Author's response to reviews

Title: A small molecular agent YL529 inhibits VEGF-D-induced lymphangiogenesis and metastasis in preclinical tumor models in addition to its known antitumor activities

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Author's response to reviews: see over
Dear editor,

We are grateful for your letter dated on 9 January, 2015, with regard to the comments for our manuscript entitled “A small molecular agent YL529 inhibits VEGF-D-induced lymphangiogenesis and metastasis in preclinical tumor models in addition to its known antitumor activities (MS: 9316125914519646)”. We thank you and the reviewers for the valuable comments.

We have thoroughly revised the manuscript and addressed all the issues. The detailed explanations are included in the manuscript marked in blue or in the following up section of this letter. We are looking forward to hearing from you at your earliest convenience time. Thanks again for your reconsideration.

Best regards

Sincerely Yours,

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Editor: (Comments to the Author)

Reviewer #1: (Major Compulsory Revisions)

Q1: While the preface to the analysis of the inhibitor YL529 is the modeling of the inhibitor and VEGFR-3 by homology modeling and molecular docking methods one could reasonably assume, given the previous history of other small molecule inhibitors to the VEGFR family, that some level of activity by the small molecule would be expected between two such highly homologous kinase domains.

A1: This is an insightful comment. We acknowledge that, due to the homology, if a small molecule interacts with VEGFR-3, it is possible that it will interact with other two homologous proteins too. But wet experiments are necessary to confirm whether it is the case. So we first used the homology modeling and molecular docking methods to analyze the interactions between YL529 and VEGFR-3, and then tested the specificity by wet experiments.

Q2: The analysis relies on the activity of one of the VEGF growth factors VEGF-D. While this is fine from the perspective of testing the inhibitor and having a generic activator of the pathway, VEGF-C which is the other ligand for VEGFR-3 and indeed VEGFR-2 should be recognised, described in the text and it would have been preferable to look at whether VEGF-C stimulation could be inhibited.

A2: Thanks for this comment. We agree with the reviewer’s comment that both VEGF-D and VEGF-C are the ligands for VEGFR-3, and some researchers have reported that VEGF-C plays an important role in the procession of lung cancer metastasis (Hanahan D and Weinberg RA, Cell 2011; Stacker SA, et al., Nat Rev Cancer 2014,); but VEGF-D as a ligand for VEGFR-3/-2 is indispensable for development of the lymphatic system. VEGF-D regulates the growth of lymphatic vessels via their receptor VEGFR-3, and partly regulates the growth of blood vessels via VEGFR-2. Consistently, tumor cells with up-regulated VEGF-C could increase the intratumoral and peritumoral lymphangiogenesis and exacerbate metastasis to local lymph nodes and distant organs. So it is true that VEGF-C stimulation could be inhibited. However,
due to the limitations of our experimental conditions, we chose to detection the expression of VEGF-D in VEGF-D-LL/2 cell line after YL529 treatment. Based on this, we revised the manuscript and added some comments in “Introduction” section to addressed that VEGF-C plays an important role in the procession of lung cancer metastasis.(Page 3, paragraph 2\textsuperscript{rd}).

**Q3:** Importantly YL529 also targets VEGFR-2 (which has been previously described) a receptor that can also be expressed on lymphatic endothelial cells, can be stimulated by VEGF-D and could be affected in biological or cellular assays where the YL529 inhibitor is being used. These caveats have not been addressed in the article. Can some of the activity in the various bioassays be attributed to VEGF-D stimulation of VEGFR-2? Can some of the inhibitory activity also be assigned to VEGFR-2? While p-VEGFR-3 alters how can we directly attribute the activity seen to VEGFR-3 rather than VEGFR-2?

**A3:** Thanks for the comment. We agree that VEGFR-2 can also be expressed on lymphatic endothelial cells, but VEGFR2 is thought to be the major mediator of angiogenesis and it mainly regulates blood vessel growth of vascular ECs, not lymphatic ECs (Cleaver and Melton, Nat Med 2003; McCarty et al., Mol Cancer Ther 2004; Gaengel et al., Arterioscl Throm Vas Biol 2009). We have reported that YL529 could block the activities of human umbilical vein endothelial cells (HUVEC) stimulated with VEGF\textsubscript{165} previously. Furthermore, other researchers in our group (Jing Wen, et al. Int. J. Cancer 2009) have found that VEGF-D mainly bind to VEGFR-3 in lymphatic endothelial cells in vivo.

