Author’s response to reviews

Title: 2-Ethoxystypandrone, A Novel Small-Molecule STAT3 Signaling Inhibitor from Polygonum Cuspidatum, Inhibits Cell Growth and Induces Apoptosis of HCC cells and HCC Cancer Stem Cells

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Author’s response to reviews:

Cover Letter

Dear Editor,

We thank you very much for giving us an opportunity to revise our manuscript. We also appreciate editor and reviewers for their constructive comments and useful suggestions on our manuscript.

We have studied editor and reviewers’ comments carefully, and We have made revisions according to their comments. All the changes have been marked in red in the text and our detailed responses to the specific comments provided by the referees are described as follows. We hope that these revisions improve the manuscript such that you and the reviewers will agree that our work is suitable to be published in BMC Complementary and Alternative Medicine. We look forward to your decision.
Yours sincerely,

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Revisions

Manuscript ID:

Title: 2-Ethoxystypandrone, A Novel Small-Molecule STAT3 Signaling Inhibitor from Polygonum Cuspidatum, Inhibit Cell Growth and Induce Apoptosis of HCC cells and HCC Cancer Stem Cells

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Editor and Reviewers' comments:

Editor’s comments and Responses:
The study is scientifically sound, however, there are several key issues:

1. Please engage professional English proofreading service
2. Please include chemical identification of compound, especially 2- (e.g. HPLC, MS etc) as supplementary data

3. Please indicate clearly the statistic analysis used in each figure.

4. Please state the culture condition for each cell line (medium used etc)

Answer: We thank you for your careful reading of our manuscript and for your constructive comments. We have made corrections according to your instructions.

The spectrums including IR, ESI-MS, HR-ESI-MS,1H, COSY, 13C, DEPT135, HSQC, and HMBC have been uploaded as supplementary data. Spectroscopic data of known compounds 2-8 have been included in the supplementary data.

The statistic analysis has been described in the figure legends and the culture conditions have been described in the Methods section.

Reviewers’ comments and Responses:

Reviewer 1:

1. You mentioned HepG2, Hep2, and HeG2 cells. Are they the same cell line? If so, what is real name? Why are HepG2/STAT3 cells resistant to 2-ethoxystypandrone? Are HepG2/STAT3 cells also resistant to pyridone 6?

Answer: We thank the reviewer for the careful reading of our manuscript. We are sorry for the mistakes and have made corrections for all the names of the HepG2 cells. HepG2 cells are human hepatocellular carcinoma cells.

We used HepG2 and HepG2/STAT3 cells in our experiments. HepG2/STAT3 cells were the parental HepG2 cells stably transfected with a STAT3-responsive firefly luciferase reporter plasmid. We used the HepG2/STAT3 cells to assay the effects of our compounds on the IL-6-induced STAT3 phosphorylation/activation which are reflected by the luciferase activity. The activation of the STAT3 was measured within a short time period (less than 6 hours) before cell
death. The HepG2/STAT3 cells are neither resistant to 2-ethoxystypandrone nor to pyridine 6. Both compounds can induce death of the HepG2 cells.

2. Is STAT3 inhibition really needed for the effects of 2-ethoxystypandrone? Have you ever investigated effects of pyridone 6 in Figs. 3 and 4?

Answer: First, our data directly demonstrated that 2-ethoxystypandrone was able to block STAT3 activation (Figure 2A) and induce cell apoptosis (Figure 3A and 3B). Second, Pyridone 6, a known STAT3 signaling pathway inhibitor, has been shown to inhibit cell proliferation and induce apoptosis of human cancer cells. (Pedranzini L. et al., Pyridone 6, a pan-Janus-activated kinase inhibitor, induced growth inhibition of multiple myeloma cells. Cancer Research, 2006, 66(19): 9714). Therefore, both our data and the literature data support that STAT3 is the necessary target for 2-ethoxystypandrone to induce cell growth inhibition.

3. Please write catalog numbers of each antibody.

Answer: We have added catalog numbers of each antibody in our revised manuscript (Page 12, lines 11-15).

4. Which methods did you use for statistical analysis? Please mention it in each figure legend. What kind of software did you use for statistical analysis?

Answer: SPSS 19.0 software was used for statistical analysis and the statistical methods (One-way ANOVA and T-Test) were mentioned in each figure legend now (page 13, lines 16-18 and page 30, lines 6 and 16).

5. Please mention details of the plasmid construct expressed in HepG2/STAT3 cells.

Answer: The HepG2/STAT3 cells was stably transfected with a STAT3-responsive firefly luciferase reporter plasmid and was a gift kindly provided by Prof. Xinyuan Fu (National University of Singapore, Singapore). Therefore, we don’t have details of the plasmid construct information.

