Peer Review File

Article information: https://dx.doi.org/10.21037/jgo-23-87

Reviewer A

Hepatocellular carcinoma (HCC) has become one of the most frequently diagnosed forms of malignancy. In the manuscript "Schisandrin B inhibits tumor progression of hepatocellular carcinoma by targeting the RhoA/ROCK1 pathway", authors determined the impact and mechanism of schisandrin B (Sch. B) on cell duplication, migration, and invasion in hepatocellular carcinoma (HCC).

Couple questions are required to be answered before it will be accepted.

(1) In the abstract, please state clearly the in vitro experiments.
"HUH-7 cell were firstly treated by Sch. B at final concentrations of 40, 30, 20, 10, 5, 1, and 0 μM and cell duplication were determined by CCK-8."

Response: Thank you for suggestion. We have added "For in vitro experiments" at Page 2, Line 18 to clearly state the in vitro experiment.

(2) In the introduction, it was proposed to add related reference (doi: 10.21037/atm-20-6109) about the function of Schisandrin B in cancer.

Response: We have added this related reference as ref. at Page 3, Line 15 in the introduction.

(3) Why to choose female mice? And how to determine the dose and concentrations of Sch. B? please state in the methods.

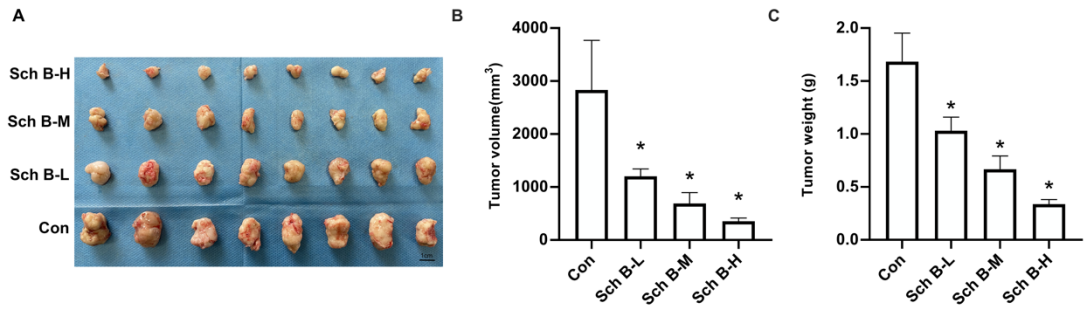
Response: We chose the female mice because they do not usually fight, but males in a group often fight together. Male mice together are usually not peaceful. And the fight may affect the results of drug efficiency. We have added the dose and concentrations of Sch.B in the methods as Page 4, Line 13-17. Mice in the different groups were treated with either saline only, 100, 200, or 400 mg/kg/d of Sch. B solution respectively, by gavage daily for 21 days. Sch. B was purchased from Chengdu Pufei De Biotech Co., Ltd. Sch.B was dissolved in DMSO, then diluted in PBS at final concentration of 5 mg/ml, 10 mg/ml and 20 mg/ml.

(4) In the text, "Cell cultivation" should be changed to "Cell culture".

Response: We have modified it accordingly.

(5) The figure 1A was not representative. It was better to provide total tumor sample each group.

Response: We have provided the total tumor sample each group accordingly.



(6) Whether there were inhibitors of RhoA/ROCK1 pathway? It was better to further test the pathway by inhibitors.

Response: We are appreciative of the reviewer's suggestion. We found Fasudil (HA-1077; AT877) is a non-specific RhoA/ROCK inhibitor accordingly. Indeed, it will be more interesting if we performed the further test the pathway by inhibitors. However, we can repeat the experiment, but it will need long time. We definitely perform the experiment in future. Therefore, we seek for the editor's tolerance and understanding. Many thanks for your kind help!

(7) In the figure 5-7 legends, please state the concentrations of used Sch. B, and RhoA.

Response: We have added the statement of concentrations of Sch. B at figure 5-7 legends. However, RhoA was overexpressed in HUH-7 cell through transfected by overexpression vector, there is no concentration of RhoA.

(8) In the ARRIVE checklist, please supplement the section title.

Response: We have modified the ARRIVE checklist

Reviewer B

The authors showed that Schisandrin B inhibits tumor progression of hepatocellular carcinoma. Using in vitro and in vivo experiments clearly demonstrate the inhibitory effect of Schisandrin B in both proliferation, apoptosis and metastasis in vitro, and in tumor growth in xenograft models. Additionally, authors also investigate the potential role of the Schisandrin B in regulating on RhoA/ROCK signaling pathway. The methods are sound, the experiments well designed and the results in the manuscript look convincing. Below are some minor and major points that need to be addressed prior publication.