We also agree with the reviewer’s comment that some of the inhibitory activity maybe be assigned to VEGFR-2, because YL529 is a multikinases inhibitor with potent antitumor activities. According to the reviewer’s suggestion, we revised the manuscript and added some comments in “Discussion” section to address the caveats. (Page 14, paragraph 1\textsuperscript{rd})

**Q4:** VEGF-D is cleaved by proteolytic digestion and forms intermediate and mature
forms of the growth factor that have variable affinity for VEGFR-2 and VEGFR-3. The SDS-PAGE/Western blot presented does not describe the form of VEGF-D presented in the gel. As VEGF-D can be cleaved into multiple forms (~52, ~30 and ~21 kD) which is these is seen in the gel. The 52 kD form is uncleaved and will have significantly reduced activity on both VEGFR-2 and VEGFR-3. The fully mature form of 21 kD has the greatest affinity for the receptors and can bind and activate both VEGFr-2 and VEGFR-3. In these particular experiments knowing which form is generated is critical to the overall interpretation of the study and the interplay between VEGFr-3 and VEGFR-2.

A4: Thanks for the reviewer’s comment. In present study, the antibody against 21 kD VEGF-D was used because the fully mature form of 21 kD has the greatest affinity for the receptors, and can bind and activate not only VEGFR-2 but also VEGFR-3. According to the reviewer’s suggestion, we added the form of VEGF-D in revised manuscript. (Page 11, paragraph 3rd, line 2th to line 6th)

Q5: The schematic diagram is an overly simplified version of the events and does not describe other participating receptors or pathways well. It is misleading to the non-expert and needs some clarity about other significant targets for YL529 in cells, particularly VEGF-D/VEGFR-2, VEGF-C/VEGFR-2 and VEGF-C/VEGFR-3.

A5: Thanks for this comment. According to the reviewer’s suggestion, we have revised the figure 7 and have described other participating receptors and associated VEGF-D/-C -VEGFR-3/-2.

Minor Essential Revisions:

Q1: Some English expression could be improved through editing

Results section, round down % reduction data eg. from 90.22% to 90% Are (A) and (B) of Figure 3 transposed?

A1: Thanks for the reviewer’s comment. According to the reviewers’ comment, we have revised throughout the manuscript and edited the language by a native-English speaker with scientific expertise, the changed part have been marked in blue in the
Reviewer #2:

(Major)

Q1: I agree with the opinion that VEGF-D is one of important regulators of lymphangiogenesis in various pathological conditions including cancers. However, in general, VEGF-C is most well-known as lymphangiogenesis-related molecule. So, I recommend adding and/or emphasis of the information on pathological significance and prognostic roles of VEGF-D in several cancers into “Introduction” section.

A1: Thanks the reviewer for the comment. According to the reviewers’ recommend, we have revised the “Introduction” section and added the related references and information on pathological significance and prognostic roles of VEGF-D in several cancers. (Page 2, paragraph 2nd, line 5th to line 19th)

Q2: In migration assay, you showed that YL529 inhibited the migration of cells compared with that of untreated cells (Figure 2A). However, unfortunately, I cannot confirm your opinion in Figure 2A. Please show clearer and enlarged Figure of cell scratch assay.

A2: Thanks for the reviewer’s comment. According to the reviewers’ comment, we have revised Figure 2A, and also provided a higher resolution figure of cell scratch assay as enclosed files for review.

Q3: In Figure 4D, Kaplan-Meier survival curves were shown. In this study, number of mice in each group was just 10. I have a senesce of discomfort with that such small data is showed by Kaplan-Meier survival curve. So, I recommend showing the relationship between YL529 and survival by the other method. I would like to know your opinion.

A3: Thanks the reviewer for the comment. In fact, this survival experiment was repeated done three times under the same conditions. The number of mice in each group was 20, half of animals (10) in each group was done as pharmacodynamics
experiment. So the animals in each group for survival analysis were 10 mice. According to the reviewer’s recommend, we have analyzed the survival data of three repeated by using Kaplan-Meier with log-rank test (because our experiment was very suitable for single factor analysis). The three results were consistent with previous results. Thanks again for the reviewer for this good comment.

**Q4:** Please add the new photo with higher magnification (X400~600) or change the Figure 6 because I am afraid that your original photo is too small to understand your results.

**A4:** Thanks for the reviewer’s comment. According to the reviewer’s recommend, we have changed the photos and revised the Figure 6.

**Q5:** In Figure 6A, I felt that pathological findings of cancer tissues was different between vehicle tissue and treated one. Please explain your opinion.

**A5:** Yes, indeed, we have observed that the pathological finding of cancer tissues was different between vehicle and treated tissues. We reason that YL529 may have the potential to repair damage (tumor) tissues, therefore play additional beneficial role for lung cancer patients.

**References**


