Author's response to reviews

Title: Effects of Astragalus polysaccharides on P-glycoprotein efflux pump function and protein expression in H22 hepatoma cells in vitro

Authors:

Qing E Tian (tqedkj@163.com)
Huan De Li (lihuande1953@126.com)
Miao Yan (yan.miao@126.com)
Hua-Lin Cai (ghostspecialist@163.com)
Qin-You Tan (tgy1013@163.com)
Wen-Yuan Zhang (zwykl@qq.com)

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Authors Response to Reviewer’s (Ashish Mehta’s) Comments

Qing E Tian, Ph.D.
Clinical Pharmacy and Pharmacology Research Institute,
The Second Xiangya Hospital,
Central South University,
139 Renmin Middle Road Changsha,
Hunan 410011, China
E-mail: tqedkj@163.com

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RE: MS-3477053768925311
Dear Ashish Mehta,

We would like to thank you for giving us constructive suggestions which would help us both in English and in depth to improve the quality of the paper, and also thank the editor for giving us a chance to resubmit the paper. Here we submit a new version of our manuscript with the title “Effects of Astragalus polysaccharides on P-glycoprotein efflux pump function and protein expression in H22 hepatoma cells in vitro” (Note: the title has been changed. The original title is Astragalus polysaccharides inhibit P-glycoprotein efflux pump function and decrease its protein expression in H22 hepatoma cells in vitro), which has been modified according to your suggestions. Efforts were also made to correct the mistakes and improve the English of the manuscript. We greatly appreciate both your help and that of the editor concerning improvement to this paper. We hope that the revised manuscript is now suitable for publication.

Sincerely yours,
Qing E Tian, Ph.D.
The following is a point-to-point response to the reviewers’ comments.

Reviewer: Ashish Mehta

General comments: Tian and coworkers suggest that addition of Astragalus polysaccharides (APS) could sensitive the effect of anti-cancer drugs by inhibiting P-glycoprotein efflux pump function and decreasing protein expression in H22 hepatoma cells. However, the study is weak and there are too many anomalies in the presented results that hamper the acceptance of the manuscript. It seems that the authors were very casual in their approach while conceptualizing the study.

Answer: Thank you very much for your detailed, professional and helpful comments, thank you very much for your time and patience. The present study was based on the following background: (1) the dried root of Astragalus membranaceus has a long history of medicinal use in traditional Chinese medicine; (2) Being an adjunct anticancer agent, APS has been most widely studied. Studies have shown that APS has anti-tumor activity for tumor cell lines in vitro, animal tumor models and clinical studies also confirmed APS has anti-tumor activity. However, drug-resistant tumor cells were treated by APS was rarely reported; (3) in clinical applications, patients with advanced cancer are often treated by APS combined with chemotherapeutic drugs; (4) reports indicate that Astragalus membranaceus compound preparations “Jiexinkang” and “Changweiqing” could reverse multidrug resistance and “preventing recurrence formula for UC” could inhibit the expression of P-gp in colon tissue; (5) APS is the main active ingredient of Astragalus membranaceus, and it is worthy of further investigation on whether APS is involved in the reversal of multidrug resistance. Thus, Rifampicin (P-GP inducer) and Verapamil (P-GP antagonist) were used as positive control, H22/ADM cells were used as a blank control group, the present study has been focused on investigating the effect of APS on P-glycoprotein function and expression in H22 hepatoma resistant to Adriamycin (H22/ADM) cell lines.

Seriously and carefully read your comments, and careful examination of our paper, we have a lot of work is indeed imperfect, according to your suggestions, we have corrected some errors. Maybe some shortcomings we have not found, look
forward to receiving your professional further comments, if so, we can do some further work regarding the paper we can do some further work regarding the paper. Thank you for giving me the opportunity to revise our paper.

Comment 1: Result section; In table 2 authors demonstrate effects of sensitizing cells with APS, why did the authors not use doses higher than 500mg/mL to evaluate if inhibition rates could be higher as in the case of DDP.

Answer: Many experiments reported cell proliferation inhibition assay in vitro, the APS concentration range of 0.2–400 mg/L (For example: Zhou RF et al., hormone sensitive breast cancer cell lines, 0.2–200 mg/L; LI Qiang et al., human hepatocellular carcinoma, 10–200 mg/L; Min Yang et al., K562 cells, 50–400 mg/L; CHEN Chao-jun et al., human dendritic cells, 50–200 mg/L; Shao P et al., dendritic cells, 10–250 mg/L), and in clinical applications, Astragalus polysaccharide injectable powder(250mg /vials) 250 mg+500mL normal saline or 5 to 10% glucose injection ( i.e., Clinical maximum dose 500mg / L), intravenous infusion, Once a day(State Food and Drug Administration, P. R. China). According to reports and Package insert, we used the APS doses range of 0.8–500 mg/L.

