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

Authors:

Wuguo Li (liwuguo@qq.com)
Qing Zhang (qzhang@simm.ac.cn)
Kaotan Chen (2446630@qq.com)
Zhenhua Sima (781775818@qq.com)
Jingli Liu (jiliu@shapb.com)
Qiang Yu (qyu@sibs.ac.cn)
Jiawei LIU (jiawei.liu@ymail.com)

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Author’s response to reviews:

Dear Editor,

We thank you very much for giving us an opportunity to revise our manuscript. We also appreciate Reviewers for their careful reading of our revised manuscript and for their helpful and constructive comments that help us further improve our article.

According to reviewers’ comments, we have made revisions. All the corrections have been marked in red in the text and our detailed responses to the specific comments are described as following. We hope that these careful 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,
Editor Comments:

Although you have addressed some of the concerns, reviewers still have some questions that need clarification.

Answer: We thank Editor and Reviewers for their careful reading of our revised manuscript and for their helpful and constructive comments that help us further improve the article. We have
studied reviewers’ comments carefully and made corrections according to their comments. Please see all the changes marked in red in the text.

Reviewers’ comments and Responses:

Reviewer 1:

Tomoharu KUBOYAMA (Reviewer 1): I yet cannot believe that 2-ethoxystypandrone shows anti-cancer effects via STAT3 inhibition. You did not show any evidences that STAT3 in HepG2/STAT3 cells were highly phosphorylated. You should cite appropriate references about HepG2/STAT3 cells, what kinds of plasmid expressed. In Fig. 2C, STAT3 was not phosphorylated without IL-6, but in Fig. 2D, STAT3 was constitutively phosphorylated. Please show that STAT3 is higher phosphorylated in HepG2/STAT3 cells than HepG2 cells. Are there correlation between STAT3 phosphorylation levels and effects of 2-ethoxystypandrone in HepG2, HepG3B, SK-HEP-1, Li-7, and Huh-7 cells? If pyridone 6 shows toxic effects in these cancer cells, please cite references in the manuscript. In HepG2/STAT3 cells, 2-ethoxystypandrone inhibited STAT3 phosphorylation within 2h and induced cell death within 6.5 h. However, in HepG2 cells, 2-ethoxystypandrone inhibited STAT3 phosphorylation within 2h, but did not induce cell death within 6.5 h, 24 h was needed to induce cell death. Why these different effects occurred?

Answer: The constitutive basal phosphorylation levels of STAT3 were the same in the HepG2 and the HepG2/STAT3 cells. The Western blots of Figure 2C and 2D were exposed for different lengths of time. The phosphorylation of STAT3 was further induced by IL-6. Both of the basal constitutive and the IL-6-induced phosphorylation of STAT3 were inhibited by 50 μM 2-ethoxystypandrone. The correlation between the level of STAT3 activation and the cell death sensitivity to JAK2 inhibitors was reported previously (Y. Wang, X.Q. Ma, S.S. Yan, S.S. Shen, H.L. Zhu, Y. Gu, H.B. Wang, G.W. Qin, and Q. Yu (2009) 17-Hydroxy-jolkinolide B inhibits STAT3 signaling by covalently crosslinking Janus kinases and induces apoptosis of human cancer cells. Cancer Res. 69 (18):7302-7310.). The HepG2/STAT3 cells and the plasmid expressed were described in the Materials and Methods. (Reporter plasmid construction: Briefly, three tandem repeats of the oligonucleotide GATCGTCGACATTCCCCTTAGAT, which contains the DNA binding sequence of STAT3, was artificially synthesized and introduced into the luciferase reporter vector pGL2-P plasmid (Promega). The pGL2-P/STAT3 construct was confirmed by sequencing. Cell transfection: The luciferase reporter plasmid pGL2-P/STAT3 was used to transfect HepG2 cells, using Lipofectamine 2000 (Invitrogen). The stable transfected
HepG2 cell line with the pGL2-P/STAT3 plasmid was obtained by screening for G418-resistant cell clones.

What kinds of t-test did you use? Two-tailed unpaired t-test? If so, please write so.

Answer: We used Two-tailed unpaired T-Test for statistical analysis by SPSS 19.0 software and added it in the legend for Figure 5. Please see Page 32, lines 18.

Yin Quan Tang, Ph.D. (Reviewer 2): General comments:

Overall is good, but minor correction is needed.

