Oridonin attenuates *Burkholderia cenocepacia* virulence by suppressing quorum sensing signaling

Xia Li, Kai Wang, Gerun Wang, Binbin Cui, Shihaoo Song, Xiuyun Sun, and Yinyue Deng

*Corresponding Author(s): Yinyue Deng, Sun Yat-sen University*

**Review Timeline:**

- **Submission Date:** May 13, 2022
- **Editorial Decision:** June 9, 2022
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*Editor: Giordano Rampioni*

*Reviewer(s): Disclosure of reviewer identity is with reference to reviewer comments included in decision letter(s). The following individuals involved in review of your submission have agreed to reveal their identity: Eunhye Goo (Reviewer #2)*

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**DOI:** [https://doi.org/10.1128/spectrum.01787-22](https://doi.org/10.1128/spectrum.01787-22)
Dear Prof. Yinyue Deng:

Thank you for submitting your manuscript to Microbiology Spectrum. Your manuscript has been evaluated by two Reviewers with expertise in the area addressed in your study and it was the consensus view of these Reviewers that your paper reports interesting data. However, both Reviewers recommend modifications before manuscript acceptance, including key control experiments to evaluate possible oridonin cytotoxicity, and to support and elucidate its specific mechanism of action. I will be glad to consider for publication in Microbiology Spectrum a revised version of your manuscript addressing all the comments raised by the Reviewers.

When submitting the revised version of your paper, please provide (1) point-by-point responses to the issues raised by the reviewers as file type "Response to Reviewers," not in your cover letter, and (2) a PDF file that indicates the changes from the original submission (by highlighting or underlining the changes) as file type "Marked Up Manuscript - For Review Only". Please use this link to submit your revised manuscript - we strongly recommend that you submit your paper within the next 60 days or reach out to me. Detailed instructions on submitting your revised paper are below.

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Sincerely,

Giordano Rampioni
Editor, Microbiology Spectrum

Reviewer comments:

Reviewer #1 (Comments for the Author):

in this work, Li et al identify oridonin among more than 1,000 natural compounds as an antivirulence and anti-quorum sensing molecule in Burkholderia cenocepacia. They claim that oridonin could be used for treating Burkholderia infections.

Major comments
There is an antivirulence screen with more than 1,000 natural products that could be made available.

Fig. 1 and 2 show several compounds that seem to have similar activity to that of oridonin. However, the choice of oridonin for follow-up studies is not justified.

Line 136-139. There is a knowledge gap between a compound's first report of activity and the identification of its putative binding target. It is not clear what knowledge or findings led the authors to hypothesize that RqpR could be a binding target of oridonin.

Fig. 3B. The EMSA results are not very clear. The free probe does not decrease with the addition of oridonin.

The literature reports several effects of Oridonin seems on many cellular processes. It seems that many of these claims could be due to unspecific binding, protein aggregation, protein precipitation. Have the authors considered this possibility?

In order to show activity, most of the assays are performed at high concentrations (100μM). There is plenty of data on the toxic effects of oridonin. Have the authors look at the toxic effects at these concentrations?

Figure 6A and 6B. It is not clear how the authors calculated the percentages. The y-axis of figure 6B is labeled % WT. It is not clear what that means.

The idea of targeting quorum sensing as an antivirulent target is not new. However, it did not so far render any promising results. The authors should exert caution on suggesting this approach as promising. Quorum sensing in Burkholderia is complex and not conserved in the different species. In addition, quorum sensing may not be relevant at chronic stages of infection.