6. In Fig. 2C,D, inhibitory concentrations of 2-ethoxystypandrone are different from IC50 in Table 2. Why?
Answer: The two data were from different assays. In Table 2, IC50 was determined at gene expression level by the STAT3-dependent luciferase reporter assay. However, the data from Figure 2C and 2D showed the inhibition effects of 2-ethoxystypandrone on STAT3 protein phosphorylation level using western blot assay.

7. Your discussion about structure-activity relationship looks imagination because you hide detail information. Is it possible to discuss SAR by referring your previous studies?

Answer: We accepted the referee’s suggestions and added more discussions on the SAR by referring our previous studies. Please see page 19, lines 11-19. We are going to explore SAR and structure optimization studies of 2-ethoxystypandrone in future work.

8. You discuss that 2-ethoxystypandrone (1) prefers to inhibit cell growth and induce apoptosis of HCC CSCs compared to regular HCC Huh-7 cells. Is it caused by difference of culture condition? Have you ever compared the effects of conventional anti-cancer drugs between HCC CSCs and regular HCC Huh-7 cells? Do your HCC CSCs show drug resistance?

Answer: We thank and agree the reviewer for his constructive comments. Although we could not rule out the possibility that different sensibilities of 2-exthoxystypandrone against HCC CSCs and regular HCC Huh-7 cell lines were due to different culture condition, previously reported studies (Ma S, et al 2008; Li J et al, 2015) however have already demonstrated that HCC CSCs isolated from Huh-7 were more resistant to conventional anticancer agents, such as doxorubicin and 5-fluorouracil than regular HCC Huh-7 cells. Therefore, we believe that the two cell lines are different.


Reviewer 2:

General comments:
Pyridone 6 as a control only was used for MTT? How about other assay?

Answer: We thank the reviewer for careful reading of the manuscript and for the useful comments. We have made corrections according to the reviewer’s suggestions.

Pyridone 6 was used as a positive control for STAT3 signaling pathway inhibitor in the present study. Literature data has demonstrated that Pyridone 6 was able to inhibit cell proliferation and induce apoptosis of human cancer cells (Pedranzini L. et al., Pyridone 6, a pan-Janus-activated kinase inhibitor, induced growth inhibition of multiple myeloma cells. Cancer Research, 2006,66(19) : 9714 ).

Abstract:

Generally, the abstract is good but minor corrections are needed for clarity.

Please use italic for "in vitro"

Suggest to replace "anti-tumour" with "anticancer" as no animal experimentation is performed in this study.

Please define" EtOAc".
Please space after a full stop.

Incomplete sentence: "The phosphorylation … western blot".

Please reconstruct, "In-vitro anti-cancer….. respectively".

Answer: We have made corrections according to the reviewer’s suggestions.

Background:

Introduction is good and sufficient.

Please include references for stating juglone analogues exhibited many biological activities.

Please define "EtOAc".

Answer: We added two references to state juglone analogues exhibiting different biological activities (page 5, lines 8 and page 24, lines 15-21).


Methods:

Please state the brand and model of machines/equipment used in this study.

Please define "TLC", "MeOH-H2O", "EtOAc", "PE- EtOAc", "EtOAc-MeOH"

May authors state identification of Dr. Peng? A qualified botanist?

Answer: We have made corrections according to the reviewer’s suggestions. The plant samples of Polygonum Cuspidatum were identified by Dr. Guangtian Peng, a botanist who has been working at Pharmaceutical School of Guangzhou University of Chinese Medicine for ten years.

Confusing sentence: "….using MeOH as eluting solvent to afford…"

Please correct the word "…to abtain resveratrol…"
Answer: We have made corrections according to the reviewer’s suggestions.

Suggest to rewrite the whole section of "Extraction and isolation" as hard to follow and understand.

Answer: We made some corrections according to the reviewer’s suggestions and detailed the extraction and fractionation procedures. The described isolation process is repeatable and easy to follow.

Please identify the cell types of "Huh-7", "Li-7" and "SK-HEP-1".

Answer: We have made corrections according to the reviewer’s suggestions. They are human hepatocellular carcinoma Huh-7, Li-7 and SK-HEP-1 cells.

Luciferase reporter assay

Please describe what are the test samples?

May authors state which promega luciferase kit is used?

Answer: We added compounds 1-8 and the promega luciferase kit (# E4550) (page 10, lines 1 and 4).

May authors explain why cells stimulated with IL-6 for 5.5hr in luciferase reporter assay and 15 min in western blot?

