Author's response to reviews

Title: Inhibitory effect of ginsenoside Rg3 combined with gemcitabine on angiogenesis and growth of lung cancer in mice

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

In the revised manuscript, we did some changes according to the comments and suggestions of referees and asked a friend majoring in English to improve the language (all modifications made are highlighted in red). In addition, we replied to the questions of the referees.

Sincerely yours,
Cheng Yi

Replies to Reviewer’s question (Cheryl Baker)

1. The authors have chosen Gemcitabine as their chemotherapeutic agent; however the study would have enhanced if they use an agent from the platinum based therapy.
Response: Gemcitabine and cispaltin are accepted as a first-line chemotherapeutic agent for lung cancer, and gemcitabine is usually combined with cispaltin to treat lung cancer. In this study, we want to know whether ginsenoside Rg3 combined with low-dose chemotherapy has effect on angiogenesis and growth of established human lung carcinoma in mice. Because in our previous study, we used gemcitabine and had some experiences about gemcitabine, so in this study we still chose gemcitabine, not cispaltin.

2. The selection of the dosing of chemotherapeutic regimen is not clear (10 mg/kg every third day). It is very important to explain how it will be compared to the normal human dosing and it require further clarifications such as the current dose is how much lower to standard chemo dose (therapeutic dose) as a single first line chemotherapeutic agent in lung cancer. Gemcitabine usually given along with Cisplatin and it requires proper justification for using Gemcitabine alone in their study with the sub-therapeutic dose mentioned in the manuscript.
Response: In this study, gemcitabine was injected ip on every 3rd day (10 mg/kg) in a total of 6 treatments, so the total dose of gemcitabine is 60 mg/kg in mice. In humans, gemcitabine iv. 800-1250mg/m^2 (usually 1000mg/ m^2) at 1st and 8th day,. the therapeutic dose of gemcitabine is about 2000mg/ m^2 (≈ 50mg/kg). Thus, from the theory, the therapeutic dose of gemcitabine in mice = therapeutic dose of gemcitabine in humans ×12.33 (conversion coefficient) =50×12.33 ≈616mg/kg. In fact, mice hardly bear therapeutic dose of gemcitabine because of considerable side effects. In this study, we evaluated the efficacy of ginsenoside Rg3 combined with low-dose gemcitabine (10mg/kg) on angiogenesis and growth of established human lung carcinoma in mice.
The reason that we chose 10mg/kg gemcitabine (1/10 therapeutic dose of gemcitabine) also based on our previous study. We chose 5, 10, 20 and 30mg/kg gemcitabine on every 3rd day in a total of 6 treatments to treat lung cancer in mice, respectively. We want to observe the effect of tumor growth and side effects in tumor-bearing mice. The results showed that treatment with 5mg/kg gemcitabine showed no obvious side effects, however, no appreciable decrease in tumor volume. Treatment with 20 or 30mg/kg gemcitabine showed an appreciable decrease in tumor volume, however, considerable side-effects which the mice could not bear occurred, and these side-effects were the main reason of death. Treatment with 10mg/kg gemcitabine could not only inhibit tumor growth but also have milder side effects. So, in this study, we still chose 10mg/kg gemcitabine.

3. Using low dose Gemcitabine as a single agent is another problem. Gemcitabine has less effective (20%) when compared to combination therapy with Cispaltin (upto 40%) if given at a therapeutic dose in NSCLC. The response of this drug will be even less if given at low dose. The study must have benefitted if the authors also include a therapeutic dose for Gemcitabine as a single agent along with sub-therapeutic (low) dose to compare the effects in a real scenario.

Using low dose for Gemcitabine could be the reason for the loss of more animals (which in fact reduced the ®@ size) and poor outcomes.

Response: It’s true that we should add a group (a therapeutic dose for Gemcitabine as a single agent). In our further study, we will consider your suggestions.

4. The methods described in this manuscript lack the proper reference to published work or protocols and results indicated for the tumor necrosis rate are non-significant.

Response: We have provided a reference for necrosis rate of tumor in methods.

5. The data showing the protein expression results for VEGF will be more supportive in addition to the results presented on the immunohistochemical staining.

Response: We provided high powered pictures in the revised paper.

6. The manuscript will be more interesting if the authors provide insight into the mechanism of synergy between Gemcitabine and Ginsenoside R3.