Reviewer #2 (Comments for the Author):

This study showed the inhibitory efficacy of oridonin against two types of quorum sensing in the pathogenic Burkholderia cepacia. The various experimental data support the conclusions, and the manuscript is written in standard English and easy to comprehend. However,

1. Lack of description of the rationale for choosing oridonin as a QS inhibitor among all the other candidates.
2. The concentration of 100 μM, which showed effectiveness as a QS inhibitor, is pretty much high. Is it economical for practical use?
3. There is no discussion about the mode of action of oridonin as a QS inhibitor. Since the chemical structures of oridonin, BDSF and C8-HSL are different, it is less likely that the oridonin is a competitive inhibitor. It can easily get the evidence through a simple experiment to determine whether the oridonin is a non-competitive inhibitor. Please test whether increasing the concentration of C8-HSL does not affect biofilm, motility, or protease activity in the cepI mutant in the absence or presence of oridonin at the IC50 value.

The following are minor points that are needed to improve the manuscript.

1) Lines 97, 215: "Burkholderia" should be italic.
2) Lines 111-113: data not shown?
3) Lines 118-121: The levels of cytotoxicity of bilirubin look similar to its theaflavin 3'3-digallate. Please clarify the percentage of cytotoxicity as the baseline for choosing the compounds.
4) Lines 128-133: Based on your data (Fig. 2A), β-hydroxylsovalerylshikonin is not an effective inhibitor against rpfF gene expression. Same as the theaflavin 3'3-digallate, protopseudohypericin, ginsenoside Rk1, theaflavin-3-digallate, and α-boswellic acid. Please consider re-select the compounds which show the significant differences.
5) Lines 200-201: Please explain the reason in the Discussion.
6) Methods: If you used a commercial product of oridonin, please mention the company of the product.
7) Figure 4B and lines 173-175: The intensities of the band of the bound probe are pretty much the same between 20 μM and 10 μM of oridonin. It seems that the amount of the bclACB promoter probe bound to the CepR did not decrease dependent on increasing oridonin concentration.
8) Method: In general, QS signal receptor proteins are insoluble when overexpressed without the cognate signals. Please describe the details of the purification process of signal receptor proteins, and indicate whether the QS signals were added to the EMSA reaction.

Staff Comments:

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Thank you for submitting your paper to Microbiology Spectrum.
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Point-to-point response to reviewers' suggestions

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Major comments

There is an antivirulence screen with more than 1,000 natural products that could be made available.

Fig. 1 and 2 show several compounds that seem to have similar activity to that of oridonin. However, the choice of oridonin for follow-up studies is not justified.

Response: Good suggestion. Among these thirteen active candidate compounds, theaflavin-3,3-digallate, thonningianin A, oridonin and acetyl-alpha-boswellic acid exhibited good inhibitory activity on both *rpfF*<sub>BC</sub> and *cepI* gene expression. However, we have only identified the direct targets of oridonin. Isothermal Titration Calorimetry (ITC) analysis showed that only oridonin bound to RqpR with an estimated dissociation constant (K<sub>D</sub>) of 8.28 ± 0.895 μM (Fig. 3, Fig. S2). So, we chose oridonin for further investigation in this study.

Line 136-139. There is a knowledge gap between a compound's first report of activity and the identification of its putative binding target. It is not clear what knowledge or findings led the authors to hypothesize that RqpR could be a binding target of oridonin.

Response: Good suggestion. Our previous study showed that the novel two-component system RqpSR directly controls the BDSF and AHL QS systems in *B. cenocepacia* (Cui et al., Molecular Microbiology, 2018). As oridonin significantly inhibited both *cepI* and *rpfF*<sub>BC</sub> gene expression (Fig. 2), we then tested whether oridonin affects the expression of *rpfF*<sub>BC</sub> and *cepI* through RqpR. These details have been described in line 136-140.

Fig. 3B. The EMSA results are not very clear. The free probe does not decrease with the addition of oridonin.

Response: Good suggestion, we have replaced the figure of Fig. 3B as suggested.

The literature reports several effects of Oridonin seems on many cellular process. It seems that many of these claims could be due to unspecific binding, protein aggregation, protein precipitation. Have the authors considered this possibility?

