Author's response to reviews

Title: Inhibition of metastasis, angiogenesis, and tumor growth by Chinese herbal cocktail Tien-Hsien Liquid

Authors:

Jean-San Chia (chiajs@ntu.edu.tw)
Jia-Ling Du (small-lyn@yahoo.com.tw)
Wei-Bin Hsu (d94445002@ntu.edu.tw)
Andy Sun (andysun7702@yahoo.com.tw)
Chun-Pin Chiang (cpchiang@ntu.edu.tw)
Won-Bo Wang (wbwang@ntu.edu.tw)

Version: 3 Date: 31 March 2010

Author's response to reviews: see over
Dear Professor Dirk Vordermark and Editors:

We appreciate the work put in by the reviewers and you in reviewing our manuscript titled “Inhibition of metastasis, angiogenesis, and tumor growth by Chinese herbal cocktail Tien-Hsien Liquid” (Manuscript 7536428613122381). We especially thank you for giving us the opportunity to revise our manuscript. We have performed some new experiments and revised our manuscript as suggested by the reviewers. Our responses to each of the points raised by the reviewers are listed below.

Response to Editorial requests:
1. All animal experiments in this study were performed following the Guidelines for Animal Experiments in National Taiwan University and were approved by the Institutional Animal Care and Use Committee in College of Medicine, National Taiwan University (IACUC Approval No: 20060184) (please refer to the Methods section, page 10, line 1-4).
2. This study was funded by Ching-Hsing Medical Foundation, a nonprofit organization in Taiwan (see Acknowledgements).

Responses to Reviewer 1 (Dr. Chendil Damodaran)

Reviewer comment 1: Lack of mechanism whether the agent inhibits its anticancer activity of through HIF-1α, if so, there is not enough results to support the authors claim. Studies such as whether inhibition of HIF-1α revert the anticancer activity was not demonstrated.
Response:
The reviewer may have misunderstood our data. The reviewer suggested us to test whether inhibition of HIF-1α would revert the anticancer activity of THL. Since THL was shown by us to be able to inhibit HIF-1α in cancer cells (see Fig. 7A), it is not possible that inhibition of HIF-1α can revert the anticancer activity of THL.

Reviewer comment 2: The authors stated that THL “inhibitory effect of THL on the migration of these cancer cells is not due to the cytotoxic effect, because the viability of these cancer cells was barely affected by THL, in the concentration range tested: (page 12), if so, how this agent induces apoptosis in xenograft models (Figure 8F)?
Response:
THL has been shown by us to be able to inhibit tumor angiogenesis (see Fig. 8E). We believe that inhibition of neovascularization in the tumors can lead to the apoptosis of tumor cells. Because in the absence of new blood vessel formation, the
tumor cells (especially those in the inner regions of the tumor) will be short of supply of nutrients and oxygen.

**Reviewer comment 3**: Lack of mechanism of action of the compound significant dampens the reviewer’s enthusiasm.

**Response:**

The mechanism by which THL inhibits tumor metastasis and angiogenesis has been discussed in the second and third paragraph of the Discussion section. The mechanisms underlying THL inhibition of cancer cell metastasis could be the following: (1) THL could inhibit the transcription and secretion of MMP2 and MMP9 in cancer cells (see Fig. 2A and newly added Supplementary Fig. S2); (2) THL could inhibit MMP2 activity directly (see Fig. 2B); (3) THL could inhibit the secretion of uPA in cancer cells (see Fig. 2C); (4) THL could inhibit, in cancer cells, the activity of ERK1/2 which has been shown to be able to promote tumor invasion and metastasis (see Fig. 2D); and (4) THL could inhibit, in cancer cells, the expression of HIF-1α (see Fig 7A), a transcription factor that promotes metastasis by regulating the expression of metastasis-related genes.

The mechanisms underlying THL inhibition of tumor angiogenesis could be the following. Firstly, THL could directly inhibit the migration, invasion and tube formation of endothelial cells (see Fig 4 and 5). The mechanism underlying this effect of THL could be that THL could inhibit the expression of MMP-2 (see Fig 4C) and uPA (see Fig 4D) in endothelial cells. Secondly, THL could inhibit the secretion of pro-angiogenic factor, VEGF-A, by cancer cells (see Fig. 7B). VEGF-A secreted from the cancer cells can attract and guide sprouting neovessels into oxygen-depleted regions of the tumor mass. The blocking of VEGF-A expression in tumor cells can thus lead to inhibition of neovessel formation in tumors. At least two mechanisms may account for THL inhibition of expression of VEGF-A in cancer cells. (1) THL could inhibit the expression of HIF-1α, which is a transcriptional activator of the vegf-A gene, in cancer cells (see Fig 7A). (2) THL could inhibit, in cancer cells, the activity of ERK1/2 (see Fig 2D), which phosphorylates the transcription factor Sp1 and causes the recruitment of Sp1 to the vegf-A promoter.

**Reviewer comment 4**: Figure-1; Rationale for selection of cell lines are missing. Similar concentration of THL could be used throughout the manuscript. Results should have been discussed in detail, rather describing in one line.

**Response:**

(a) The reason for choosing MDA-MB-231, H1299, PC-3, and CT-26 cell lines
in our study is that these cancer cell lines have been shown to be highly invasive and metastatic in previous reports (see references 57-60). (b) Similar concentrations of THL have been used in all in vitro experiments. (c) Results have been discussed in the second paragraph of the Discussion section.

**Reviewer comment 5:** Figure-2A, 2B, 2c; not visible.

**Response:**

The contrast between the gel background and the bends has been adjusted. The bends now are more visible.