Response: We added the possible mechanisms of synergy between gemcitabine and ginsenoside R3 in revised paper.

Minor errors and types:

1. The last sentence in the first paragraph of background looks incomplete.

Response: We revised the last sentence in the first paragraph of background.

2. The term {Cum}± in ®Cum survival rate;± should be corrected to {Cumulative} in all over the text or at least when it appeared in the text for the first time.
Response: We have corrected the term Cum to cumulative in all over the text.

3. Correct the symbols (showing as boxes) in the last paragraph in page 8 and also in page 12.
Response: We checked the symbols.

4. Page 14; Discussion; Correct the word 4therapeutic.
Response: We have corrected the word 4therapeutic to therapeutic.

Quality of written English: Needs some language corrections before being published.
Response: We asked a friend majoring in English to improve the language.
Replies to Reviewer’s question (Wanju Kim)

1. Kaplan-Meier survival curve with longer and more samples for support synergic inhibitory effect on the louse lung cancer.
Response: In our further study, we would consider your suggestions.

2. MVD data include more high power data such as > 14~30 vessels/field.
Response: In the revised manuscript, we provided high power immunohistochemical photos of CD31 and VEGF.

3. Authors strongly had show that immuno blot about cell cycle, apoptosis, necrosis, angiogenesis and signaling.
4. Need immunoassaying with TUNEL assay in tumor tissues and caspase assay data.
5. In vivo data such that invasion assay and MTT assay.
3-5 Response: Your suggestions such as cell cycle, signaling and TUNEL assay, etc are advisable for us. In our further study, we will consider your suggestions. Thanks for your advice.

6. Authors must check the mark used figure lend and text.
Response: We have checked the mark used in figure legend and text.

Detail (Minor)

Fig 1B.
1. How many times repeat?
Response: inhibitive rate of tumor (%) = (1− average tumor weight in treated group/average tumor weight in control) × 100%.

Fig. 2.
1. Authors had to shown paralleling gemcitabine and control group, and color data is better.
Response: We added the pictures of gemcitabine and control group in Fig 2. All pictures are colorful.

2. In the bar graph, group name is better instead of numbering.
Response: We accepted your suggestion in Fig 2. We also did the same change in Fig 4 and Fig 5.

Fig 3.
1. In the Bar graph, need type on the graph. Also in figure legend, I can not see I, II, III.
Response: In Fig 3, we typed “E”.
In Fig 3 legend, there are I, II, III. I, minimal blood flow (one or two dot-like or a
thin- and short-like blood flow signals detected within the tumor); II, moderate blood flow (up to three dot-like blood flow signals or one longer blood flow signals detected within the tumor); and III, abundant blood flow (more than five dot-like blood flow signals or two longer blood flow signals detected within the tumor).

Line graph is better for explaining clearly the data instead of bar graph.  
Response: We think bar graph is better than line graph. Some references also used bar graph.

Fig 5.
1. I can not see yellow brown color in data (Not color) 
2. Author must show high powered data as insert form 
Response: We accepted your suggestion and provided high powered pictures in the revised paper.

Fig. 6
Author must show high powered data or need insert
Response: We provided high powered pictures in the revised paper.
Reviewer's report:

The manuscript ‘Inhibitory effect of ginsenoside Rg3 combined with gemcitabine on angiogenesis and growth of lung cancer in mice’ by Liu and others is an interesting in vivo study illustrating combination therapy directed at cytotoxicity and angiogenesis may prove to be beneficial. This group possesses the most experience in the world on Rg3. Though the study is compelling there are several issues that must be addressed to strengthen the manuscript.

Below are some key points…

1) Title: No problems

2) Abstract: No problems

3) Introduction: Both Avastin and were approved the same year. Avastin was approved for the treatment of cancer whereas Macugen was associated with macular degeneration.
   Response: It's true that Avastin (Bevacizumab) was approved by FDA in 2004 for the treatment of colorectal cancer whereas Macugen (Pegaptanib sodium) was associated with AMD. We revised the introduction about Macugen.

4) Last paragraph of introduction is worded oddly. Please consider revising to flow better and to get your point across better.
   Response: We revised the last paragraph of introduction.

