Peer Review File

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Reviewer A

1. Comments to Authors

Authors did not describe concerning the exist of the mice without metastasis in preventive model. This is very important. If there were no mice without metastasis, I think portal oridonin administration was no effect on prevention of liver metastasis.

<Answer>

To some degree, you are right. If there were no mice without metastasis, the preventive effect of intraportal oridonin on liver metastasis seem to be doubtful. However, the fundamental point about the effect of intraportal oridonin on prevention of liver metastasis was made in our manuscript for the following reasons: i) As our manuscript points out, in response to the formation process of liver metastasis, killing these cancer cells that have spread to liver and inhibiting the development of neovascularization are the keys to preventing the occurrence of CRCLM. Oridonin was found to display an effective anti-angiogenesis effect by blocking the Notch signaling(1). This study further confirmed that intraportal oridonin can effectively kill both recently metastasized cells and established tumor cells by the induction of

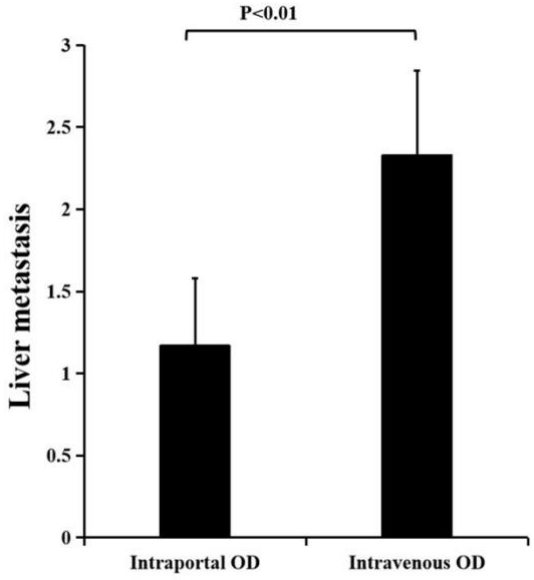
apoptosis. Obviously, these findings provide evidence for the preventive value of intraportal oridonin on CRCLM. ii) Admittedly, all of mice in our preventive group were found small liver metastasis nodule. What's more, although the mice in the treatment group have a larger metastatic liver nodules compared with those in the preventive group, the differences were not statistically significant. All of the results indicate as if only of therapeutic value of intraportal oridonin was found in our study. Nevertheless, tumor prevention and treatment is interlinked. In a way, treatment is equal to prevention. It was found that the low solubility(2) and rapid plasma clearance(3) of oridonin cause it to be poor bioactivity, which partly accounted for the result of our preventive group. Thus, if the anti-tumor effect of oridonin is increased tenfold or more by the various strategies including structural modification and new pharmaceutical formulations, it's preventive value will be more perfectly demonstrated. Interestingly enough, compound **C7**, an oridonin derivative synthesised by Shen et al(4), has been found to significantly suppress the proliferation of HCT116 cells and it was about 43-fold more efficacious compared with oridonin. Based on these reasons above, we think intraportal oridonin can exert a preventive effect on CRCLM, even if it is not perfect enough.

2. Comments to Authors

In order to demonstrate the effect of portal administration, the significant difference should be shown compare with peritoneal administration or venous injection.

<Answer>

I totally agree with you. In the preliminary design, we made a plan to compare the difference between venous injection and intraportal administration, and established a administration group via tail vein. Interestingly, the anti-metastasis effect of intraportal oridonin was significantly stronger than that of venous injection (see supplementary figure). But in order to make the manuscript more concise and readable, the relative data was not presented. Now, i supplement it into our manuscript as a supplementary material. As for peritoneal administration, it was hardly applied in clinical. Thus, we did not preliminarily consider to compare it with intraportal injection.



Supplementary figure. Liver metastasis scores between intraportal and intravenous OD. OD, oridonin.

3. Comment to Authors

In order to demonstrate non-hepatotoxicity by intraportal oridonin, other controls i.e, Sham operation (After hemispleen operation, mice given medium without HT 29 cells) should be provided for oridonin prevention and therapeutic model.

<Answer>

That’s really a good suggestion. In our preliminary design, we also considered it. Because of the aforementioned reason, we did not display these data. Thanks to your advice, we realize it to be very important for the demonstration of our point. Thus, i have replenished it in our revised manuscript.

Reviewer B

1. Comments to Authors

In the method section, oridonin was administrated intraportally. Please explain why oridonin was administered specifically to intraportal.

<Answer>

Colon cancer cells spread to the liver mainly through the mesenteric veins and portal system. The newly growing liver metastases less than the size of 0.5 mm receive their blood supply via the portal branches, and they are partly supplied by hepatic artery until the size of micrometastasis is greater than 0.5 mm(5). Therefore, postoperative

intraportal infusion chemotherapy could be an efficient way to prevent the occurrence and progression of liver metastases, by delivering higher concentrations of anticancer drug to local tumour cells at the initial phase of metastatic invasion. Additionally, in our preliminary experiment, it was found that the anti-metastasis effect of intraportal oridonin was significantly stronger than that of venous injection (see supplementary figure). That's why oridonin was administered specifically to intraportal.

2. Comments to Authors

These experiments need more detailed analysis and the mechanistic signaling known to be exerted by oridonin. Did oridonin have any effects to cytokine? Please show data about cytokines.

