Author's response to reviews

Title: Anti-tumor effect of Liqi, a Traditional Chinese Medicine prescription, in tumor bearing mice

Authors:

Ji Deng-Bo (jidengbo.peking@yahoo.com.cn)
Ye Jia (yejia@bjmu.edu.cn)
Qian Bo-Wen (qianbowen@gmail.com)
Jiang Yi-Min (jiangyimin@bjmu.edu.cn)

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Author's response to reviews: see over
Dear Dr. Sabina Alam,

Thank you very much for your email dated Oct 14, 2008 and the referees’ reports for our manuscript entitled “Anti-tumor effect of Liqi, a Traditional Chinese Medicine prescription, in tumor bearing mice” (manuscript ID 1205479634216499). Based on your comments and requests, we have made extensive modification on the original manuscript. We have consulted with a native English speaker with scientific knowledge in this field to check the text. Here, we attached the revised manuscript. A list of answering every question from the referees was also summarized and enclosed. We hope that the revised manuscript is acceptable for publication.

Thank you.

With best wishes,

Jia Ye

Replies to Reviewers

We would like to thank the reviewers for the constructive and positive comments.

Replies to Reviewer #1: jong-hwei pang

Major Compulsory Revisions:

1. Author needs to explain their experimental designs such as:
   - why three different animal models? Why data were collected at different time period (day 12 & 21)? Why cell cycle was studied only for SGC-7901? No dose study for Figure 1?

   **Response:** The aim that we choose three different animal models is to investigate the effect of Liqi on different tumors. Mouse Sarcoma 180 tumor cells and Lewis lung carcinoma cells are mouse source cells and human gastric carcinoma cell line SGC-7901 is human source cell. The results indicate that Liqi has antitumor effect both on mouse tumor and on human tumor.

   Three weeks is a course of clinic treatment in Chinese medicine. Our preliminary test showed that Liqi significantly inhibited the tumor growth in tumor bearing mice when Liqi was administrated for 21 days. However, Liqi has already significantly regulated the immune function on day 12.

   SGC-7901, which is human gastric carcinoma cell line, is more close to tumor in
human body.

The effect of Liqi on mouse Sarcoma 180 tumor and Lewis lung carcinoma in tumor bearing mice showed that the most effective dose was 50g/kg. Our preliminary test showed that Liqi slightly inhibited human gastric carcinoma growth in implanted SGC-7901 nude mice at dose 12.5, 25g/kg and that there was no significant difference between Liqi treated group and model group. We now add the result in figure 1.

2. The inhibition of tumor growth in nude mice with destructed immune functions by Liqi could reach 38.69% that was similar to two other animal models with normal immune functions (34.54% and 42.54%). Does it mean that the anti-tumor effect of Liqi is not involved very much with the immune responses? The result in Fig. 1 might be simply against what the author claimed in this manuscript.

Response: For Sarcoma 180 tumor and Lewis lung carcinoma implanted mice, the inhibition 34.54% and 42.54% represented the inhibition of tumor weight. For SGC-7901 implanted nude mice, the inhibition of tumor weight was 27.42% which is lower than the other animal models. The inhibition 38.69% represented the inhibition of tumor volume.

3. As we know that Immune-related anti-tumor reaction involves the induction of apoptosis, however, in Table 3, data showed no subG1 (apoptotic cells). Why?

By the way, the data in Table 3 was indeed the opposite to what author discussed in the manuscript.

Response: In this experiment, we did not investigate the effect of Liqi on cell apoptosis. In addition, subG1 phrase commonly appears when apoptotic cells exist, but it dose not appear inevitably. In Table 3, we typed the results inversely. We have corrected the error.

4. It is better and more meaningful to measure the IL-2 and TNF-alpha activities directly from tissues, not after the simulation by Con A or LPS.

Response: It’s good idea. However, in tumor bearing mice, the time point of IL-2 and TNF-alpha releasing peak was difficult to be determined. After stimulated by ConA and LPS, it was stable and easy to be determined.
5. It is better that data from normal control should be added to Figure 4, 5 and 6.

**Response:** According to your comments, normal control was added to figure 4 and 6. Figure 6 shows that effect of Liqi on lung metastasis of tumor cells, no tumor colony was found in the lungs of normal control. In our experiment, we measured TNF-alpha activities after the stimulation by LPS. The healthy animal without tumor xenograft which was stimulated by LPS could not be taken as normal control. It is no meaning to compare the model group/ the treated group with the healthy animal without tumor xenograft and LPS stimulation.

