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Piceatannol attenuates RANKL-induced osteoclast differentiation and bone resorption by suppressing MAPK, NF-κB and AKT signalling pathways and promotes Caspase3-mediated apoptosis of mature osteoclasts

Liuliu Yan, Lulu Lu, Fangbin Hu, Dattatrya Shetti and Kun Wei

Article citation details
R. Soc. open sci. **6**: 190360.
http://dx.doi.org/10.1098/rsos.190360

Review timeline
Original submission: 26 February 2019
Revised submission: 10 May 2019
Final acceptance: 13 May 2019

Note: Reports are unedited and appear as submitted by the referee. The review history appears in chronological order.

Review History

RSOS-190360.R0 (Original submission)

Review form: Reviewer 1

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Yes

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
This study investigated that PIC inhibited RANKL-induced osteoclastogenesis and bone resorption by suppressing MAPK, NF- κ B and AKT signaling pathways and promoted caspase3-mediated apoptosis of mature osteoclasts. The previous study declared that PIC inhibits the NF- κ B, MAPK and PI3K pathways. PIC also inhibits the formation of osteoclasts (doi: 10.1096/fj; doi: 10.1016/j), so the innovation of this research is relatively insufficient. At the same time, some problems should be addressed in this study.

Major concerns:
1. The osteoclastogenesis assay not only implemented by RAW264.7 cells, but also should use BMMCs.
2. The toxicity of PIC on RAW264.7 cells should be declared. In Figure 5A, the TRAP staining showed that number of osteoclasts were decreased by PIC, it also may be caused by toxic effect of PIC.
3. The Figure 5 showed that PIC promoted apoptosis of osteoclasts and decreases the number of osteoclasts. Please explain that why the number of osteoclasts not change in cell viability assay (Figure 1B & C).
Minor concerns:
1. The negative control group should be added in Figure 1D.
2. The TRAF6 trigger NF- κ B, MAPK pathway, and the C-Src trigger PI3K pathway. (line 52-58)

Review form: Reviewer 2

Is the manuscript scientifically sound in its present form?
Yes

Are the interpretations and conclusions justified by the results?
Yes

Is the language acceptable?
Yes

Is it clear how to access all supporting data?
Not Applicable

Do you have any ethical concerns with this paper?
No

Have you any concerns about statistical analyses in this paper?
No

Recommendation?
Accept with minor revision (please list in comments)

Comments to the Author(s)
The submitted manuscript claims to treat the bone-destructive diseases by naturally occurring organic polyphenolic stilbene compound called Piceatannol. This compound is present in many foods as a strong antioxidant and anti-inflammatory effect. There was no toxicity effect of Piceatannol on RAW264.7 cells insisting its safe to use them as a drug to inhibit the osteoclast formation and bone resorption. Piceatannol successfully suppressed the MAPK, NF-κB and AKT signaling pathway which considered to be a major molecular pathway in osteoclast formation and bone resorption. Further, it induces apoptosis in mature osteoclast by caspases 3 dependent pathway. Overall, the research article nearly covers all the significant area which is required to address to treat the bone-destructive diseases.

1)I highly recommend this research article to be accepted.
2)The research article clearly depicts the importance of the Piceatannol in treating bone-destructive diseases by covering most significant research area
3)The research article is novel because it uses the naturally occurring compound to treat bone disease with no cytotoxicity effect
4)The minor suggestion is to write a conclusion in the end also discussion part can be written more accurately

Decision letter (RSOS-190360.R0)

07-May-2019

Dear Mr Yan

On behalf of the Editors, I am pleased to inform you that your Manuscript RSOS-190360 entitled "Piceatannol Attenuates RANKL-Induced Osteoclast Differentiation and Bone Resorption and Promotes Caspase3-Mediated Apoptosis of Mature Osteoclasts" has been accepted for publication in Royal Society Open Science subject to minor revision in accordance with the referee suggestions. Please find the referees' comments at the end of this email.

The reviewers and handling editors have recommended publication, but also suggest some minor revisions to your manuscript. Therefore, I invite you to respond to the comments and revise your manuscript.

• Ethics statement
If your study uses humans or animals please include details of the ethical approval received, including the name of the committee that granted approval. For human studies please also detail whether informed consent was obtained. For field studies on animals please include details of all permissions, licences and/or approvals granted to carry out the fieldwork.

