Author's response to reviews

Title: EphB4 as a therapeutic target in mesothelioma

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Thank you for consideration of our manuscript for publication in your journal. We have reviewed the above manuscript according to your reviewer’s comments.

Reviewer #1: Sally Stephenson

Minor Essential Revisions
1. The authors should explain their choice of H2373 and 211H as the model cell lines for different parts of the study.
   
   We chose to use cell lines H2373 and 211H due to the more difficult to treat pathologic variants. On page 6, paragraph 3, we noted H2373 is a sarcomatoid mesothelioma cell line and added “Sarcomatoid is also a category of mesothelioma that is the most difficult to treat”. On page 7, paragraph 1, we added “(211H is derived from a patient with) biphasic histology, a hard to treat category of mesothelioma”.

2. The authors describe the loss of pAkt, pS6 and pSrc as “persistent through the length of the study (25 days)” but they cannot really say this given analysis was only performed after 25 days of treatment. This should be reworded.
   
   On page 6, paragraph 4, we reworded it to “inhibition of phosphorylation of Akt, S6, and Src was observed at the end of the 25-day treatment”.

3. In Figure 4, the co-localisation of CD31 and NG2 is obscured by the addition of the DAPI staining. Can the authors include a panel without the DAPI?
   
   Yes. This is added.

4. Please include images of the positive control cells used in the immunohistochemistry experiments – 293T expressing EphB4, with a comparison to the parental 293T cells as validation of the specificity of the antibody staining.
   
   A figure showing the EphB4 antibody used in this study stains EphB4 is documented by staining wild type and ectopically expressing EphB4 293T clones. We have added this data in Fig. 1C, confirming the specificity of the antibody. We have also referenced our previous study (27), documenting the specificity of the antibody and lack of cross reactivity to any of other EphB receptors.

Discretionary Revisions
1. The data showing loss of pAkt, pS6 and pSrc could have been supported by in vitro studies.
   
   The reviewer has made important suggestion. This experiment will require co-culture of tumor cell and endothelial cells which may not replicate in vivo environment. We thus chose to conduct these analyses with the in vivo tumor tissues.

2. ephrin-B2 signaling is also mediated through PI3K and Src and sEphB4-HSA may be preventing ephrin-B2 activation. Confirmation that the loss of immunoreactivity seen in the tissue was from the tumour cells and not the supporting cells (endothelial cells and pericytes) would be valuable.
After sEphB4-HSA treatment, endothelial cell number was reduced to only a small fraction of the whole tumor (Fig. 2B) thus the contribution of endothelial cell to the overall outcome of PI3K or Src signaling status is minimal.

Several typographical errors were noticed including
1. Page 3 Background should be Background
   Changed accordingly
2. Page 3 kinasekinase should be kinase
   Changed accordingly
3. Page 4 BD biosciences should be BD Biosciences
   Changed accordingly
4. Page 6 phosphylated should be phosphorylated
   Changed accordingly
5. Page 12 Bevacixumabon should be Bevacizumab
   Changed accordingly

Reviewer #2: Xiaofeng Han

Minor Essential Revisions
1. For the statistics in Figure 2, the coverage percentages were used for measuring tumor density, apoptosis or other activities. Were these coverage percentages normalized by DAPI coverage? Because the areas of each small figure are the same, but the numbers of cells are maybe different, normalizing by DAPI coverage will be more accurate for these statistics.
   TUNEL, CD31 and Ki67 coverage were normalized to DAPI coverage. Clarification has been added in the figure legend of Fig.2.

2. In figure 3, individual sEphB4-HSA and Bevacizumab treatment showed similar effect on tumor size, but in Figure 4, Bevacizumab treatment was not that significant comparing with the control. The discussion of this part is not enough; please add more discussion to analyze this mechanism.
   The tumors were only treated for a week in the experiment shown in Fig. 4, which may explain the lack of apparent efficacy of Bevacizumab. We have added the following discussion on Page 7: “Since tumors went to complete remission after combinatorial treatment of sEphB4-HSA and Avastin, we had no tumor tissues for analysis. Therefore, we performed a one-week treatment of 211H tumors to analyze the mechanism of the combinatorial effects of sEphB4-HSA and Bevacizumab (Figure 4). After one week treatment, sEphB4-HSA and Bevacizumab each alone reduced vessel density (P < 0.01 and P < 0.05, respectively). sEphB4-HSA had much greater inhibition of tumor vessel density than Bevacizumab, which is consistent with our previous study in Kaposi’s Sarcoma [14]. However, tumor growth inhibitions after 3 weeks of treatment with sEphB4-HSA and Bevacizumab were similar (Figure 3). This is very likely due to Bevacizumab’s effects on tumor cells directly - mesothelioma is one of only few tumor types that express VEGFR2, produce VEGF and thus have an autocrine loop [33].”

3. Have authors tried other EphB4 inhibitors on mesothelioma other than sEphB4-HSA?
Yes. We have tested EphB4 specific siRNA in vitro and antisense oligonucleotide in vivo (Xia et al., 2005), which is noted in Page 6, paragraph 2. We have not conducted studies with kinase inhibitors due to the lack of availability of a highly specific such inhibitor.

Besides the above responses, we also made adjustments in the email address of one author (Yue Zhou) and also in the acknowledgement section.