Author's response to reviews

Title: Screening of protein kinase inhibitors identifies PKC inhibitors as inhibitors of osteoclastic acid secretion and bone resorption

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Author's response to reviews: see over
Dear Editor of BMC musculoskeletal diseases

Thank you for the positive review of our manuscript entitled “Screening of protein kinase inhibitors identifies Rottlerin as a potent inhibitor of osteoclastic acid secretion and bone resorption”.

We wish to thank the reviewers for their constructive criticism. We would also like to highlight that 3 out of 4 reviewers found the article important and interest and as one of the reviewers stated “An article of importance in its field”.

In addition, we have stated in the Cell culture methods from where the blood was received and that the ethical approval for receiving and using the blood is held by the blood bank by Dr. M.H. Dziegiel.

We have also changed the title of the manuscript to:”Screening of protein kinase inhibitors identifies PKC inhibitors as inhibitors of osteoclastic acid secretion and bone resorption” after a suggestion for one of the reviewers.

All the issues raised by the reviewers are answered in the paper and has been marked in red, and in the point-by-point comments in the rebuttal letter below.

Reviewer: Fraser Coxon

Level of interest: An article whose findings are important to those with closely related research interests

Major Compulsory Revisions:

1) Comment: In the acid influx assay, are the compounds acting through PKC inhibition or another effect, such as directly on the machinery responsible for acidification? Since these are completely cell-free experimental systems (and not incubated with cytosolic extracts), the latter seems quite likely, therefore the involvement of PKC needs to be demonstrated, or the conclusions from the data modified.

Answer: We thank the reviewer for this important question. We have done Western blotting both on osteoclast lysate and on the membranes from the osteoclasts using a PKC antibody, and an antibody against the V-ATPase B2 as a control. The Western blot shows that PKC is indeed in the membranes of the human osteoclasts and also in the lysate. In addition, V-ATPase B2 is also present in both preparations as expected showing that the Western blot was done properly. The Western blot has been added as Figure 5 (figure legend: page 28-29), in Methods (page 9, line 4-19), in Results (page 14, line 6-12) and in the Discussion (page 18, line 16-20).

2) Comment: It is not clear why some compounds, which were extremely effective at inhibiting bone resorption and acid influx, had no discernible effect on acidification in intact osteoclasts. This conundrum should be given more attention in the discussion.
Answer: We thank the reviewer for this important question. The bone resorption experiments and the acid influx experiments were done with different time lines and using different concentrations and this affects the effect of the compounds. In addition, the bone resorption assay was done using mature human osteoclasts seeded on bone slices, as opposed to the acid influx assay which is based on microsomal membranes isolation from mature osteoclasts. The influx assay and the bone resorption assay have been used before in studies of the effect of different inhibitors (e.g. Henriksen et al. Calcif Tissue, 2008; Henriksen et al, BBRC, 2009), and in these studies differences were observed as well. We have added a paragraph in the discussion (page 18, line 3-15) about the discrepancies between the acid influx assay and the bone resorption assay.

3) Comment: On page 11, it is stated that “however for GF109203X and Hypericin (figure 4A and B) and Palmitoyl-DL Carnitine Dl (figure 4E) there was a clear distinction between inhibition of resorption and reduction of cell viability, although Ro31-8220 and Sphingosine exhibited toxic effects (Figure 4C and 4D)”. However, for Hypericin and Palmitoyl-DL Carnitine Dl this difference was vastly smaller than for GF109203X, while sphingosine appeared to reduce viability at concentrations that had little effect on resorption. These subtle but important differences in the data should be clearly stated and discussed.

Answer: We apologize for the confusion of the data and that it has not been clarified that we see differences between them, and we have now clarified these subtle differences in detail. We have in the manuscript added a paragraph in the Result section about the inhibitor effect and toxicity seen in the bone resorption experiment. Changes have been made in the text on page 13, line 19-24.

4) Comment: In the discussion it is stated that rottlerin was the most potent inhibitor, but from the data, GF109203X looks to be at least as potent, certainly in terms of inhibition of resorption. This needs to be clarified, while the title might be better describing the effects of PKC inhibitors more generally, rather than concentrating on Rottlerin.