Major comments:

Introduction:

#1) The introduction is missing a hypothesis.

Reply: we have added the hypothesis at introduction part.

Changes in the text: we have modified the manuscript at P3, L33 to P4, L1, and highlight in green.

"Based on those researches, we propose a scientific hypothesis that Sch. B performs the therapeutic impact on HCC progression in vivo and in vitro, which is related with the RhoA/ROCK signaling pathway."

#2) Providing some more introductory context for Schisandrin B and RhoA/ROCK signaling pathway (which is not described at all in the Introduction) would help with contextualizing the study goals and experimental goals.

Reply: We have modified it accordingly.

Changes in the text: From P3, L18 to P3, L32.

"Schisandrin B (Sch. B), a potent dibenzocyclooctadiene derivative extracted from the fruit of the traditional Chinese medicinal plant Schisandra chinensis (Turcz.), has been reported to have significant inhibitory effects on a variety of solid malignancies, including colon, cervical, and breast cancers(3-5). Recently, He et al. reported that Sch. B inhibits the duplication of gastric cancer cells and promotes *5-fluorouracil* (5-FU) drug sensitivity(6). Yan et al. found that Sch. B promotes docetacxel-induced inhibition of cervical cancer progression(4). Sch. B has also been shown to reduce neoplastic changes in osteosarcoma(7). However, there are fewer studies reporting on the suppression effect of Sch. B on HCC cells.

The RhoA/rho-associated protein kinase 1 (ROCK1) pathway has been found to participate in regulating duplication and metastasis in HCC cells and also promoting lung cancer formation (8). It has been suggested that targeting the RhoA/ROCK1 signaling pathway may be an effective strategy for inhibiting HCC and breast cancer progression (9, 10). However, there is little reported in the existing literature about the role of Sch. B in regulating the RhoA/ROCK1 pathway."

Methods:

#1) Why did authors use only female, not half female half male?

Reply: Thank you for your suggestion. Sorry for our mistakes, we used the half female half male. We have modified the manuscript.

Changes in the text: changes at P4, L8.

"A total of thirty-two, half female and half male, 4–6-week-old, Balb/c nude mice were obtained from Liaoning Changsheng Biotechnology Co."

Discussion:

#1) Some discussion is warranted for how Schisandrin B regulates HCC progression? Did other mechanism participated?

Reply: There are many reports about Schisandrin B regulates various cancer cell. We have discussed this issue, which is: Liu et al. found Sch. B performed an inhibitory role in gastric cancer(13). He et al. reported that Sch.B increased the efficacy of chemotherapy drug 5-FU in gastric cancer cell(6). In addition, Sch. B has been shown to inhibit epithelial-mesenchymal transition and stemness in lung cancer by regulating the NF-κB and p38 MAPK pathways, thereby exerting an inhibitory effect(14). Sch. B has also been reported to block the Wnt-β-catenin and PI3K-Akt pathways to inhibit progression in osteosarcoma cells together with inducing apoptosis(7). Furthermore, in prostate cancer cells, Sch. B promotes cell apoptosis and oxidative stress as an effective agent for cancer therapy (15). Dai et al. found that Sch. B was effective for inhibiting triple negative breast cancer through regulation of STAT3 (16). Jiang et al. demonstrated that Sch. B could block the progression of glioma cells by regulating the HOTAIR–micoRNA-125a–mTOR axis (17). However, there is limited research on anti-

HCC effect of Sch. B. In this study, we found Sch. B performed a promising anti-HCC role, which depends on RhoA/ROCK1 pathway. Our results demonstrated that Sch.B regulates on RhoA/ROCK1 pathway to inhibit the progression of HCC cells.

Therefore, we discussed the RhoA/ROCK1 pathway in regulating on cancer progression.

The description is "The RhoA/ROCK1 pathway is one of most important pathways in tumor cell motility and morphogenesis, which is associated with cell metastasis (18, 19). RhoA belongs to the Rho family of GTPases, which contributes to F-actin polymerization forming stress fibers to regulate actin movement and cellular contractility. In addition, the activation of ROCK1 by RhoA has been shown to promote the formation of local adhesion of tumor cells, thus allowing their movement, with the result that the RhoA/ROCK1 signaling pathway affects tumor cell migration and invasion (9, 19). During tumor cell migration and invasion, cell adhesion capacity, cytoskeletal alterations, and the polarization of cells are associated with a stimulated RhoA/ROCK1 pathway. Many studies have shown that over activating the RhoA/ROCK1 pathway in HCC could induce migration and invasion ability (9, 20). Similar results were reported in breast and gastric cancer cells, where the stimulation of the RhoA/ROCK1 signaling pathway was found to promote cell migration (21, 22). Therefore, inhibition of RhoA/ROCK1 signaling pathway activity is considered an effective way to inhibit HCC progression.