Comment 2: Does N.S stand for “Normal Saline”. Please add the same in figure legend for table 2.

Answer: According to your recommendations, N.S stand for “Normal Saline” was added the same in figure legend for table 1. (The original table 1 has been deleted, the original Table 2 into Table 1)

Comment 3: Table 2, It is not clear how did the authors calculate the percentage inhibition. Looking at the formula provided in the material and methods the resultant values don’t match. For eg. for APS 0.8mg/ml, (1-0.71(test)/3.918 (control))x100 = (1-0.181)x100= 8.19% . This value is nowhere close to -0.67% as reported by the authors. Author need to clarify?

Answer: We appreciate the detailed, professional and helpful comments from referees.
We carefully checked the experimental data and the original manuscript, corrected the error (DDP 0.1 mg / L, OD Value 2.09 ± 0.62 → 0.29 ± 0.02; NS OD value 4.018 ± 1.06 → 1.018 ± 1.06; DMSO 0.1mg / L OD Value 3.918 ± 0.134→ 0.918 ± 0.134), blue mark, and re-calculated the inhibition rate. (The original table 1 has been deleted, the original Table 2 into Table 1)

Comment 4: Since the IC50 for APS was calculated as 278 mg/mL, did the authors observe reduction in cell viability with the addition of 500mg/mL of APS. What was the loss of percentage of live cells? How did the authors normalize the following difference when calculating the % inhibition as well as evaluating results?

Answer: Under your proposal, we carefully checked the experimental data and the original manuscript, corrected the error (DDP 0.1 mg / L, OD Value 2.09 ± 0.62 → 0.29 ± 0.02; NS OD value 4.018 ± 1.06 → 1.018 ± 1.06; DMSO 0.1mg / L OD Value 3.918 ± 0.134→ 0.918 ± 0.134), blue mark, and re-calculated the inhibition rate. Addition of 500mg/mL of APS, cell inhibition rate 62.40%, which is 37.6% cell survival.

In fact, for the IC50, there is not exhibit a clear linear correlation between drug concentration and inhibition rate, but between the probability of inhibition rate and Logarithmic concentration of drugs.

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population’s response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. The yellow tetrazolium MTT (3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) is reduced by metabolically active cells, in part by the action of dehydrogenase enzymes, to generate reducing equivalents such as NADH and NADPH. The resulting intracellular purple formazan can be solubilized and quantified by spectrophotometric means. The MTT Cell Proliferation Assay measures the cell proliferation rate and conversely, when metabolic events lead to apoptosis or necrosis, the reduction in cell viability.

Absorbance values that are lower than the control cells indicate a reduction in the
rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Rarely, an increase in proliferation may be offset by cell death; evidence of cell death may be inferred from morphological changes.

When the growth inhibition rate is different from the evaluation results, troubleshooting as the following:

1. Problem: MTT Reagent is blue-green.
   
   Cause: Contamination with a reducing agent or cell/bacterial contamination. Excessive exposure to light.
   
   Remedy: Discard.
   
   Remove aliquots of new MTT Reagent using sterile technique. Store solution in the dark at 4°C.

2. Problem: Blanks (medium only) give high absorbance readings.
   
   Cause: The medium is contaminated with cells/bacteria/yeast (visible under microscope).
   
   Remedy: Discard. Check medium before plating. Use sterile technique for cell plating in biological hood.
   
   Use sterile 96-well plate. The medium contains ascorbic acid.
   
   Incubate plate in the dark. Find alternative medium if possible.

3. Problem: Absorbance readings too high.
   
   Cause: Cell number per well too high. Decrease cell density at plating. Contamination of culture with bacteria or yeast.
   
   Remedy: Discard. View wells prior to addition of MTT Reagent to check for contamination.

4. Problem: Absorbance readings are too low.
   
   Cause: Cell number per well is too low.
   
   Incubation time for reduction of MTT intracellularly too short. No purple color visible in cells when viewed under microscope.
   
   Cells not proliferating due to improper culture conditions or inadequate time of recovery after plating.
   
   Remedy: increase cell density at plating.
Increase incubation time with MTT Reagent until purple color is evident inside cells when viewed under microscope. Longer incubation of up to 24 hours may be required for some cell types. Incubation time for solubilization of formazan dye too short (intact cells with intracellular dye visible when viewed under the microscope). Increase incubation time with Detergent Reagent or incubate at 37°C. View under microscope to ensure no crystals remain out of solution. Check that culture conditions (medium, temperature, humidity, CO2, etc.) are appropriate. View cells periodically to check condition. Increase time in culture after plating for cell recovery.

