Author's response to reviews

Title: Incarvine C suppresses proliferation and vasculogenic mimicry of hepatocellular carcinoma cells via targeting ROCK inhibition

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Author's response to reviews: see over
Authors’ Response

Thank you sincerely for giving us the opportunity to improve our manuscript. We have made changes according to your instructions and reviewers’ comments. Besides, we have carefully checked the whole manuscript. All the changes in the revised manuscript are in red color.

Response to Editorial requests:

>Comment 1: We recommend that you copyedit the paper to improve the style of written English.

If this is not possible, you may need to use a professional language editing service.

Response: Thanks for your advice. Edanz has gone through and polished the manuscript to achieve acceptance for publication.

>Comment 2: Please provide a continuous line numbering (no page breaks).

Response: Thanks for your advice. We have made a corresponding revision in the paper.

Response to Reviewer 1

Major concerns:

>Comment 1: The authors provide misinterpretation for the anti-VM effect of IVC.

In the author’s previous report (ref. 17; PloS one 2014, 9(9):e107661), the authors clearly showed that ROCK inhibition by Y27632 (50 microM) could inhibit only cell migration, invasion, and VM formation of MHCC97H cells without apoptosis induction (24 h) or inhibiting cell proliferation (48 h). This indicates that ROCK does not essential for survival and proliferation of MHCC97H cells.

In this manuscript, the suppression of cell proliferation and apoptosis induction by IVC were clearly demonstrated at a concentration range of 15-60 microM. As shown in Figure 3, IVC at
15-30 microM significantly induced apoptosis after 24 h treatment. IVC also inhibited cell proliferation (48 h) as shown by IC50 value of 35 microM in Figure 1B.

In this manuscript, the inhibitory effects of IVC (at 15 and 30 microM) on MHCC97H cell motility, invasion, and VM formation are parallel with cytotoxicity of IVC. These results of IVC are difference from the results of the Y27632 positive control ROCK inhibitor which showed in the author’s previous report (ref.17). Taken together, these results indicated that the inhibitory effects of IVC on cell migration, invasion, and VM formation are associated with cytotoxicity of the compound. Any cytotoxic compound such as doxorubicin can also inhibits migration or VM at its cytotoxic concentrations, due to the cellular damage.

Thus it is not clear that the inhibitory effects of IVC are due to ROCK inhibition or cytotoxicity of the compound. If the authors can provide the results demonstrate that ROCK inhibition leads to suppression of cell proliferation and apoptosis in the MHCC97H cells, the conclusion that IVC inhibits VM through ROCK pathway will be accepted although it contrast to the author’s previous report.

**Response:** Thanks for your valuable advices. We feel so sorry for any inappropriate statements that confused you.

Initially, Reviewer 1 thinks that ROCK does not essential for survival and proliferation of MHCC97H cells based on our previous report [1], which is not the intention of our experiments. Our previous results only proved that Y27632 at the concentrations up to 50 µM was little cytotoxic to MHCC97H cells. It is known that ROCK play a regulative role in cell apoptosis and proliferation in different cell lines [2]. For example, Y27632 and fasudil are the most representative ROCK inhibitors. Abe et al [3] demonstrated that the long-term treatment with
fasudil improved pulmonary vascular remodeling with suppression of vascular smooth muscle cells (VSMC) proliferation and enhancing of VSMC apoptosis, which is similar to the result of IVC (our manuscript) but is different to Y27632 (our previous report), indicating that each compound has various functions with respective or unique mechanism. Furthermore, Y27632 has been reported to have opposite effects on cell motility. Koga et al [4] reported that Y27632 promoted human trabecular meshwork cells in adhesion, contraction and motility, which was inconsistent with our or other reports [1, 5, 6], implying that same compound has a wide variety of effects on different cell line. Moreover, growing number of ROCK inhibitors have been reported, however, their pharmacological effects varied in different diseases [7].