**Reviewer comment 6:** Figure 2D; down regulation of total levels of Akt suggest the compound might be toxic: b-action seems to be not equally loaded so densitometer results are required. So inclusion of normal cell lines may strengthen the manuscript.

**Response:**

(a) The effect of THL on the expression of AKT in normal gingival fibroblast was tested. As shown in the figure attached below, the expression of AKT in gingival fibroblast was not down-regulated by THL, suggesting that THL is not toxic to normal cells. (b) The level of phosphorylated ERK has been quantitated by densitometer and the data (relative level of phosphorylated ERK) have been included in the Fig. 2D.

![THL (%) 0 0.25 0.5 1 Akt GAPDH](image)

**Reviewer comment 7:** Figure-3: The concentration of the agent, duration and concentration of treatment is missing. It is not clear whether any statistical difference were seen, because of morphological and pathological results there is not much difference between groups. Please include higher magnification of Figure 3c.

**Response:**

(a) The dosage of THL and treatment protocol can be found in the Methods section (subtitle: Pulmonary metastasis assay). (b) Statistical difference between water and THL-treated groups was significant ($P < 0.05$, see Fig. 3B). (c) Histological examination of the lung sections clearly shows that the lung section of the
water-treated mouse was filled with metastasized tumor cells while that of the THL-treated mouse contained much less metastasized tumor cells (see Fig. 3C).

**Reviewer comment 8**: Once again figure 4C,D E; not visible. How these results were normalized with control? What controls were used?

**Response**:

(a) The contrast between the gel background and the bends has been adjusted. The bends now are more visible. (b) The conditioned media were concentrated and the concentration of the total protein was determined. Equal amounts of concentrated conditioned medium (which contain equivalent amount of total protein) were loaded on the gel.

**Responses to Reviewer 2 (Dr. Yew Hoong Cheah):**

**Major Compulsory Revisions**

**Reviewer comment 1**: Could the authors explained/justify the different dosage being used for "pulmonary metastasis assay" (200 ul solely THL) and "MDA-MB-231 breast cancer xenograft model" (1:1 v/v dilution)? Is there any optimization of doses being done prior to the experiment.

**Response**:

The optimal dosage of THL for both the pulmonary metastasis assay and the MDA-MB-231 breast cancer xenograft model was determined in our preliminary experiments. The dosage used in this study showed no adverse effect to the mice (see page 11, line 15-17 for newly added sentence). In MDA-MB-231 breast cancer xenograft model, we intraperitoneally injected PBS-diluted THL. The reason for using diluted THL is that THL is too thick to inject. After diluted with PBS (1:1 dilution), the diluted THL can be easily injected into the mice.

**Reviewer comment 2**: In the grouping of animal in breast cancer xenograft model (page10/paragraph3/line6), the reviewer would like to why the mice were divided based on the body weight but not the starting tumor volume?

**Response**:

The wording of the original sentence (One week after tumor cell inoculation, the mice with similar body weight were randomly divided into two groups) may be misleading. The sentence has been changed to: One week after tumor cell inoculation, the mice were randomly divided into two groups. The weight of the mice in these two groups was similar at this time point (please refer to page 11, line 12-14).

We did not divide the mice based on the starting tumor volume, because the
tumors at this time point are too small to measure.

**Reviewer comment 3:** Wound healing assay procedures should be discussed in the Methodology not in the Results section

**Response:**

The wound healing assay has been described in the section of Methods (see page 8, paragraph 1). The description of wound healing assay in the legend of Fig. 1 has been removed (see page 29, line 22).

**Minor Essential Revisions**

**Reviewer comment 1:** please provide reference for the statement "It is safe for cancer patients with no adverse effect" page 5;paragraph 3;line 8

**Response:**

The statement “It is safe for cancer patients with no adverse effect” has been deleted. This statement was based on the responses from many patients. The reference documenting the safety of THL is lacking.

**Reviewer comment 2:** Under the subtitle "Human MDA-MB-231 breast cancer xenograft model", line 6 the word 'boy' should be body

**Response:**

The sentence has been modified.

**Reviewer comment 3:** Under the subtitle "THL inhibits the growth of human MDA-MB-231 breast cancer xenografts in SCID mice' line 3 the word 'latter' should be 'later' - page 20; paragraph 1; line 8. 'Secondly' should be 'Secondly'

**Response:**

(a) The word “latter” has been changed to “later”.

(b) It is “Secondly” now.

**Discretionary Revisions**

**Reviewer comment 1:** Please mention whether the animal studies conducted is being approved by the ethical committee or the animal care committee

**Response:**

Yes, all the animal experiments were approved by the Institutional Animal Care and Use Committee in College of Medicine, National Taiwan University. The following sentence has been added to the Methods section. All animal experiments in this study were performed following the Guidelines for Animal Experiments in National Taiwan University and were approved by the Institutional Animal Care and
Use Committee in College of Medicine, National Taiwan University (IACUC Approval No: 20060184) (please refer to page 10, line 1-4).

**Reviewer comment 2:** Could the authors explained the both flanks injection of MDA-MB-231 cells in the xenograft model as this will likely to induce multi tumor growth in the flanks

**Response:**

We had routinely injected tumor cells into both flanks of the *NOD-SCID* mice. No multi-tumor growth was observed 40-50 days after tumor implantation.

We would like to thank you again for your kind help in editing our manuscript. Please let us know if, in any way, our manuscript can be further improved.

Sincerely,

Won-Bo Wang, Ph. D