Our data demonstrated RhoA and ROCK1 expression levels were promoted in tumor tissues of mice, indicating the RhoA/ROCK1 pathway was markedly activated, which is consistent with the results of a previous study (8). After Sch. B treatment, RhoA and ROCK1 levels in tumor tissues were significantly reduced, suggesting Sch. B treatment in liver cancer could be related to inactivation of the RhoA/ROCK1 pathway. You et al. investigated that Schisandrin A inhibit the RhoA/ROCK1 pathway to promote pulmonary capillary endothelial(23). Other study found that Schisandrin A recover erectile dysfunction through regulating RhoA/ROCK1 pathway in type 1 diabetes mice(24). However, the regulatory role of Sch. B on RhoA/ROCK1 pathway was not reported. To further verify the detailed molecular mechanism of Sch. B treatment in HCC, we constructed Huh-7 cells overexpressing RhoA in vitro. Our data demonstrated that RhoA and ROCK1 levels were markedly increased in Huh-7 cells after transfection with RhoA overexpression viral-based vector, indicating that the RhoA/ROCK1 signaling pathway was markedly activated. After Sch. B treatment, RhoA and ROCK1 levels were significantly reduced, which was consistent with the results in vivo. Interestingly, Sch. B could partially reverse the activation of RhoA/ROCK1 signaling pathway by overexpression of RhoA. In addition, the proliferation, migration, and invasion abilities under Sch. B treatment were significantly reduced, and apoptotic cells were also increased. These results suggested that Sch. B could partially reverse the impact of RhoA overexpression in HCC cell proliferation, apoptosis, migration, and invasion, indicating that Sch. B suppressed the proliferation and metastasis of HCC by downregulating the RhoA/ROCK1 pathway. There is a limitation that we did not examine other mechanisms in Sch.B regulating on HCC cell. And we will explore this in a follow-up study.

Changes in the text: P10, 25-29; P11, L8-L10.

Minor comments:

#1) Add antibodies catalogue information.

Reply: We have modified the manuscript accordingly.

Changes in the text: P5, L3-L4; P7, L2-3.

#2) How were the tissues sectioned? Provide equipment

Reply: We have modified the manuscript accordingly.

Changes in the text: P4, L27-28.

Reviewer C

1) First, the abstract needs some revisions. The background did not describe the knowledge gaps on the pharmacological mechanisms of Sch.B in HCC and what the clinical significance of this research focus is. The methods need to describe the method for grouping the 32 mice and the purposes of these experimental procedures. The results need to quantify the findings by reporting statistics and P values such as the expression levels in different groups. The conclusion needs comments for the clinical implications of the findings.
   Reply: Thank you for the critical suggestion. We have modified the introduction part accordingly.
   Changes in the text: P2, L3-P3, L8

2) Second, in the introduction of the main text, it is necessary to provide evidence on the effectiveness of Sch.B for HCC. Without such evidence, it is meaningless to examine the pharmacological mechanisms. It is also necessary to review known pharmacological mechanisms of available medications for HCC to inform the current study.
   Reply: Thank you for the critical suggestion. Before we perform the experiment, we searched NCBI and google scholar, did not find the report about Sch. B inhibiting HCC progression. We only found that Wang et at reported that Schisandrin B suppresses liver fibrosis in rats by targeting miR-101-5p through the TGF-β signaling pathway. Therefore, we performed this study to prove it and explore the molecule mechasnisms. Moreover, we added some pharmacological mechanisms of available medications accordingly.
   Changes in the text: P3, 25-27.

3) Third, in the methodology of the main text, please first have an overview of the experimental procedures and the corresponding questions to be answered by these procedures. In statistics, please clearly describe the outcome variables to be compared across the subgroups and indicate P value for statistical significance.
   Reply: we have modified the methodology of the main text. We added an overview of the experimental procedures and the corresponding questions in the first sentence of each method paragraph. In statistics issue. We added the sentence: $P < 0.05$ was considered statistically significant. And we put the subgroups information in the figure legends.
   Changes in the text: P7, L32.

Reviewer D

1. Please check all abbreviations in the abstract and main text, such as below. All abbreviated terms should be full when they first appear.

