combination with rapamycin treatment after 72 h reduced both cell viability and proliferation. The authors should clarify the causes of cell viability and proliferation reduction, especially 72 h after treatment.

➢ Thank you for this comment, which was mentioned in similar fashion by reviewers 1 and 3. We assume that the effects seen in the WST-1 cell viability assay are mainly caused by an inhibition on cellular proliferation, since the proliferation markers PCNA and Ki-67 are down regulated. Since the WST-1 assay measures cellular metabolic activity, changes in metabolism as seen on transcriptional level for PGK and GLUT-1 might also play a role. We did not observe changes in the amount of dead cells in the Annexin V/PI-assay. To confirm these results we performed LDH cytotoxicity assays which were found to be negative as well. 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). When checking for induction of senescence we found that the proportion of senescent cells was elevated after alpelisib treatment. In light of this, we 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.

In adipocyte differentiation in 2D and 3D models, the authors showed that alpelisib treatment attenuated adipocyte differentiation and decreased the size of 3D lipoma spheroids during 10 days. There is no explanation in the manuscript what phenomena was induced on LipPD1 cells by alpelisib treatment. When adipocyte differentiation was inhibited by alpelisib, did LipPD1 cells keep immature state such as mesenchymal stem cell or induce cell senescence? The authors should perform further experiments to reveal what type of cellular reaction was induced by alpelisib.

➢ We want to thank the reviewer for this very valuable comment. We performed additional qPCR experiments on adipogenesis (PPARγ, aP2, adiponectin), senescence (p16) and stem cell markers (CD44, CD90) for alpelisib treated and control cells after 10 days in differentiation medium. We found that differentiation markers PPARγ, aP2 and adiponectin were down regulated after 10 μM alpelisib treatment. This is in line with literature findings, where PPARγ is regulated through the PI3K-pathway (insulin signaling). We added the results (page 8, lines 201-221), restructured Figure
9, added the Figures 10, 11 and supplementary Figure S11 and discussed the results (page 12, lines 317-324). Furthermore, we compared the stem cell markers CD44 and CD90 after differentiation and found CD44 upregulated and CD90 down-regulated after alpelisib treatment. CD90 downregulation is associated with senescence in mesenchymal stem cells [Martini 2019 #55]. Although considered a mesenchymal stem cell marker (Ramos 2016, #29), CD44 was also found to be a senescence induced cell adhesion gene (Mun 2010, #30). The senescence marker p16 was upregulated as well, indicating induction of senescence instead of differentiation. We also performed β-galactosidase senescence staining after 72 h alpelisib treatment in culture medium and found elevated numbers of senescent cells, which is in line with the attenuated proliferation we observed. To show these results we added the section 2.5 to the results (page 9, lines 237-262, Figure 12 and 13) and added supplementary Figure S12 and S13. We discussed the findings on page 12, lines 326-337.

The authors analyzed the effects of alpelisib with the concentration ranges from 1mM to 100mM. How the authors determine these concentration ranges? The data of cytotoxicity test of alpelisib against LipPD1 cells should be demonstrated.

- We performed an initial WST-1 assay with concentrations ranging from 0.1 μM to 100 μM and found no effect below 1 μM, thus we chose this as a minimum concentration. Alpelisib was soluble in cell culture medium at no higher concentration than 100 μM, which is why we could not use higher concentrations.

In Western blotting, an internal control such as b-actin or GAPDH should be included.

- The GAPDH controls as well as all Western blots are provided in the supplement, we added an indication to that in the results section (page 6, lines 173-174).