As mentioned by Reviewer 1, we also noticed that these results of IVC are different from the results of the Y27632 as positive control in our previous study, which is still an important issue that needs further researches. In respect of these differences, it is well known that ROCK have two subtypes: ROCK1 and ROCK2. However, Y27632 targets the highly conserved kinase domain of ROCK, which does not distinguish between ROCK1 and ROCK2 isoforms. Inhibition of ROCK1 resulted in a decreased proliferation, whereas inhibition of ROCK2 had the opposite effect, significantly enhancing proliferation relative to the control cells and regulating cyclin D1 [8] [9, 10] to mediate the canonical Wnt/TCF pathways involving β-catenin. Therefore, the relative contribution of these kinases to the effects of Y27632 treatment differ in different cell types and cellular processes. This is an important consideration in developing specific therapeutics tailored to distinct cellular responses and diseases. Taken together, our manuscript showed that Y27632 and IVC have distinct effects on cell proliferation and apoptosis, further investigation will be performed to explain what's different.
Besides, Reviewer 1 thinks that the inhibitory effects of IVC on cell migration, invasion and VM formation are associated with cytotoxicity of the compound. Initially, we performed cell proliferation and apoptosis experiment for more than three times and the results kept stable, so the results were reliable. Furthermore, the results of reduced level of p-MYPT-1 and distribution of stress fiber formation demonstrated that IVC was a potential mediator of ROCK. Moreover, the inhibitory effects of IVC with different concentrations on cell migration and invasion, ROCK activity and VM formation were below IC$_{50}$ value of 35.7 ± 4.7 µM in order to avoid potential toxicity. Thus, we speculate that IVC could effectively exert a regulated impact on cell migration, invasion and VM formation, but are hardly associated with cytotoxicity of the compound.

Comment 2: Although IVC could inhibit ROCK activity but it may affect other cellular targets at the same time. The cell cycle arrest and apoptosis induction of IVC may caused by effect of compound on the other targets. This point is an interesting to explore further.

Response: Thanks for your advices. According to your comments, it is really true that IVC may affect other cellular targets at the same time when it inhibits ROCK activity. Owing to the regulated functions of ROCK on VM and IVC executes a significant inhibitory effect against VM, hence, we paid more attention to the relationship between ROCK and IVC, deducing that ROCK may play an important role in the formation of VM mediated by IVC. Moreover, it is important that the cell cycle arrest and apoptosis induction of IVC may be caused by effect of compound on the other targets as you mentioned. Therefore, we will concern about further specific and comprehensive researches related to other relevant targets and prepare to apply for National Natural Science Foundation of China to explore it further.

Minor concerns:
>Comment 1: Abstract/line 9: 7-AADassay (typographical error)

Response: Thanks for your advices. We have made a corresponding revision in the manuscript.

>Comment 2: Figure Legends: The authors should inform how long the time that cells were incubated with IVC before harvesting for analysis in Figure 4A, Figure 6C, and Figure 6E.

Response: Thanks for your advice. We are so sorry for such confusions. Cells were treated with increasing concentrations of IVC for 24 h. We have made a corresponding revision in the Materials and Methods of our manuscript.

Response to Reviewer 2

Minor Comments:

Abstract:

>Comment 1: Please add the full name before abbreviations:

Row 6: Cell Proliferation Cytotoxicity Assay Kit or Cell Counting kit-8 (CCK-8).

Row 9: Rho-associated, coiled-coil-containing protein kinase (ROCK)

Response: Thanks for your advice. We feel so sorry for our carelessness. We have added the full name as you mentioned and checked other similar abbreviations in our manuscript.

>Comment 2: Row 13: consider rephrasing the first sentence.

Response: Thanks for your advice. We feel so sorry for the inappropriate statement that confused you. We have made a corresponding revision in the manuscript.

Methods:

>Comment 3: Matrigel Invasion assay: row 17: "the number of cell migrating through the Matrigel… was determined" How? Which method was used?
Response: Thanks for your pivotal advice. We feel so sorry for the missing informations that confused you. We have added relevant methods in our manuscript.

Results:

Comment 4: Please indicate if the same effect (except proliferation) was observed in other HCC cell lines. In addition it would be elegant to provide data supporting that IVC is act in vivo as well (HCC mice model).

Response: Thanks for your advice. Our findings of colony formation assay and the expression of p-PMYT-1 by western blot analysis in other HCC cell lines (data not shown) were consistent with the results of cell proliferation. MHCC97H cells have the capacity of VM formation. For simplicity and clarity, we used MHCC97H cells as a model to study the anti-VM potency and mechanism of IVC. When this manuscript was completed and submitted, the animal experiment has just begun. Now, the results of tumor size and invasion in nude mice are consistent with the data in vitro. We will continue to carry out other experiments and hope to release our results in *BMC Cancer* in future.

Discussion:

Comment 5: Please consider adding potential future plan.

Response: Thanks for your advice. According to your valuable comments, we have added the “future plan” in our manuscript.

Reference

2. STREET CA, BRYAN BA: Rho Kinase Proteins-Pleiotropic Modulators of Cell Survival