Answer: The expression level of luciferase reaches its peak value after 5-6 h stimulation by interleukin (IL)-6. The phosphorylation of STAT3 protein only needs 15 min after stimulation.

Confusing sentence: "……was controlled by equal seeding."

Answer: we have deleted this confusing sentence.

Cell Viability

Please write full of MTT in the first time use.
Please describe what are the "compounds"?

Please state the reason for incubating the cells for 3 hrs after treatment?

Answer: We have made corrections (page 10, lines 13 and 16).

The MTT solution was added to incubate the cells for 3 h at 37°C in order to form MTT-formazan crystals by metabolically viable cells.

HCC Cancer Stem cells Culture

Please re-check the citation for [17] as they not involving in stem cell culture selection.

Answer: We have changed a reference as followed:


Author should explain why Huh-7 is selected for stem cell culture selection?

Answer: Previously published data (Suetsugu A et al, 2006, Li J et al, 2015) had demonstrated that Huh-7 cells contained CD133(+) cells and liver cancer stem cells could be isolated from Huh-7 cells by Flow cytometry or with stem cell culture selection.


Incomplete sentence, "….the cells with the high-stemness………"

Answer: This sentence has been modified as followed:

“After 10-14 days of culture, the most Huh-7 cells died and the cells grew up as nonadherent, three-dimensional sphere clusters.”
Tumorsphere passage and tumorsphere formation assay

Please use italic for "in vitro"

Please state the version of ImageJ is used.

Answer: We have corrected them.

Western Blot analysis

Please state the range of different concentrations

May authors explain why cells stimulated with IL-6 for 15 min in western blot but 5.5hr in luciferase reporter assay?

Answer: The expression level of luciferase reached its peak value after 5-6 h stimulation by interleukin (IL)-6. The cells were stimulated with IL-6 for 15min since the phosphorylation of STAT3 protein appeared in the 15 min.

Please state the percentage of gel used.

Please state the secondary antibody is anti-mouse as all primary antibodies are from mouse.

Suggest to include catalogue number and brand of antibodies used in this study.

Answer: We have corrected them according to reviewer’s suggestions, please see page 12, lines 11-15.

Flow Cytometric analysis

"…..of cuspidatone (1)…” Please state clearly whether compound 1 is 2-ethoxystypandrone or cuspidatone.

Suggest to re-construct "The dots in …..respectively"

There is no "+" and "-" superscript in Annexin V and PI.

Please define "PI"

For analysis of apoptosis and cell cycle of HCC CSCs, no Annexin V/PI staining is required? 4-6.
Answer: We have corrected them according to reviewer’s suggestions.

Statistical analysis

Author did not mention which types of statistical tests were used for different experiments.

Answer: SPSS 19.0 software was used for statistical analysis, statistical methods (One-way ANOVA and T-Test) were mentioned in each figure legend, please see page 30, lines 6 and 16-17.

Results:

Isolation and structural elucidation

Please write "Figure 1" in text instead of Fig.1.

Please state the identification of compound 1 before the rest.

Please cite the articles/website from where the chemical structure of compounds 1-8 obtained.

Inhibitory activity pathway.

"HepG2/STAT3-luciferase cells…for 6.5h and…" But in Material section, authors stated cells were treated for 1h. Please check.

Statement "HepG2/STAT3-luciferase cells…for 6.5h and MTT assay..." is unacceptable as in both assays, cells were treated with compounds for different times; 1hr (luciferase assay) and 72 hr (MTT assay).

Answer: We have carefully checked and corrected them according to the suggestions by reviewer.

HepG2/STAT3 cells were HepG2 cells stably transfected with a STAT3-responsive firefly luciferase reporter plasmid. The expression level of luciferase reached its peak value after 5-6 h stimulation by interleukin (IL)-6. HepG2/STAT3 luciferase cells were pretreated with 2-ethoxystypandrone at indicated concentrations for 1 h, and the luciferase activity was measured following stimulation of IL-6 (10 ng/mL) for 5.5 h (cells were treated with 2-ethoxystypandrone for total 6.5h). 2-Ethoxystypandrone inhibited the IL-6-induced STAT3 expression, but did not inhibit the viability of cells for the same treat time (showed in Figure 2A and 2B). We used MTT assay to measure the cell growth inhibitory activities of 2-ethoxystypandrone on different human hepatocellular carcinoma cells. The cells were treated with 2-ethoxystypandrone for 72h. The results showed that it inhibited the cells viability.
2-Ethoxystypandrone……..apoptosis in HCC Cancer Stem Cells.