6. How to rule out the possibility that LDH may also be released from effector cells, especially the ratio of effector cells: target cells is as high as 100:1.

**Response:** In this experiment, the percentage of NK cell cytotoxicity was calculated using the formula: cytotoxicity (%) = (Experimental release − Effector spontaneous release − Target spontaneous release)/(Target maximum release − Target spontaneous release) × 100. Experimental release stood for LDH release resulting from cocultures at an E/T ratio of 100:1, Effector spontaneous release or Target spontaneous release was spontaneous LDH release from effector or target cells incubated with medium alone, and Target maximum release was obtained from target cells lysed with the lysis solution. We rule out LDH release from effector cells by measuring Effector spontaneous release.

Replies to Reviewer #2: kazumi yagasaki

Major points:
This manuscript describes anti-tumor effect of Liqi and some aspects of its modes of action in vivo using tumor-bearing mice. The aim of study seems somewhat sound. However, effective dose of Liqi is extremely high (25-50 g/kg), indicating Liqi should be ingested as food. Authors do not refer to toxicity of such high dose of Liqi. Furthermore, Liqi contains many kinds of components so that effective chemical entity cannot be identified because of diverse effects by diverse components. Authors should indicate that TNF-alpha possesses cytotoxicity against Sarcoma 180, and add evidence that Liqi can enhance TNF production in tumor infiltrating macrophages from mice treated with Liqi.
Anyhow, the major problem with this manuscript is high effective dose of Liqi.

**Response:** In our study, animal dose was dose of crude drug. In the practice of Chinese medicine, human dose of *Poncirus trifoliate* (L.)Raf, *Akebia Trifoliata* Koidz, *Citrus medica var. sarcodactylis* Swingle and *Saussurea lappa* was 9-12, 12-15, 18 (refer to clinical dose of Pro. Bo-Wen Qian), 9-12g/60kg body weight daily respectively. According to Chinese traditional medicine pharmacology experimental methods, when translated into mice dose, the dose calculation between human and mice is 60 times. In our experiment, mice dose of above 4 herbs should be 9, 12, 18, 12g/kg respectively, and the amount was 50g/kg. According to your comments, we indicate that TNF-alpha possesses cytotoxicity against Sarcoma 180 in section Discussion.

Minor points:
1) There are many inconsistencies in wording, for instance, antitumor and anti-tumor, C57BL, C57BL and C57BL/6, China and P. R. China, and so on.

**Response:** according to your comments, we have modified the inconsistencies.

2) Page 4, lines 5-1 from bottom: ##mice weighing 18-20 g---#. Age of mice should be shown (cf. Ppage 5 line 2). What was the composition of animal diet (food) ? Is there any possibility that the diet contained active principles of Liqi?

**Response:** according to your comments, we have indicated the age of the mice (6 to 8 weeks old). The composition of animal diet was normal diet without Liqi.

3) Page 7, lines 13: specific radioactivity (ex. Bq/mmol) of \[^{3}H\]TdR should be described.

**Response:** specific radioactivity of \[^{3}H\]TdR was $7.4 \times 10^{11}$ Bq/mmol. According to your comments, we indicate it in manuscript.

4) Page 9, lines 3-1 from bottom: this sentence is not consistent with results shown in Table 3. A representative histogram of cell cycle analysis should be shown with Table 3.

**Response:** In Table 3, we typed the results inversely. We have corrected the error.

Others:

There are many typographical errors such as CO2, lack of space such as 0.5mCi and 0.6ng/mL etc.

**Response:** according to your comments, we have corrected these errors.
Replies to Reviewer #3: József Molnár

**Reviewer's report:**

Many herbal extracts used in the Chinese clinical practice are able to inhibit the growth of tumor cells. In the present work, extracts of mixtures of 4 selected herbs were prepared with boiling water and administered to mice bearing xenografts of different tumor cell lines. The antitumor effect was demonstrated by various methods including cytotoxicity, immunological and physical methods e.g. electric impedance. The idea and structure of protocol is properly worked out based on a special kind of formal logic. As an initiative authors collected publications on the effect of “liqi” in vitro to define the main aims of the in vivo study.

If liqi prescription is from TCD, authors should mention the source by reference. By quoting the already known in vitro effects, the system was ready for screening anticancer effects of Liqi, a Traditional Chinese Medicine preparation in in vivo studies. In this aspect Deng-Bo Ji’s paper has a good perspective.

**Response:** Liqi prescription is from the commonly used anti-tumor drugs in the Chinese clinical practice of Pro. Bo-Wen Qian, a famous traditional Chinese physician. No research has been conducted on its anti-tumor effect in vivo and in vitro. Only single component researches are found. We mentioned it in Background.