5) Materials and Methods: Was animal experiment approved by an animal care committee?
   Response: All animal procedures were approved by the Animal Care and Scientific Committee of Sichuan University.

6) Can delete last sentence of first paragraph of Materials and Methods. It does not add anything.
   Response: We accepted your suggestion and the last sentence of first paragraph of Materials and Methods (The rest chemicals were of the highest grade available) was deleted.

7) What is the meaning of the following sentence from the Materials and Methods section…The tumor tissue from Lewis lung carcinoma mice were triturated and made into cell suspensions (dilution 1:5 with normal saline). Were these cells from xenograft tumors or were they grown in vitro?
   Response: These cells were from xenograft tumors.
8) A daily dose of 20 mg/kg of Rg3 was administered. Is this achievable in humans? How is it metabolized and excreted?
Response: Phase I clinical trials of Rg3 in humans showed that \( t_{1/2} \) of Rg3 was (4.84 ± 1.41)h and Rg3 concentration time profile conformed to a one-compartment pharmacokinetic model.

In humans, the dose of Rg3 from 0.8∼3.2 mg/kg/day is safe and effective (gavage). Thus, a daily dose of Rg3 in mice = dose of Rg3 in humans × 12.33 (conversion coefficient) = (0.8∼3.2)×12.33 ≈10~40 mg/kg (gavage) may be safe and effective.

To cancer patients: daily dose of Rg3 is 0.8 mg/kg~1.6 mg/kg (gavage)
a daily dose of Rg3 in mice = dose of Rg3 in patients × 12.33 ≈10~20 mg/kg (gavage)

In some references, the dose of Rg3 in mice is different, such as 20, 10, 5, 3 mg/kg of ginsenoside Rg3 daily by gavage or daily intraperitoneal injections of ginsenoside Rg3 (3.0 mg/kg). Our previous experiments suggest that 20 mg/kg/day of Rg3 in mice (gavage) is better than 10 or 5 or 3mg/kg/day. Thus, in this study, 20 mg/kg/day of Rg3 in mice was used. Our data indicate that the dose of 20 mg/kg/day of Rg3 is safe and effective.

9) If I understand correctly, cells were injected and 7 days later treatment began for 18 days. This is a very short experiment. How big were the tumors when you began treatment? How big were they at the end of treatment?
Response: Cells were injected and 7 days later treatment began for 18 days. When we began treatment the tumors were about 50mm³, at the end of treatment the tumors were about 1631 mm³ (control group), 712 mm³ (ginsenoside Rg3 group), 962 mm³ (gemcitabine group) and 408 mm³ (combination group), respectively.

10) Check manuscript for grammar and spelling.
Response: We have checked manuscript for grammar and spelling.

11) ‘Quality of life’ was measured. A lot of what we know of quality of life in humans is subjective, thus impossible to assess in a mouse. For example, psychosis was measured. I think in animals this is near impossible to measure. Perhaps instead of saying quality of life can say you are measuring or assessing whether the animal is distress.
Response: Quality of life in mice is subjective and relative. Side effects of mice such as change in behavior and feeding, reaction to stimulation, ruffling of fur and psychosis (distress) were observed not measured.

12) I have never heard of the inhibitive rate of tumor. Please provide a reference for the assay. Same for necrosis rate of tumor. Necrosis is important but must consider
proliferative rate and apoptosis as well.
Response: We have provided reference for the assay of inhibitive rate of tumor and necrosis rate of tumor, respectively.

Tumor volume and necrosis rate of tumor are usually used to evaluate treatment effect of chemotherapy. So, we added tumor volume (tumor growth curve) in the revised manuscript.

Thanks for your suggestions. In the further study, we would consider proliferative rate and apoptosis.

13) 7 MHz ultrasound was used to assay the tumors. This size probe is routinely used to image larger organs, i.e. prostate. Though the pictures included look impressive, would a higher MHz be more effective?
Response: We examined the pictures again and found there was a mistake in writing the frequency of ultrasound. In our study, 5 MHz ultrasound was used to assay the tumors, not 7 MHz. Thank you for your professional question.