<Answer>

Obviously, your suggestion is of great value for us. Our manuscript has pointed out that oridonin was found to have a anti-cancer effect on CRC by targeting BMP7/p38 MAPK/p53 signaling(6) and inhibiting glucose metabolism via downregulating the protein levels of GLUT1 and MCT1(7). Meanwhile, oridonin displayed inhibitory effect on tumor growth and metastasis through anti-angiogenesis by blocking the Notch signaling(1). Therefore, oridonin can effectively kill off colon cancer cells through targeting multiple signaling pathways and inhibit the development of

neovascularization. All of these findings indicated oridonin to be able to target multiple signal molecules. However, our study mainly wanted to preliminarily investigate the preventive and therapeutic effect of intraportal oridonin on CRCLM. As for the data about cytokines, we did not perform relative in vitro and in vivo experiments to reveal them. We know this is very important for a scientific research. Thus, as indicated in the discussion of our manuscript, we will carry a further study to clarify the specific anti-metastasis mechanism and reasonably present the quantitative index. Your suggestion hardened our resolve to reveal the anti-metastatic mechanism of oridonin on CRCLM. I am truly and sincerely obliged to you for your help.

3. Comments to Authors

Please describe some side effects of oridonin in the manuscript.

<Answer>

Thanks for your suggestion. You know, oridonin has been previously reported to confer a number of advantages compared with conventional anti-cancer agents, one of which is reduced toxicity. On the other hand, the clinical application of oridonin is limited because of its low solubility, poor bioavailability(2) and rapid plasma clearance(3). Thus, the clinical side effect of pure oridonin was not nearly reported. However, surfactants and organic solvents were usually added in the injection formulation to overcome these drawbacks. For example, aminopterin was synthesized

to induce remissions in children with acute leukemia using oridonin(8). But the unpredictable side effects of aminopterin such as mucosal toxicity, myelosuppressive effect, tumor lysis syndrome, etc, caused it to be withdrawn from the market in the early 1950s(9). Therefore, the side effect of oridonin was often caused by these surfactants and organic solvents. According to your suggestion, i have revised our manuscript.

Reviewer C

1. Comments to Authors

The metastasis score, the method for evaluating the liver metastasis is vague. You showed us only one picture of the liver in Figure 2. Can you offer more liver 's pics of control group and study group to recognize the inhibition effects?? And please add the box plots for the results of metastasis scores.

<Answer>

Thanks for your suggestion. The significant difference was not seen between the OD-P and OD-T group, whereas detected between the mice treated with intraportal oridonin and the mice receiving the control injection. What’s more, table 2 summarized all of quantitive indicators from gross appearance to TUNEL staining among four groups. So we only presented one representative figure of liver. But your suggestion are undoubtedly right, one figure of liver was not enough. So I have added

our scores figures of liver metastasis into our revised manuscript. In addition, we have supplemented the box plots for the results of metastasis scores according to your suggestion.

2. Comments to Authors

You explained many kinds of statistics methods. Please clarify which method was used for which comparison and explain one by one from a non-parametric statistical point of view.

<Answer>

Thanks for your suggestion. There were three data types in our study including measurement, categorical and ranked data. Now, we clarify these specific statistical approach one by one from measurement data to ranked data. i) The indicators in table 2 (including liver weight, depth of infiltration, cell death ratios, apoptotic index), table 3 (including serum liver enzymes and tumor markers) and figure 2 (including cell viability) were measurement data and analyzed by Student's *t* test, whilst the one-way ANOVA followed by Bonferroni's post hoc test was used to evaluate statistical differences among multiple. ii) The indicators in table 2 (including irregularly distributed cells and cured scars) were categorical data and evaluated for significance using fisher's exact test followed by Bonferroni adjustment. iii) The indicators in

figure 5 (including liver metastasis scores) were ranked data and analyzed using Kruskal-Wallis test followed by Dunn's test.

3. Comments to Authors

Since the liver weight of control group is smaller than treatment group, I doubt that OD have effects on, not only tumor, but also normal liver cell. Please show the results of in vivo experiments; proliferation assay with normal liver cells and toxicity assay with tumor cells. You can compare the amount of OD you need to kill cells.

<Answer>

I am so sorry for that. Our expression was not clear enough so that you had the aforementioned misunderstanding. I am so sorry for that again. Obviously, the liver weight of control group is larger than treatment group (2.05±0.20 v.s. 1.61±0.10, $P<0.05$). Otherwise, oridonin has also been demonstrated to selectively induce tumor cell apoptosis without affecting other vital organs, including the bone marrow, liver and kidney(10). In our study, intraportal oridonin was also demonstrated to possess non-hepatotoxicity by the normal serum levels of ALT, AST and ALP of mice in the blank control group(11). Furthermore, CCK-8 and MTT assay were performed to investigate the inhibitory effect of oridonin on HT29 cells. It was found that oridonin reduced the viability of HT29 cells in a concentration and time dependent manner

($P<0.001$). The IC_{50} values of oridonin were calculated to be 30.309 μmol/l for HT29 cells.

4. Comments to Authors

In my impression, Oridomin is one of old drugs, and some companies and researchers have given up on the clinical application of Oridomin. Please add more comments about recent trends and evidences regarding Oridomin in discussion section.

<Answer>

You really have an encyclopedic knowledge. You are right, aminopterin was firstly synthesized to induce remissions in children with acute leukemia using oridonin(8). But the unpredictable side effects of aminopterin such as mucosal toxicity, myelosuppressive effect, tumor lysis syndrome, etc, caused it to be withdrawn from the market in the early 1950s(9). Thus, many strategies are used to improve the pharmacological effect of oridonin by structural modifications and build-up of drug delivery systems over recent years. I have added many comments about recent trends and evidences regarding oridonin in discussion section.

Your comments give us great help to modify our manuscript. Special thanks from our heart to you for your good comments. We have carefully and thoroughly revised our manuscript according to your comments. After adopting your suggestions, we believe a great improvement can be seen in our revised manuscript. We appreciate for your

warm work earnestly, and hope that the correction will meet with approval. Once again, thank you very much for your comments and suggestions.

Yours sincerely,

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