Author's response to reviews

Title: Genetically engineered Endostatin-Lidamycin Fusion Proteins Effectively Inhibit Tumor Growth and Metastasis

Authors:

Wen-guo Jiang (jiangwg@gmail.com)
Xin-an Lu (luxinan09@gmail.com)
Bo-yang Shang (byshang25@163.com)
Yan Fu (fuyan@mail.tsinghua.edu.cn)
Sheng-hua Zhang (drug@caca.sina.net)
Dai-fu Zhou (zhdaifu@gmail.com)
Liang Li (Liang771124@163.com)
Yi Li (liyi0108@163.com)
Yong-zhang Luo (yluo@tsinghua.edu.cn)
Yong-su Zhen (zhenys@cae.cn)

Version: 3 Date: 25 August 2013

Author's response to reviews: see over
Dear Prof. Oliver Stoeltzing,

Thank you very much for your letter and advice. We have revised the manuscript, and would like to re-submit it for your consideration. We have addressed the comments raised by the reviewers, and the amendments are highlighted in red in the revised manuscript. Point by point responses to the reviewers’ comments are listed below this letter.

We hope that the revised version of the manuscript is now acceptable for publication in your journal.

I look forward to hearing from you soon.

With best wishes,

Yours sincerely,

Yong-su Zhen
Yongzhang Luo
We would like to express our sincere thanks to the reviewers for the constructive and positive comments.

Replies to Reviewer 1

1. **The data are fine but I don't think they can say much about why the fusion protein works better. The mechanism they propose is plausible, but they provide little data to support their proposed mechanism. I do not want to be too hard on the authors. However, I think they should at least tone down their claims concerning mechanism since they do not have the data to support those claims.**

   **Answer:** The mechanism of the fusion protein mainly includes 2 aspects, the targeting activity and the combined activity.

   First, the targeting activity of ES moiety is well documented. In addition, ES-LDP could bind human lung cancer tissue by tissue-microarray analysis *in vitro*, and have the capacity to accumulate into the tumor area in nude mice bearing human PG-BE1 xenograft. It is uncertain why ES-LDP works better than LDP-ES and what its specific target molecular is. So we put forward some assumptions according to the results. Several sentences have been changed in the Discussion (page 21, paragraph 1) in the revised version to address this issue.

   Secondly, the proof of the combined activity to tumor vasculature and tumor cell by respective ES and LDM moiety of fusion protein was not sufficient enough. Several sentences have been changed in the Discussion (page 21, paragraph 2; page 23) and Conclusion (page 3; page 23) in the revised version to address this issue.

2. **I also did not like the Introduction and Discussion about ES. There is a vast literature of different ideas about ES functions their narrative is overly selective.**

   **Answer:** ES inhibits 65 different tumor types and modifies 12% of the human genome to down-regulate pathological angiogenesis. We mainly cited the references about the targeting activity. Now, we simplify them. Then we add other references of other groups to explain the function of ES in Introduction. Several sentences have been changed in the Introduction (page 4; page 5, paragraph 2) in the revised version to address this issue. This reference has been quoted as ref.2-6 and 16 in the revised version.

   We mainly introduced some ES-based fusion proteins in Discussion, which include ES-cytosine deaminase protein, prolactin antagonist-ES, anti-HER2 IgG3-ES, ZBP-ES (Endostar), and Fc-ES. They amended some defects of ES from different aspects. Additionally, we cited another ES-based fusion protein, a cell-permeable ES protein (HM73ES).

3. **Although U.S. trials were discontinued due to poor clinical responses, a modified ES (ZBP-endostatin) is being evaluated in China, together with conventional chemotherapeutic agents. ZBP-endostatin reported to have higher anti-angiogenesis and anti-tumor activities than native ES proposed several reasons for the difference including maintenance of N-terminal sequence integrity, enhanced zinc binding, improved solubility and/or recovery of E. coli–expressed recombinant proteins. However, a paper published recently about**
cell-permeable endostatin (CP-ES) suggests another mechanism to explain the enhanced activity of the recombinant ES protein as compared to native ES—enhanced protein uptake. This is very important information to explain why cytoplasmic uptake of ES is an important functional determinant with regard to both angiogenesis and anti-tumor activity, and why the plain ES fails in clinical trials and cell penetrating domain-tagged ES (ex, ZBP-endostatin or CP-ES) shows higher clinical efficacy in terms of intracellular delivery. This paper should add this critical mechanistic insight in Discussion of the manuscript.

Answer: The CP-ES exhibited enhanced tissue penetration and suppressed the growth of human tumor xenografts to a significantly greater extent than unmodified ES. Those results suggest another important mechanism to explain the enhanced activity of ZBP-ES and CP-ES. Several sentences have been changed in the Discussion (page 20, paragraph 1) in the revised version to address this issue. This reference has been quoted as ref.32 in the revised version.

Replies to Reviewer 2

1. The authors should denote the source where they cloned the LDP and ES and provide the sequence of LDP and ES they cloned or the references.

Answer: The vector pET30(a)-ldp contained the LDP-encoded fragment. ES sequence was cloned from the cDNA of A549 cell. Several sentences have been changed in the Materials and Methods (page 7, paragraph 2) in the revised version to address this issue.

2. Since the in vitro data show that fusion proteins inhibited the sprouts of human vascular endothelial cells with more potency compared to ES, the authors should provide the scenario of vasculature in tumors from tumor-bearing mice treated with these proteins. It will be better if the status of tumor cells treated with fusion proteins in vivo is provided.

Answer: We agree. Certainly it will be more informative if corresponding in vivo data are obtained. Actually, we excised out the tumor after the animals were sacrificed to detect the CD31, a specific endothelial marker and the Ki-67, a proliferative marker strongly linked to cell cycle control. This should be performed in the future study.

3. For Figure 7, the authors should describe how to calculate the surface metastasis of 4T1 tumor in lung and best provide the macrophotograph of lung from treated mice or alternately image of HE stained lung section.

Answer: The surface metastases of 4T1 tumor in lung were counted by direct visualization using a stereomicroscope. The total number of metastases per lung section was counted and averaged among the animals. Because 4T1-Luc mouse model could be evaluated intuitively through in vivo imaging evaluation of the pulmonary metastases, and the lung of the mouse has been divided into 5 separate lobes and mixed together as counting the surface metastases, we regretted our inability to provide the macrophotograph of lung from treated mice or alternately image of HE stained lung section. Several sentences have been changed in the Materials and Methods (page 12, paragraph 1)
in the revised version to address this issue.

4. The statement "Therefore, we reasoned that endostatin-lidamycin (ES-LDM) fusion proteins upon energizing with enediyne chromophore may have both specificity and potency toward tumor vasculature" at 30-33 line and similar sentence at 460-462 line should be revised since the core of this study is based on the combined targeting tumor vasculature and tumor cell by respective ES and LDM moiety of fusion protein.

Answer: Several sentences have been changed in the Abstract (page 2, paragraph 1) and in the Conclusion (page 23, paragraph 2) in the revised version to address this issue.