14) By scanning the field for CD31 hotspots, bias may be introduced.
Response: MVD was assessed by immunohistochemical analysis with antibodies to the endothelial marker CD31 and determined according to the method of Weidner and colleagues. Briefly, the immunostained sections were initially screened at low magnifications (40 and 100×) to identify hot spots, which are the areas of highest neovascularization. Any yellow brown stained endothelial cell or endothelial cell cluster that was clearly separate from adjacent microvessels, tumor cells, and other connective tissue elements was considered a single, countable microvessel. Within the hot spot area, the stained microvessels were counted in a single high-power (200×) field, and the average vessel count in 3 hot spots was considered the value of MVD. All counts were performed by three investigators in a blinded manner. Microvessel counts were compared between the observers and discrepant results were reassessed. The consensus was used as the final score for analysis.
15) Results: The Table is not helpful. Consider describing only in text and removing table.
Response: We accepted your suggestion and removed the table.

16) To better illustrate survival, survival curves, Kaplan Meier curves should be utilized (Figure 1 A).
Response: In the revised manuscript, we used Kaplan-Meier curves in Fig 1.

17) Again not sure what the inhibitive rate of tumor is showing. Please plot tumor growth curve over time for each group.
Response: We accepted your suggestion and plotted tumor growth curve over time for each group.

18) Considering doing apoptosis and KI-67 or PCNA for IHC to better elucidate the mechanism behind Rg3. In my experience when high rates of necrosis are evident, there may be a higher incidence of toxicity, though none was reported here.
Response: Thank you for your suggestion. In the further study, your suggestion will be taken into our consideration.
It’s true that when high rates of necrosis are evident, there may be a higher incidence of toxicity when we used conventional chemotherapy schemes for the treatment of cancer. In the present study, ginsenoside Rg3 may potently decrease side effects of therapy and improve quality of life of tumor-bearing mice. We believe that its effect may be attributed to its wide spectrum of medicinal effects, which endow ginsenoside Rg3’s special predominance differing from other angiogenic inhibitors.

19) Discussion: Nicely stated discussion. Please incorporate some of the issues raised above. In addition, I am unsure of what a one-class new drug means in China? Has it been used in clinical trials? If so what has been the outcome?
Response: There are some information about Class (Class 1) new drug in China.
NEW DRUG CLASSIFICATION: From a regulatory perspective, new chemical entities (NCEs) fit into three classes of new drug classification. The statute defines “new drug” as any drug that has not yet been manufactured in China. Marketed drugs with a new dosage form, route of administration or indication, and marketed drugs that are new combinations, are also subject to new drug regulatory review. New drugs are divided into five classes. NCEs fit into three classes. Under this definition, an imported NCE that has been sold in the marketplace but not manufactured in China is generally considered a new drug by local manufacturers. In the five classes of new drug classification, class 1, class 2, and class 4 are related to NCE review (Table). Class 1 NCEs are true NCEs that have not been marketed in the world, Class 2 NCEs are new drugs that have been marketed abroad, but are not part of foreign pharmacopeia and have not been imported into China, Class 3 compounds are new combinations of already approved drugs, Class 4 compounds include the following: 1. NCEs that have been listed in the foreign...
pharmacopoeia of developed countries/regions (ie, the United States, the European Union, and Japan), or have
previously been imported into China, 2. New dosage forms of approved compounds, or 3. New routes of administration for approved compounds, and
Class 5 compounds are new indications for approved NCEs.

Table  Rationale for Classification of New Chemical Entities in China

<table>
<thead>
<tr>
<th>Class</th>
<th>Marketing Abroad</th>
<th>Foreign Pharmacopeia</th>
<th>Importation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Class 1</td>
<td>No</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Class 2</td>
<td>Yes</td>
<td>No</td>
<td>No</td>
</tr>
<tr>
<td>Class 4*</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
</tr>
</tbody>
</table>

* If the drug is either in a foreign pharmacopoeia or imported, the drug is class 4.

If you want to know the details, you can search the paper from Google: Rongling Deng and Kenneth I Kaitin. The Regulation and Approval of New Drugs in China.

In 2000, Rg3 appeared in the market as a new anti-cancer drug called “Shen-Yi Capsule” in China. Now, Ginsenoside Rg3 has been applied into clinical therapy as a class 1 new drug in China.

Now, Shen-Yi Capsule has showed therapeutic effects on lung cancer, liver cancer and breast cancer in patients. Actually, many other cancer patients also take Shen-Yi Capsule in China.