Author's response to reviews

Title: Zuo Jin Wan reverses P-gp-mediated multidrug resistance by inhibiting activation of the PI3K/Akt/NF-kappaB pathway

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Author's response to reviews: see over
Dear Editors and Reviewers:
Thanks for your letter and the reviewers’ critical comments. Based on these comments and suggestions, we have made careful and necessary modification on the original manuscript in a point-to point manner. All changes are in red so that they are easily identified.
We shall look forward to hearing from you at your earliest convenience.

Yours sincerely,
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Point-by-point responses to the reviewers’ comments

Reviewer #1: Comments to the Author
Thank you for your critical comments and we totally agree with your suggestions which might be of great help to improve the quality of our manuscript.

1. Figure 1 showed increased cytotoxicity by ZJW of L-OHP in MDR cells. How was in HCT116 cells.
Response: Thank you for helpful comment, and we have made some supplements in the revised manuscript and Fig 1B.

2. In several results, cells were exposed to LY294002 for 2 hrs. Is it true that P-gp has declined within 2 hrs (Fig 3A)? If ZJW down-regulates MDR through inhibiting PI3K/Akt signaling pathway, the exposure time of LY and ZJW should be same, namely 2hr or 48 hr.
Response: We used LY294002 for 2 hrs in the inhibition study based on the data from the literature and our own findings. John et al. have shown that LY294002 significantly blocked PI3K/Akt signaling activation for 2 hrs (Cancer Res 2004;64:8397-8404). Previously, we reported a JNK signaling inhibitor SP600125, has downregulated the level of P-gp for 2 hrs with 20 µM (Carcinogenesis, 2011,32:667–675). In the present study, cells were randomized into 4 groups. ZJW group was exposed to ZJW for 48 hrs; LY294002 group was exposed to LY294002 for 2 hrs when ZJW treatment for 46 hrs, which to ensure cell cycle consistency.
How was the effect of another inhibitor wortmannin?
Response: We used LY294002 to inhibit PI3K/Akt signaling activation based on the data from the literature and our own findings (Clin Cancer Res 2009, 15: 4538-4545). The main difference between wortmannin and LY294002 is the effect of intracellular calcium ion concentration. We chose LY294002 because this study aim to explore the activation of PI3K/Akt signaling and its downstream target, not involved with intracellular calcium ion concentration. However, for serious consideration, we could be willing to added wortmannin as the control for LY294002.

3. In Discussion “In our study, a remarkable activation of phosphorylation AKT and NF-kB was detected in HCT116/L-OHP cells, which also have up-regulation of P-gp.” Where those data are shown? Authors have to show those data in Figure 3 and 4.
Response: Thank you for pointing out the inappropriate places in writing. Actually, we want to show that a remarkable up-regulation of p-AKT, p-NF-kB and P-gp was detected in control group, which as HCT116/L-OHP cells. In the revised manuscript, we have provided supplement data in revised Fig 5C.

4. ChIP assay data shown in Figure 5 indicates association of NF-kB with ABCB1 gene. How was in HCT116 cells, and how was the effect of Zuo Jin Wan.
5. Though authors think that Zuo Jin Wan reverses P-gp-mediated MDR by inhibiting expression of P-gp. Does not Zuo Jin Wan compete for transport of L-OHP?
Response: In the present study, we elucidated the reversed MDR effect of ZJW from the view of the ATP-binding cassette (ABC) family transporters. We found the molecular mechanisms of the ZJW reversed MDR was concerned with PI3K/Akt/NF-κB/P-gp/MDR signaling. Actually, ZJW not only increased L-OHP accumulation and sensitivity, but also including other chemotherapeutic drug, such as 5-Fu, DDP and MMC (ECAM 2013, doi.org/10.1155/2013/957078). However, it is very worthy of discussion and research that whether ZJW has the function of competing for L-OHP. We appreciate the very professional advice from reviewer, and will make some research as suggested.

6. Student t-test should not be used for comparison of multiple groups.
Response: Thank you for pointing out our writing error, which has been corrected in the revised version.
Reviewer #2: Comments to the Author

Thank you for your critical comments and we totally agree with your suggestions which might be of great help to improve the quality of our manuscript. Meanwhile, we have corrected those language errors in red.

1. Title: Zuo Jin Wan reverses P-gp mediated multidrug resistance -> Zuo Jin Wan reverses P-gp-mediated multidrug resistance
Response: Thank you for pointing out the standardization error, which has been corrected in the revised version.

2. Abstract: oxaliplatin (L-OHP) induced cell apoptosis -> oxaliplatin (L-OHP)-induced apoptotic cell death
Response: Thank you for pointing out the standardization error, which has been corrected in the revised version.

3. Authors used the test compound, ZJW, was formulated by Rhizoma Coptidis and Evodia in a ratio of 6:1#. What is the main component of ZJW?
Response: ZJW, a typical traditional Chinese medicine (TCM) formula, mainly composed of two herbs, that Rhizoma Coptidis and Fructus Evodiae. In addition, our previous studies and Wang’s study have used this formula in their reports (ECAM 2013, doi.org/10.1155/2013/957078; J Ethnopharmacol. 2012, 141(1):377-85).

4. Authors measured the apoptotic cell death using Anexin-V-FITC/PI assay. The rate of apoptotic cell death (%) increased in a concentration-dependent manner. However, late stage of apoptotic cell population was slightly increased. Authors should be measured the sub-G0 population of cell after treatment with ZJW.
Response: As you observed, it was indeed a slightly increased in the late stage of apoptotic cell population. We also tested cell cycle analyses by Flow cytometric analysis in revised Fig 1D, and the results showed that there was no significantly change in any phase arrest, especially in S phase, when cells in response to treatment with ZJW only compared with control group. All of these observations suggest that ZJW did not alter cell cycle in its lowest dosage of the IC_{10}. In the cell proliferation, apoptotic and cell cycle experiments, we used the dose of ZJW was bellowed its IC_{10} to eliminate barriers from ZJW itself. We found it was a greatly enhanced in MDR cells sensitivity of chemotherapeutic agents with synergistic effect of ZJW. In Figure 3, however, higher than IC_{10} was used to indicate there was a down-regulation in the expression of ABCB1 mRNA with a dose-dependent manner. It is an experiment to research the molecular mechanism of ZJW in reversing MDR, which based on functional data previously.

5. Authors indicated “ZJW inhibits P-gp expression and the effect of the PI3K/Akt pathway” in Figure 3. As you know, the PI3K/Akt signaling pathway plays an important role in many aspects of cellular homeostasis, it is necessary concern that
ZJW, probably act as a PI3K inhibitor, would interfere with the survival and proliferation of critical populations of normal cells and show unacceptable toxicity. Please confirm this experiment.

Response: We were quite sympathetic to toxicity dose experiment in normal cells exposed to ZJW. In our previous studies (ECAM 2013, doi.org/10.1155/2013/957078), therefore, we have used three MDR cells treatment with ZJW (0-600 µg/mL), and the survival and proliferation was measured by CCK-8. These results suggest that concentrations of ZJW belowing 50 µg/mL are not toxic to the three MDR cell. According to this comment, we have conducted additional experiments to check on the effects of ZJW in tumor sensitive cell, such as HCT-116 (revised Fig 1B), and the effects of ZJW in cell cycle (revised Fig 1D). These results implied Zuojinwan did not affected cell proliferation, cell cycle etc in its lowest dosage of the IC_{10}. 