Suggest to use "the root ethyl acetate (EtOAc) extract of P.cuspidatum" instead of "the ethyl acetate (EtOAc) extract of the roots of P.cuspidatum"

Please include standard error mean (SEM) value for each IC50.

Please define "NMR, MS, UV and IR analysis" as can't find inside the text.

Need explanation on how 2-ethoxystypandrone (1) can block cell cycle progression at the S and G2/M phases, and how this blockage can led to apoptosis.

Answer: We thank Reviewer for his careful reading and for his constructive comments. We have studied these comments and made corrections. Please see all the responses as following:

Abstract:

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

Suggest to use "the root ethyl acetate (EtOAc) extract of P.cuspidatum" instead of "the ethyl acetate (EtOAc) extract of the roots of P.cuspidatum"

Please change "...by flow cytometry measurement, respectively." to "flow cytometric analysis."

Isn't it cell proliferation and cell survival are the same? Please recheck the write up of ".... inhibited cell proliferation and cell survival of HCC...."
Please recheck the sentence "….fits the Lipinski's rule of five…." as this no meaning without further explanation.

Answer: We have made corrections according to reviewer’s suggestions, Please see pages 2, line 9, line 19, line 21 in Abstract Section.

We agreed on Reviewer and deleted this sentence “…fits the Lipinski's rule of five” in Abstract Section. Please see pages 3, lines 13-14.

Background:

Introduction is good and sufficient.

Please standardize either using "HCC CSCs' or "CSCs in hepatocellular carcinoma" as both meaning are the same.

Suggest to amend "……target for novel anti-cancer drugs." to "……target for development of novel anti-cancer drugs."

Please include standard error mean (SEM) value for each IC50.

IC50 has an exact value  SEM, and not the range (IC50 = 3.69-20.36uM). Please recheck the IC50 of 2-ethoxystypandreone stated here.

Answer: According to reviewer’s comments, we standardized the term “HCC CSCs” throughout the manuscript, included standard error mean (SEM) for each IC50 value. Please see pages 4, lines 8-22, pages 5, lines 13-17 in Background Section.

Methods:

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

Answer: We added the brands and model of machine/equipment used in the present study. Please see all the changes marked in red in the text in Method Section.

Incomplete sentence: "2-Ethoxystypandreone (1): yellow acicular crystal in acetone. mp: 153-154°C."
Answer: We corrected them. Please see page 8, lines 22.

Author mentioned in abstract that "….including NMR, MS, UV and IR analysis and….", but author didn't explain much about these analysis except putting the some numbering and ions which hard to understand (under section "Identification of 2-ethoxystypandrone (1)").

Answer: We actually described the detailed structure elucidation of 2-ethoxystypandrone (1) on the basis of analysis of NMR, MS, UV, IR spectroscopic data and by their physico-chemical properties in Results Section. Please see page 14, lines 12-24 and page 15, lines 1-12 in the Isolation and Structure Determination Section. Therefore, it is unnecessary to repeat these explanations and descriptions in Method Section.

In addition, we defined respectively the NMR, MS, UV and IR in Abbreviation Section (Please see pages 25, lines 1-2). Therefore, These standard abbreviations of chemical terms such as NMR, MS and IR make Abstract simpler for the readers to understand.

Please define "FCS" and indicate the brand as well.

Author mentioned tumorspheres were photographed and counted. How author define/count one tumorsphere? Based on size? Because tumorsphere size can vary sometimes.

Answer: We thank Reviewer’s constructive suggestions. The brand name of FCS was added (see Page 9, line 19). Tumorspheres larger than 50 µm in diameter have been counted using software ImageJ 1.45., we added this sentence to describe how to count number of tumorsphere in Method Section, Please see page 12, lines 2-4.

Under the "Flow Cytometric Analysis" section,

- Author define upper left quadrant is damages cells. Please define these damaged cells.
- Author showed the results of cell cycle analysis, but never mentioned how this assay was conducted. Please add the methodology for this part.

Answer: We added the dot data to describe the percentage of damaged cells in the upper left quadrant, please see Figure 3B and 5A, and also the cell cycle analysis approach in the Method Section, Please see page 13, lines 14-22.
Results:

Please include standard error mean (SEM) value for each IC50.