• Data accessibility
It is a condition of publication that all supporting data are made available either as supplementary information or preferably in a suitable permanent repository. The data accessibility section should state where the article's supporting data can be accessed. This section should also include details, where possible of where to access other relevant research materials such as statistical tools, protocols, software etc can be accessed. If the data has been deposited in

an external repository this section should list the database, accession number and link to the DOI for all data from the article that has been made publicly available. Data sets that have been deposited in an external repository and have a DOI should also be appropriately cited in the manuscript and included in the reference list.

If you wish to submit your supporting data or code to Dryad (http://datadryad.org/), or modify your current submission to dryad, please use the following link:
http://datadryad.org/submit?journalID=RSOS&manu=RSOS-190360

• Competing interests
Please declare any financial or non-financial competing interests, or state that you have no competing interests.

• Authors' contributions
All submissions, other than those with a single author, must include an Authors' Contributions section which individually lists the specific contribution of each author. The list of Authors should meet all of the following criteria; 1) substantial contributions to conception and design, or acquisition of data, or analysis and interpretation of data; 2) drafting the article or revising it critically for important intellectual content; and 3) final approval of the version to be published.

All contributors who do not meet all of these criteria should be included in the acknowledgements.

We suggest the following format:
AB carried out the molecular lab work, participated in data analysis, carried out sequence alignments, participated in the design of the study and drafted the manuscript; CD carried out the statistical analyses; EF collected field data; GH conceived of the study, designed the study, coordinated the study and helped draft the manuscript. All authors gave final approval for publication.

• Acknowledgements
Please acknowledge anyone who contributed to the study but did not meet the authorship criteria.

• Funding statement
Please list the source of funding for each author.

Please ensure you have prepared your revision in accordance with the guidance at https://royalsociety.org/journals/authors/author-guidelines/ -- please note that we cannot publish your manuscript without the end statements. We have included a screenshot example of the end statements for reference. If you feel that a given heading is not relevant to your paper, please nevertheless include the heading and explicitly state that it is not relevant to your work.

Because the schedule for publication is very tight, it is a condition of publication that you submit the revised version of your manuscript before 16-May-2019. Please note that the revision deadline will expire at 00.00am on this date. If you do not think you will be able to meet this date please let me know immediately.

To revise your manuscript, log into https://mc.manuscriptcentral.com/rsos and enter your Author Centre, where you will find your manuscript title listed under "Manuscripts with Decisions". Under "Actions," click on "Create a Revision." You will be unable to make your revisions on the originally submitted version of the manuscript. Instead, revise your manuscript and upload a new version through your Author Centre.

When submitting your revised manuscript, you will be able to respond to the comments made by the referees and upload a file "Response to Referees" in "Section 6 - File Upload". You can use this to document any changes you make to the original manuscript. In order to expedite the processing of the revised manuscript, please be as specific as possible in your response to the referees. We strongly recommend uploading two versions of your revised manuscript:

1) Identifying all the changes that have been made (for instance, in coloured highlight, in bold text, or tracked changes);
2) A 'clean' version of the new manuscript that incorporates the changes made, but does not highlight them.

When uploading your revised files please make sure that you have:

1) A text file of the manuscript (tex, txt, rtf, docx or doc), references, tables (including captions) and figure captions. Do not upload a PDF as your "Main Document";
2) A separate electronic file of each figure (EPS or print-quality PDF preferred (either format should be produced directly from original creation package), or original software format);
3) Included a 100 word media summary of your paper when requested at submission. Please ensure you have entered correct contact details (email, institution and telephone) in your user account;
4) Included the raw data to support the claims made in your paper. You can either include your data as electronic supplementary material or upload to a repository and include the relevant doi within your manuscript. Make sure it is clear in your data accessibility statement how the data can be accessed;
5) All supplementary materials accompanying an accepted article will be treated as in their final form. Note that the Royal Society will neither edit nor typeset supplementary material and it will be hosted as provided. Please ensure that the supplementary material includes the paper details where possible (authors, article title, journal name).

