Author's response to reviews

Title: Indirubin derivative E804 inhibits angiogenesis

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Author's response to reviews: see over
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Andrzej K. Bednarek
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Dear Dr. Andrzej K. Bednarek,

, Reviewer-1

Thank you very much for your letter of March. 6, 2012 (1777019890634851) concerning the status of our manuscript entitled, “Indirubin derivative E804 inhibits angiogenesis”. We deeply appreciate the reviewers’ constructive comments. We have revised the manuscript to incorporate these comments, as indicated in the following point-by-point responses:

1. Abstract:
   -Methods: replace “on the backs”
     Per se the reviewer’s suggestion, we replaced “on the backs” in revised manuscript.
   
   -Results: omit the 3rd sentence (redundancy).
     Per se the reviewer’s suggestion, we omitted the 3rd sentence in revised manuscript.

   -Conclusions: sentence needs to be re-written.
     Per se the reviewer’s suggestion, we rewrite the conclusion in revised manuscript.

2. Background:
   -2nd paragraph, 5th sentence: Signaling through R1 and R2 are important for embryonic development.
     Thank you for the comments. We explained the role of VEGFR1 and R2 on embryonic development in revise manuscript.

   -Page4, 2nd phrase: it is quite unusual to refer to a figure here. I would omit Fig 1 since the structure is published.
     Per se the reviewer’s suggestion, we omitted the Fig.1 in revised manuscript.

3. Methods:
   -In vivo model: injection dose needs to be displayed otherwise (not molar but in micrograms).
     What is the vehicle?
     Per se the reviewer’s suggestion, we provided the information of vehicle and the amount of IDR-E804 at microgram per animal in revised manuscript.
4. Results:
- Omit first two sentences.
Per se the reviewer’s suggestion, we omitted first two sentences in revised manuscript.

- Omit Fig 1.
Per se the reviewer’s suggestion, we omitted the Fig.1 in revised manuscript.

- Page 11: it is not sure whether IDR-E804 reduces tumor growth directly by inhibiting angiogenesis or indirectly.
Since we agree with the reviewer’s comment, we omitted “directly” in revised manuscript.

- Page 12, line 8: “anti-angiogenic effects on endothelial cells” should be replaced by more accurate terms, eg. anti growth-effects, pro-apoptotic, etc.
Per se the reviewer’s suggestion, we replaced more accurate terms in revised manuscript.

- Page 12, last line: I wouldn’t say “dramatically”.
Again, we omitted “dramatically” in revised manuscript.

Figures: it is not clear why vehicle has a “star”. This result is not discussed.
Since we compared VEGF-treated condition verse other condition including vehicle-treated, vehicle has significant difference.

Reviewer-2

1. The data presented by Shin et al. describe the effect of indirubin derivate E804 on angiogenesis. The authors used various in vitro and in vivo assays. Although the results obtained in this study are with great interest, various points should be addressed prior publication. The major points are the absence of data on the effect of E804 alone; the majority of the experiments done in this study are a combination between vegf and E804.

Thank you for the reviewer comments. We agree that the effect of IDR-E804 alone on angiogenesis is very important issue therefore we investigated the effect of IDR-E804 on angiogenesis with or without VGEF from the beginning of the present study. Although we had the several data to show the effect of IDR-E804 alone we decided to orient VEGF-induced angiogenesis in the present study because IDR-E804
inhibited VEGFR-2 kinase activity (Figure 4). It would be very happy if the reviewers understand our point of view in the present study.

2. Fig. 2A. The data described in this figure showed that VEGF had no effect or very weak effect on huvec cells proliferation (vehicle/0), thereby the inhibitory effect of E804 on cell proliferation cannot be attributed only to its effect on VEGF-induced cell proliferation but through other mechanism including vegf. It will be appreciated if the authors included experiments showing only the effect of E804 on cell proliferation. The obtained data will be compared to those shown in this figure.

Lots of published data showed that VEGF had relatively weak effect on HUVECs proliferation similar as our present data [a, b] and the significant difference was shown between vehicle-treated HUVECS and VEGF treated HUVECs. As shown in below, IDR-E804 alone also has inhibitory effect of HUVECs proliferation at 5 and 10 µM.


3. Fig. 2B or 3. Here again data on the effect of E804 along on cell migration will be appreciated. The wound distance shown in the bar graph at 0 µM E804 doesn’t reflect the experimental result shown in the upper panel at 0 µM E804. In the latter it seems there is no difference between vehicle and 0 µM E804.

Again, as shown in below, IDR-E804 alone also has inhibitory effect of HUVECs migration. Although the wound distance shown in upper panel did not clearly
represent the differences between vehicle and VEGF alone, the statistical difference was reached.

4. The name of the figure should be adjusted. In the figure legend this figure corresponds to fig2c and on the figures the number figure 4 is mentioned. We are very sorry about it. We checked all the figure legend and adjusted in revised manuscript.

5. The effect of E804 alone on capillary-like tubule formation should be addressed. The data presented here show a discordance between the upper figures and there quantification values. Date in vehicle are similar to those obtained with 0, 0.1 µM E804, but the measurement of the mean tube area indicates a difference. Again, as shown in below, IDR-E804 alone also has inhibitory effect of tube formation of HUVECs at 5 and 10 µM HUVECs. We calculated tube area with HCS system ad described in materials and methods based on the figure.
6. Figure 3 of figure 5. The name of the figure should be adjusted. In the figure legend this figure corresponds to fig3 and on the figures the number figure 5 is mentioned. We are very sorry about it. We checked all the figure legend and adjusted in revised manuscript.

7. Figure 4 or figure 6. The effect of E804 alone on VEGFR2 signaling is required in order to understand the effect of E804 on complete inhibition of pERK and pAKT whereas VEGF effect is weak. As reviewer’s concern, it is possible that IDR-E804 alone inhibited the phosphorylation of AKT and ERK without VEGF signaling. As shown in below, 10 μM of IDR-E804 slightly inhibited AKT and ERK phosphorylation but not 1 and 5 μM. These results indicated that IDR-E804 inhibited VEGF-induced phosphorylation of AKT and ERK, at least 1 and 5 μM.

As we mentioned above, we did not include these data in revised manuscript since the present study focused on the effect of IDR-E804 on VEGF-induced angiogenesis. We are really eager to reviewer’s understanding at this point.

We thank the editor and reviewers for this careful evaluation of our work, and we hope that our paper is now suitable for publication in *BMC Cancer*. 
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