Please remove "…(3-(4,5-dimethyl-2-thiazolyl)-2,5-diphenyl-2-H-tetrazolium bromide, Thiazolyl Blue Tetrazolium Bromide)…" from the sentence.

Please include standard deviation value for apoptosis percentage derived from three independent experiments using flow cytometry.

Author proposed the linkage of cell cycle arrest and apoptosis by stating (…. 2-ethoxystypandrone (1) can induce apoptosis of HCC CSCs by blocking cell cycle progression at the S and G2/M phases." But author didn't further explain on this in discussion section. Please explain on this phenomenon.

Answer: Many thanks for reviewer’s constructive comments. According to Reviewer’s suggestions, we corrected them. Please see page 15, lines 21 and 24; page 16, line 3, 4, 8 and 24; page 17, lines 1-13.

We also accepted the referee’s suggestions and added the related paragraphs and sentences in Results Section to describe the results of the cell cycle progression and cell apoptosis. Please see page 18, lines 10-25, and pages 19, lines 1-7.

No statistical analysis on IC50 across different hepatoma cell lines (HepG2, HepG3b, SK-HEP-1, Li-7 and Huh-7).

Are the IC50 of 2-Ethoxystypandrone on HepG2, HepG3b, SK-HEP-1, Li-7 and Huh-7 cell lines are significant different each other? If yes, the IC50 on Li-7 (IC50=5.58uM) and Huh-7 (IC50=3.36uM) cells are different despite both cell lines are constitutively activated STAT3? Any explanation regarding this statement.

No indication (asterisk) for significant statistical differences among the groups in Figure 2 (A and B), Figure 3 (A) and Figure 4 (C).

Answer: We used IC50 values to evaluate the sensibility of 2-ethoxystypandrone (1) against different HCC cell lines such as HepG2, HepG3b, SK-HEP-1, Li-7 and Huh-7. However, these IC50 values have been determined by using GraphPad software automatically fit a dose-response curve. Therefore, It is confusing to perform the statistical analysis on IC50 across these different HCC cell lines.
Moreover, the difference between the IC50 values on Li-7 (5.58uM) and Huh-7 (3.36uM) cells is within a certain range, two values are close to each other. The measurement system errors could result in the difference between the IC50 values on Li-7 and Huh-7 cells.

In addition, it is unnecessary to indicate statistical analysis among the groups in Figure 2A, 2B, F3A, and Figure 4C.

Discussion:

Discussion is acceptable with strong support from the data. But need further clarification on some findings.

What does author mean "….to clarify SAR and ….."?

Authors should explain what is Lipinski's rule of five instead of stating it in text without explanation.

Answer: we deleted these sentences such as “...to clarify SAR and ...”“...fits the Lipinski's rule of five” in Discussion Section and rewrote the related sentences to describe them, Please see pages 20, lines 19-22.

Authors stated "2-Ethoxystypandrone (1) prefers to inhibit cell growth and induce apoptosis of HCC CSCs compared to regular HCC Huh-7 cells". Isn't the stemness of HCC CSCs make these cells less susceptible to any treatment or "stronger" than regular HCC Huh-7 cells?

Answer: The experimental data showed that 2-Ethoxystypandrone (1) was a little more sensitive to HCC CSCs compared to parent HCC Huh-7 cells. The “stemness” of HCC CSCs refers to self-renewal and differentiation capacity, it doesn’t mean that HCC CSCs are less susceptible to any drug treatment or stronger than regular and parent HCC Huh-7 cells.

We also reorganized and rewrote the related sentences to describe them in Results Section. Please see page 22, lines 11-17.

"…….2-ethoxystypandrone (1) might be proposed to block cancer stem cell activity and suppress the tumorsphere….. " What does author mean block CSC activity?
Answer: In order to describe them correctly, this sentence was rewritten. Please see pages 23, lines 16-17.

No explanation on how 2-ethoxystypandrone (1) can block cell cycle progression at the S and G2/M phases.

No explanation on how the blocking cell cycle progression at the S and G2/M phases by 2-ethoxystypandrone (1) can led to apoptosis of HCC CSCs.

Answer: Many thanks for reviewer’s constructive comments. We accepted the referee’s suggestions and added the related paragraphs and some sentences in Discussion Section to talk about the cell cycle progression and cell apoptosis induced by 2-ethoxystypandrone (1). Please see page 22, lines 18-22, and pages 23, lines 1-14.