Response: Good suggestion. In this study, we have identified two direct targets of oridonin, RqpR and CepR (Fig. 3 and Fig. 4). However, it is possible that there are other unknown targets of oridonin, or other mechanisms employed by oridonin to
affect the functions of *B. cenocepacia*, which needs further investigation.

In order to show activity, most of the assays are performed at high concentrations (100uM). There is plenty of data on the toxic effects of oridonin. Have the authors look at the toxic effects at these concentrations?

Response: Thanks for your good suggestion. In this study, we found that oridonin could significantly reduce the production of quorum sensing signals, inhibit the biofilm formation, motility and protease activity of *B. cenocepacia* at a final concentration from 20-100 μM (Fig.3, Fig.4 and Fig.5). In addition, we also found that oridonin remarkably attenuated *B. cenocepacia* virulence, while exerted nontoxic effect towards A549 cells at a final concentration from 12.5 to 100 μM (Fig. 6).

Figure 6A and 6B. It is not clear how the authors calculated the percentages. The y-axis of figure 6B is labeled % WT. It is not clear what that means.

Response: Good suggestion, we have revised all the relevant y-axis of Figures in this manuscript as suggested.

The idea of targeting quorum sensing as an antivirulent target is not new. However, it did not so far render any promising results. The authors should exert caution on suggesting this approach as promising. Quorum sensing in Burkholderia is complex and not conserved in the different species. In addition, quorum sensing may not be relevant at chronic stages of infection.

Response: Good suggestion, we have revised the sentence as suggested (Page 1, Line 89). Our study just showed that oridonin inhibited QS of *B. cenocepacia* H111 and reduced virulence and inflammation caused by *B. cenocepacia* H111, but whether the quorum sensing systems play a role in the chronic infection of *B. cenocepacia* still needs further study.

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Response: Good suggestion. We have added the biofilm formation experiments as suggested. Our results showed that BDSF and C8-HSL increased the biofilm formation of _rpfFBC_ and _cepI_ mutants, respectively, in a dose-dependent manner in the absence of oridonin (Fig.S6, S7). Exogenous addition of 50 μM BDSF and C8-HSL fully rescued the impaired biofilm formation of _rpfFBC_ and _cepI_ mutants, respectively, in the absence of oridonin, while exhibited no any restored effects on the biofilm formation of the signal-minus mutants in the presence of 100 μM oridonin (Fig.S6, S7). These results suggest the complicated action mechanisms and multiple targets of oridonin in _B. cenocepacia_, which needs further investigation. We have added these results in the revised version of our manuscript.

The following are minor points that are needed to improve the manuscript.
1) Lines 97, 215: "Burkholderia" should be italic.
Response: We have revised it as suggested.

2) Lines 111-113: data not shown?
Response: We have revised it as suggested.

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Response: Thanks for your good suggestion. We have modified this sentence.

4) Lines 128-133: Based on your data (Fig. 2A), β-hydroxyvalerylshikonin is not an effective inhibitor against rpfF gene expression. Same as the theaflavin 3’3-digallate, protopseudohypericin, ginsenoside Rk1, theaflavin-3-digallate, and α-boswellic acid. Please consider re-select the compounds which show the significant differences.
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6) Methods: If you used a commercial product of oridonin, please mention the company of
the product.
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oridonin in the Methods section as suggested.

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much the same between 20 μM and 10 μM of oridonin. It seems that the amount of the
bclACB promoter probe bound to the CepR did not decrease dependent on increasing
oridonin concentration.
Response: Good suggestion, we have repeated the EMSA experiments and replaced
the picture.

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reaction.
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as suggested.
July 4, 2022

Prof. Yinyue Deng
Sun Yat-sen University
Guangzhou 510642
China

Re: Spectrum01787-22R1 (Oridonin attenuates *Burkholderia cenocepacia* virulence by suppressing quorum sensing signaling)

Dear Prof. Yinyue Deng:

Your manuscript has been accepted, and I am forwarding it to the ASM Journals Department for publication. You will be notified when your proofs are ready to be viewed.

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