19 were firstly treated by Sch. B at final concentrations of 40, 30, 20, 10, 5, 1, and 0 μM
20 and cell duplication were determined by CCK-8. HUH-7 cells were put into a control

8 with 22 ± 1 ℃ temperature and 45% ± 5% relative humidity. The entire animal
9 protocol was approved by the IACUC of China Medical University (CMU2021633).

16 Chengdu Pufei De Biotech Co., Ltd. Sch.B was dissolved in DMSO, then diluted in
17 PBS at final concentration of 5 mg/ml, 10 mg/ml and 20 mg/ml. After the final drug

Response: we have modified the manuscript accordingly. And the edited words were highlighted in yellow.

2. There are two reference lists in your manuscript. Please remove the first one.
Response: We have removed the first reference list.

3. Reference 5 and reference 16 are the same. Please revise
5. Dai X, Yin C, Guo G, et al. Schisandrin B exhibits potent anticancer activity in triple negative breast cancer by inhibiting STAT3. Toxicol Appl Pharmacol. 2018;358:110-119.
16. Dai X, Yin C, Guo G, et al. Schisandrin B exhibits potent anticancer activity in triple negative breast cancer by inhibiting STAT3. Toxicology and applied pharmacology. 2018;358:110-119.
Reply: We have modified it accordingly.

4. Please check if any more references need to be added in the below sentence since you mentioned "Studies", but only one reference was cited.

15 Our data demonstrated RhoA and ROCK1 expression levels were promoted in tumor
16 tissues of mice, indicating the RhoA/ROCK1 pathway was markedly activated, which
17 is consistent with the results of other studies [9]. After Sch. B treatment, RhoA and

Response: We have modified the manuscript accordingly

5. Figure 1:
1) Figure 1 you submitted to us is different from Figure 1 inserted in your manuscript.
Response: we have inserted the edited Figure 1 in the manuscript

2) There is "C" part in your Figure 1 but Figure 1C legend is missing and Figure 1C is not cited in your main text.
Response: we have inserted the edited Figure 1 in the manuscript, which contains Figure 1C

3) There is no # in your Figure 1 but mentioned in the legend.

21 Figure 1 The weight and volume of tumor in each group. (A) Representative pictures
22 of tumors. (B) Tumor weight and volume data. *P<0.05 vs. Con group; #P<0.05 vs. Sch.
23 B-L group.

Response: we have deleted it accordingly.

4) The main texts below don't match with your Figure 1. "P>0.05" is wrong. Figure 1B is

"tumor volume", not "tumor weight".

Response: Sorry for our mistake, we have modified it accordingly at P7, L24-27.

and treated with different doses of Sch. B. As demonstrated in Figure 1A and Figure 1B, tumor weight in the Sch. B-L, Sch.B-M and Sch.B-H group were statistically reduced compared with that of control group (P>0.05). Moreover, as showed in Figure 1B, tumor volume in the Sch. B-L, Sch.B-M and Sch.B-H group were also remarkably decreased compared with that of control group (P>0.05).

6. Figure 2:

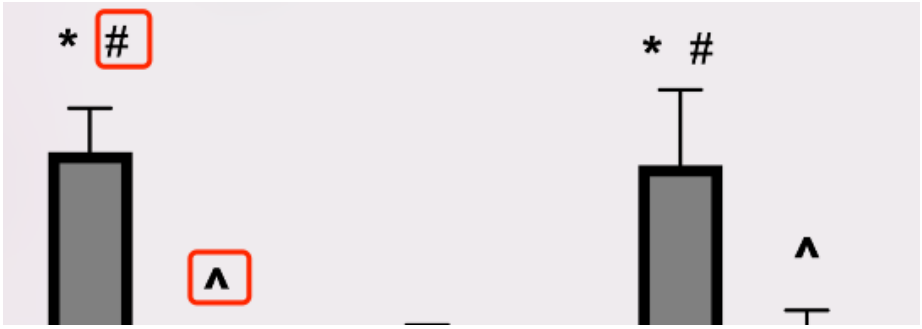
1) Please indicate the meaning of ns, # in the legend.

2) Please indicate the full name of "DAPI", "TUNEL", "IHC" in the legend.

Response: we have modified the legend accordingly.

7. Figure 5:

Please indicate the meaning of #, ^ in the legend.



Response: we have modified the legend accordingly.

8. Figure 7:

Please indicate the full name of "PI-A", "FITC-A" in the legend.

Response: we have modified the manuscript accordingly. And the edited words were highlighted in yellow.

9. Figure 8:

Please describe the observation method and magnification in Figure 8 legend.

Response: we have modified the manuscript accordingly at P17, L1-3