The Ms entitled “Antitumor effect of Liqi, a Traditional Chinese Medicine prescription, in tumor bearing mice” was revised in details. Some questions could be clarified.

Authors’ idea was good for the study but the presented methodology needs more details. Details of chromatography and references are not included, chromatography data are not presented to give evidence that the hypothesized flavonoids and terpenoids or saponins can be found in the hot water extracts or decoctums, since these compounds have poor water solubility. Consequently, authors measured the effect of water soluble compounds if the decoctum was dissolved in distilled water? The description of the applied main biological methods, from preparation of liqi, tumor cell culturing and cell cycle analysis, platelet aggregation assay needs some further details. Authors have to
revise the concentrations e.g. RNaseA 200 mg/ml?? or propidium iodine final concentration 100 mg/ml???. These concentrations are extremely high! The flow cytometry of T lymphocyte subsets also need more detailed description by giving proper information for readers to repeat the experiments.

**Response:** our method of preparation of Liqi was consistent with the route of administration in the Chinese clinical practice. The decoctum or extract was mixture which contains kinds of components. These components include some materials which posses solubilizing effect to make flavonoids, terpenoids or saponins dissolved in distilled water. According to your comments, we add some details in the description of methods. We typed incorrectly the unit of RNaseA and propidium iodine final concentration which should be 200µg/ml and 100µg/ml respectively, and now we have revised them.

Since the mice were treated with 50g/kg liqi during the 12 days, which is great dose, however, authors could reduce the doses and increase the biological effect if they change the route of the of administration: apply intravenous, or subcutaneously given injection for comparison.

**Response:** In our study, animal dose was dose of crude drug. In the practice of Chinese medicine, human dose of *Citrus medica var. sarcodactylis Swingle, Akebia Trifoliate Koidz, Poncirus trifoliata(L.)Raf and Saussurea lappa.* was 18 (refer to clinical dose of Pro. Bo-Wen Qian), 12, 10, 10g/60kg body weight daily respectively. According to Chinese traditional medicine pharmacology experimental methods, when translated into mice dose, the dose calculation between human and mice is 60 times. In our experiment, mice dose of above 4 herbs should be 18, 12, 10, 10g/kg respectively, and the amount was 50g/kg. In addition, Liqi was administrated by gavage to mice, which was consistent with the route of administration in the Chinese clinical practice.

The evaluation procedure, including dose dependence and untreated controls (called „models”) needs some re-evaluation by using the unpublished results of authors’ experiments if there are. The reason of my suggestion is that the tumor growth reduction was close to 30% or 40% in the liqi-treated animals, but there was no significant
difference between the body weights of liqi-treated and non-treated groups.  

**Response:** yes, in our experiment, there was no significant difference between the body weights of liqi-treated and non-treated groups. The tumor growth reduction was close to 30% or 40% in the liqi-treated animals, but the body weight of animal is about 18～20g, the weight of tumor is only about 1～2g which is too small to affect the body weight. The results indicated that Liqi did not affect the body weights of animals.

Some data, probably measured by the authors are missing for comparison e.g. the preliminary toxicity or the suggested therapeutic window.  

**Response:** in our experiment, Liqi showed no toxicity to normal animal, and have not shown toxicity in the practice of Chinese medicine.

The effect of liqi on T-lymphocyte subpopulations, IL-2 activity, NK cell activity, metastasis formation, platelet aggregation and thromboxane level give supporting evidences for the immune-modulatory effects of liqi on tumor bearing animals.

In case of the discussion of TNF interference with LPS induced TNF alpha, authors should take into consideration the direct electron charge transfer complex formation between LPS and flavonoids or terpenoids.

**Response:** After treated by Liqi for 11 days, tumor bearing BALB/c mice received 20µg/0.2ml of LPS iv without giving Liqi on day 12.

From the convincing discussion, the same logic leads the reader the final evaluation of experimental results. However, the mentioned „systemic immune suppression” is more complex than changes in T subpopulations and NK cell activity.  

**Response:** According to your comments, we delete “systemic immune suppression”.

Authors may find references, on the role of macrophages, cytokines and enzymes as mediators of invasion. The Figures need legends to explain briefly what is the difference between the columns. Instead of using the word „model”, authors should write untreated control of tumor transplanted animals. The word „Normal” used in some figures probably means healthy animal without tumor xenograft for the comparison.  

**Response:** according to your comments, we modified the legends of figures.