Supplementary files will be published alongside the paper on the journal website and posted on the online figshare repository (https://rs.figshare.com/). The heading and legend provided for each supplementary file during the submission process will be used to create the figshare page, so please ensure these are accurate and informative so that your files can be found in searches. Files on figshare will be made available approximately one week before the accompanying article so that the supplementary material can be attributed a unique DOI.

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Once again, thank you for submitting your manuscript to Royal Society Open Science and I look forward to receiving your revision. If you have any questions at all, please do not hesitate to get in touch.

Kind regards,
Andrew Dunn
Royal Society Open Science Editorial Office
Royal Society Open Science
openscience@royalsociety.org

on behalf of Dr John Dalton (Associate Editor) and Catrin Pritchard (Subject Editor)
openscience@royalsociety.org

Associate Editor Comments to Author (Dr John Dalton):
Associate Editor: 1
Comments to the Author:
The paper was well received and though of as novel and well performed. However, one reviewer had a number of relative major and several minor comments that should be addressed before the paper can be accepted.

Reviewer comments to Author:
Reviewer: 1

Comments to the Author(s)
This study investigated that PIC inhibited RANKL-induced osteoclastogenesis and bone resorption by suppressing MAPK, NF- κ B and AKT signaling pathways and promoted caspase3-mediated apoptosis of mature osteoclasts. The previous study declared that PIC inhibits the NF- κ B, MAPK and PI3K pathways. PIC also inhibits the formation of osteoclasts (doi: 10.1096/fj; doi: 10.1016/j), so the innovation of this research is relatively insufficient. At the same time, some problems should be addressed in this study.

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Minor concerns:
1. The negative control group should be added in Figure 1D.
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Reviewer: 2

Comments to the Author(s)
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foods as a strong antioxidant and anti-inflammatory effect. There was no toxicity effect of Piceatannol on RAW264.7 cells insisting its safe to use them as a drug to inhibit the osteoclast formation and bone resorption. Piceatannol successfully suppressed the MAPK, NF- κ B and AKT signaling pathway which considered to be a major molecular pathway in osteoclast formation and bone resorption. Further, it induces apoptosis in mature osteoclast by caspases 3 dependent pathway. Overall, the research article nearly covers all the significant area which is required to address to treat the bone-destructive diseases.

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4)The minor suggestion is to write a conclusion in the end also discussion part can be written more accurately

Author's Response to Decision Letter for (RSOS-190360.R0)

See Appendix A.

Decision letter (RSOS-190360.R1)

13-May-2019

Dear Mr Yan,

I am pleased to inform you that your manuscript entitled "Piceatannol Attenuates RANKL-Induced Osteoclast Differentiation and Bone Resorption and Promotes Caspase3-Mediated Apoptosis of Mature Osteoclasts" is now accepted for publication in Royal Society Open Science.

You can expect to receive a proof of your article in the near future. Please contact the editorial office (openscience_proofs@royalsociety.org and openscience@royalsociety.org) to let us know if you are likely to be away from e-mail contact. Due to rapid publication and an extremely tight schedule, if comments are not received, your paper may experience a delay in publication.

Royal Society Open Science operates under a continuous publication model (http://bit.ly/cpFAQ). Your article will be published straight into the next open issue and this will be the final version of the paper. As such, it can be cited immediately by other researchers. As the issue version of your paper will be the only version to be published I would advise you to check your proofs thoroughly as changes cannot be made once the paper is published.

On behalf of the Editors of Royal Society Open Science, we look forward to your continued contributions to the Journal.

Kind regards,
Royal Society Open Science Editorial Office

Royal Society Open Science
openscience@royalsociety.org

on behalf of Dr John Dalton (Associate Editor) and Catrin Pritchard (Subject Editor)
openscience@royalsociety.org

Appendix A

Dear Editors and Reviewers:

Thank you for your letter and for the reviewers' comments concerning our manuscript entitled "Piceatannol Attenuates RANKL-Induced Osteoclast Differentiation and Bone Resorption by Suppressing MAPK , NF-κB and AKT Signaling pathways and Promotes Caspase3-Mediated Apoptosis of Mature Osteoclasts". Those comments are all valuable and very helpful for revising and improving our paper. The main corrections in the paper and the responds to the reviewer's comments are as flowing:

Reviewer comments to Author:
Reviewer: 1
Reviewer comment 1: The osteoclastogenesis assay not only implemented by RAW264.7 cells, but also should use BMMCs.