Please use italic for "in vitro"

Please choose and standardize the using either the "HCC cancer stem cells (CSCs)" or "HCC CSCs" word.

Contradicting statement: Author stated no more than 20 passages of sphere will be used under the Material section. But in discussion section, tumorsphere were passaged more than 30 generations. Please check properly.

Answer: We thank the referee for careful reading of the manuscript. We have corrected them. Tumorspheres have been used to passage no more than 20 generations to sustain their stemness property of HCC CSCs isolated from Huh-7 cells in our laboratory.

Authors stated Huh-7 cells were treated with 6 different concentrations, but in Figure 4B, there were 7 different concentrations of 2-Ethoxystypandrone tested.

Tumorsphere or spherogenesis?

From Figure 4(C), IC50 is 2.42um? Please check.

Answer: We have carefully checked and modified them.

Discussion:

What does author mean "the complete SAR data will be published elsewhere"?

Answer: Further studies are underway to clarify SAR of 2-ethoxystypandrone in our laboratory. We have rewritten and reorganized some sentences to talk about the SAR in the Discussion section. Please see page 19, lines 11-19.

Discussion is rather weak although with good data.

Authors are keep repeating about the result without further/depth discussion of obtained result except the second paragraph.

Please include more published articles to support the hypothesis.

Answer: Many thanks for reviewer’s constructive comments. The related paragraphs and sentences have been reorganizes and rewritten to talk about the obtained results in Discussion
Section. Please see page 18 lines 14-18, page 19 lines 11-18, page 21 lines 8-21, pages 22 lines 1-4, and page 22 lines 13-17.

Tables and Figures:

No standard deviation in IC50 value in Table 2
Answer: We added standard deviation in IC50 value in Table 2.

Figure 2:
(B): Misleading chart title and no x-axis title.
(C) and (D): 2-Ethoxystypandrone or cuspidatone? Please check.
Answer: We have corrected them, please see Figure 2.

Figure 3:
(A): Please correct "live" Y-axis and no standard deviation for IC50 values
(B): Please add "Propidium iodide - PE"
Answer: We have corrected them. Please see Figure 3.

Figure 4:
(C): Please explain how authors determined IC50=2.42um from the graph?
Answer: We used GraphPad softwar and Prism can automatically fit a dose response curve to determine IC50 value.

Figure 5:
(A) Please check the labelling because cells were treated with 0, 2, 4 and 8um as mentioned in text.
Answer: We have corrected them.
Reviewer 3

The authors report on a novel STAT3 inhibitor, 2-ethoxystypandrone, extracted from roots of P. cuspidatum. 2-Ethoxystypandrone blocked STAT3 signaling activation and inhibited cell growth of hepatocellular carcinoma cells, tumorspheres formation. These results suggest that 2-ethoxystypandrone could be a lead compound for novel STAT3 inhibitor for HCC cells.

There are only a few minor comments for this manuscript.

1. In this manuscript, the word "2-ethoxystypandronec" and "2-Ethoxystypandron" are used in several times respectively. The manuscript should be checked.

Answer: We thank the referee for careful reading of the manuscript and his useful comments. We have corrected them.

2. Page 9, line 16, Does the word "a-MEM" mean "α-MEM"? The word should be checked.

Answer: We have corrected it, please see page 9, line 14.

3. In the discussion part, the authors described that 2-ethoxystypandrone showed 2-fold increased potency compared with 2-methoxystypandrone. Could the authors provide more commentary for comparison of 2-methoxystypandrone and 2-ethoxystypandrone in inhibition of cell growth and apoptosis induction.

Answer: Many thanks for reviewer’s constructive comments and useful direction. The current manuscript focused on isolation, characterization and preliminary bioactivity studies of 2-ethoxystypandrone as a new STAT3 signaling inhibitor from Polygonum Cuspidatum. At the present study, we examine cell growth inhibitory effect of 2-ethoxystypandrone on HCC cells and HCC CSCs cells.

Our previous studies showed 2-methoxystypandrone as STAT3 inhibitor block cell growth of breast cancer cell lines (MDA-MB-453, MDA-MB-468 and MDA-MB-231) with IC50 values ranging from 2.7 to 7.8 μM (Liu J, Zhang Q, Chen K, Kuang S, Chen W, Yu Q: Small-molecule STAT3 signaling pathway modulators from Polygonum cuspidatum. Planta medica 2012, 78(14):1568-1570.). We didn’t examine cell growth inhibitory effect of 2-methoxystypandrone against HCC cell lines. It would be inexplicable for readers to compare cell growth and apoptosis induction effects of 2-methoxystypandrone and 2-ethoxystypandrone on different cancer cell lines from different tissues.