5. **Problem:** Replicates have different values. **Cause:** Inaccurate plating or pipetting. **Remedy:** Increase accuracy of cell plating, check accuracy of pipette.

**Comment 5:** It would be better if the authors convert table 3 in a graphical format for better understanding of the data. Secondly, some of the data does not look statistically significant as indicated by the authors. Authors need to re-evaluate the same. Eg, VCR (control vs 0.8mg APS) does not seem to significant.

**Answer:** We appreciate the detailed, professional and helpful comments from referees. According to your suggestion, table 3 is converted to a graphic format (Figure 1), and found that we do a better understanding of the data. We carefully checked the experimental data and the original manuscript, and re-evaluated the data, corrected the errors you pointed out (VCR (control vs. 0.8mg APS) does not significant.). Thank you for your time and professional guidance, so that we get a lot of help.

**Comment 6:** Looking at the MTT results (OD values) in table 2, it seems that addition of 0.8mg/mL of APS could significantly reduce cell proliferation (0.71) as compared to controls (3.9). How would authors then co-relate this significant loss of cells especially when added other drugs like ADM etc to calculate IC50 values. It
seems that there is already more than 90% cell loss after addition of APS? 

**Answer:** According to your views, we carefully checked the experimental data and the original manuscript, corrected the error (DDP 0.1 mg / L, OD Value 2.09 ± 0.62 → 0.29 ± 0.02; NS OD value 4.018 ± 1.06 →1.018 ± 1.06; DMSO 0.1mg / L OD Value 3.918 ± 0.134→0 .918 ± 0.134), blue mark, and re-calculated the inhibition rate. (The original table 1 has been deleted, the original Table 2 into Table 1). After correct the error, addition of 0.8mg/mL of APS could not significantly reduce cell proliferation (0.71) as compared to controls (0.918). Absorbance values that are lower than the control cells indicate a reduction in the rate of cell proliferation. Conversely a higher absorbance rate indicates an increase in cell proliferation. Addition of 0.8mg/mL of APS, cell inhibition rate 1.11%, which is 98.9% cell survival.

**Comment 7:** What concentration of anti-cancer drugs did the authors use in not mentioned in the table 3? How did the authors select the following doses too is not mentioned?

**Answer:** The concentration of anti-cancer drugs were mentioned in the original table 1, According to your recommendations, table 1 has been omitted and the doses have been included in the material and method section. Now the concentration of anti-cancer drugs were mentioned in the “Materials and methods---- MTT assay the sensitivity of chemotherapeutic drugs to H22/ADM cell” Section. 10μl the following concentrations of chemotherapy drugs were added into each well: ADM(0.625μg /ml, 1.25μg /ml,2.5μg /ml, 5μg/ml, 10μg/ml), 5-Fu(25μg/ml, 50μg/ml, 100μg/ml, 200μg/ml, 400μg /ml),DDP(5μg/ml, 10μg/ml, 20μg/ml, 40μg/ml, 80μg/ml), VP-16(75μg/ml, 150μg /ml, 300μg/ml, 600μg/ml, 1200μg/ml), VCR(1.25μg/ml, 2.5μg/ml, 5μg/ml, 10μg /ml, 20μg/ml), CTX(100μg/ml, 200μg/ml, 400μg/ml, 800μg/ml, 1600μg/ml) , respectively.

**Comment 8:** Figure 1, Authors state that compared to the controls, the RFP
fluorescence shifted to the left. However, this is not clear in the figure. The values presented by the author in table 4 for control vs RFP group at 24h do not match with the figure?

**Answer:** Rh-123 accumulation assay was repeated three times, the results show good overall trends, but occasionally individual dose trend is not clear in the figure. Have been carefully checked, we re-select a relatively good graphics in figure 2 (the original Figure 1 into Figure 2), and expect to meet the requirements that the values presented in table 2(The original table 1 has been deleted, table 3 is converted to Figure 1, the original Table 4 into Table 2) for control vs. RFP group at 24h match with the figure. We appreciate the detailed, professional and helpful comments from referees.

**Comment 9:** For gene expression studies, what was used to normalize the test data is not shown. Are the values presented relative to control or RFP group?

**Answer:** We appreciate the detailed, professional and helpful comments from referees. RFP (P-GP inducer) and VER (P-GP antagonist) were used as positive control; H22/ADM cells were used as a blank control group. These missing sections have been added into “Materials and methods---- Western blot analysis, and Quantitative real time RT-PCR” Sections. Thank you very much for your time and patience

**Comment 10:** Table 1 can be omitted and the doses can be included in the material and method section.

**Answer:** According to your recommendations, Table 1 has been omitted and the doses have been included in the material and method section. Thank you for your suggestions.