Author reply: Thank you for your great suggestion. We are deeply aware of the necessity of both RAW264.7 cells and BMMCs for osteoclastogenesis research. Due to lack of time, I cannot do the additional experiment on BMMCs but I will consider your precious advice in future for my experiments.

Reviewer comment 2: The toxicity of PIC on RAW264.7 cells should be declared. In Figure 5A, the TRAP staining showed that number of osteoclasts were decreased by PIC, it also may be caused by toxic effect of PIC.

Author reply: Thank you for your advice. It was our negligence that we didn't explore the minimum concentration of PIC which caused toxicity. As we mentioned in section 2.1 and 2.2, PIC was dissolved in DMSO and stored at -20 °in a concentration of 50mM. Thus, the maximum concentration used in our subsequent experiments couldn't exceed 50μM to insure the highest concentration of DMSO was below 0.1% during the experiments. However, we have done Pre-Experiments several times to insure that the maximum concentration we could use (50μM) showed no cytotoxic effects in RAW264.7 cells (supplemental Fig1). Considering the above reasons, we finally use 40μM as the maximum concentration.

As we mentioned in section 3.5—"We found that PIC treatment attenuated the survival of mature osteoclasts in a dose-dependent manner(Figure 5A, B). **To investigate whether the decrease in mature osteoclast survival was accompanied by apoptosis, LDH release for cell necrosis and Hoechst 33258 staining for nuclear fragmentation were performed** as described in the methods. As shown in Figure 5C, mature osteoclasts didn't release significant LDH after 24h exposure to PIC. On the other hand, an increasing nuclear fragmentation was observed in the PIC treated cells compared to the control, indicating that PIC treatment enhanced apoptosis of mature osteoclasts.(Figure 5D). Consistent with its pro-apoptotic effect, **addition of PIC increased caspase-3 activity and induced the cleavage of the caspase-3 precursor** (Figure 5E, F).", the number of mature osteoclasts were decreased by PIC was due to

apoptosis shown by LDH release assay，Hoechst 33258 staining ,Caspase-3 activity assay and Caspase-3 protein expression.

Reviewer comment 3: The Figure 5 showed that PIC promoted apoptosis of osteoclasts and decreases the number of osteoclasts. Please explain that why the number of osteoclasts not change in cell viability assay (Figure 1B & C)

Author reply: Thank you for your good comments. Figure 1B&C only represent the cell viability of RAW264.7 cells rather than the mature osteoclasts. As we mentioned in section 2.8—"RAW264.7 cells were cultured in DMEM complete medium supplemented with RANKL (20ng/ml) and M-CSF (10ng/ml)] for 4 days to differentiate into mature osteoclasts", our results showed that PIC had no effect on the cell viability of RAW264.7 cells, but when RAW264.7 cells differentiated into mature osteoclasts, it induced apoptosis of mature osteoclasts.

Reviewer comment 4: The negative control group should be added in Figure 1D

Author reply: Thank you for your mention. We added the negative control group in supplementary data (supplemental Fig2).

Reviewer comment 5: The TRAF6 trigger NF-κB, MAPK pathway, and the C-Src trigger PI3K pathway. (line 52-58)

Author reply: Thank you for your great suggestion. We are very sorry for our incorrect writing "which then triggers the activation of several downstream signaling pathways including NF-κB, MAPKs(ERK, JNK and p38) and PI3K/AKT". It is noteworthy that several reviews report that Src/PI3K/AKT pathway is one of the downstream signaling pathways of RANKL/RANK/TRAF6 (DOI: 10.1038/nature01658; DOI: 10.1016/S0006-291X(03)00695-8; DOI: 10.1016/j.intimp.2016.04.024). In view of this, we modified the sentence into "which then activates several downstream signaling pathways including NF-κB, MAPKs(ERK, JNK and p38) and Src/PI3K/AKT".

Special thanks to you for your good comments.

Reviewer comments to Author:
Reviewer: 2
Reviewer comment 1: The minor suggestion is to write a conclusion in the end also discussion part can be written more accurately

Author reply: Thank you for your great suggestion. We will write a conclusion in the end.