Answer: We thank the reviewer for this comment. Yes, GF109203X is as potent as Rottlerin in both the influx assay and the bone resorption assay, and in addition it has no toxic effect up to 10µM as seen with Rottlerin. Because of this we have changed the title and focus on PKC inhibitors in general and not only Rottlerin. However, there are some differences between the two compounds in relation to the time of incubation required to get an effect in the cell-based acridine orange assay, as GF109203X inhibits the acidification in intact osteoclasts at the 45 min time point only, while Rottlerin inhibits at all time points tested. We have changed the Discussion to include these data in more detail (page 16).

Furthermore, the fact that GF109203X appears to be more selective towards PKC than rottlerin, strengthens the argument that PKC is involved in acidification in osteoclasts. This has now been mentioned in the manuscript as well (Page 16-17).

5) Comments: Rottlerin decreases RANK expression in osteoclasts, most likely by a PKC-independent pathway (Kang et al 2004, Mol Cells 30, 438). This could therefore mediate some of the anti-resorptive effects of this compound and should be cited.
The article by Kang et al. 2004 has been added to the reference list and discussed (page 17, line 7-10). However, the system used in the Kang study was done on U937 cells, which were used to study the differentiation from monocytes to macrophages. Thus, since we were working specifically with mature human osteoclasts, a close comparison between the mature human osteoclasts that we use and the U937 cells is difficult.

Minor Essential Revisions:

1) **Comment:** Background, line 11. “shown to have a very promising mode of action” would be better as “shown to have promising effects”.

**Answer:** The sentence has been corrected.

2) **Comment:** Background, 2nd para, line 6: remove the word “indicated”.

**Answer:** The sentence has been corrected.

3) **Comment:** How exactly were the Alamar blue assays carried out? Using the osteoclasts seeded on to the bone discs at the end of the culture period? This should be clarified.

**Answer:** The AlamarBlue assay was used at the end of the culture period to measure the cell viability. This was done on human osteoclasts seeded on bone slices and is an assay which is carried out when we end the bone resorption experiment to determine the toxic effects of a given compound. The AlamarBlue assay has been described in more detail in the method paragraph (page 7-8). In addition, the AlamarBlue assay has been used in many studies to measure cell viability. The result for the AlamarBlue correlates with the counted number of cells in these studies (Karsdal et al. Am J Pathol, 2005; Sørensen et al. JBMR, 2007).

4) **Comment:** Discussion, 2nd paragraph, last part of sentence on genistein “…however, in alignment with our assays...” is repetitive and should be removed.

**Answer:** The sentence has been rewritten.

5) **Comment:** Discussion, last paragraph. “Thus, indicating” should be changed to “This indicates”; “thus questioning” should be changed to “question”.

**Answer:** The sentences have been changed.

6) **Comment:** Legend to fig. 1 is repetitive and should be shortened.

**Answer:** We have shortened the text in the legend to figure 1. We hope that it is clearer now.

7) **Comment:** Legend to Fig. 2 is the same as the Fig. 1 legend and therefore needs to be corrected.
**Answer:** We apologize for this mistake with the legend to figure 2. The mistake has been corrected so the legend to figure 2 now describes figure 2.

8) **Comment:** It would be helpful to plot the anti-resorptive potency of the compounds against the potency in the acid influx assays. A good correlation would support this effect as a likely mechanism for inhibition of resorption. In this respect, a comparison with the ability of the compounds to inhibit their supposed target enzymes would also be beneficial.

**Answer:** We have done the plot of the anti-resorptive potency of the compounds against the potency in the acid influx assay. However, due to the high number of compounds inhibiting resorption due to toxicity, and therefore not providing useful data, the correlation does not show anything, and therefore we have not included it in the paper, although it was a good idea. If required for publication we will include the correlation. Furthermore, the same applies to the comparison to their effects on the target enzymes the number of compounds with specific effects on both resorption and acid influx is too small to get useful data.

9) **Comment:** HBDDE was able to inhibit acid influx but did not bone resorption. Why might this be the case? Is this compound less able to act in whole cells than the other compounds?

**Answer:** We thank the reviewer for this important comment on the results for HBDDE. The explanation for the inhibition in the acid influx assay lies with the intrinsic quenching of the green fluorescence by the compound itself, which explains why an effect of HBDDE is seen in the acid influx assay but not in bone resorption. Table 1 has been corrected to show that HBDDE quenches the signal and we apologize for the mistake made with HBDDE.

**Reviewer: Teun de Vries**

**Level of interest:** An article whose findings are important to those with closely related research interests

**Comment:** The results are very much for a specialist audience. As mentioned in the title, this is really a screening-of-agents-article rather than an article concerning major cell biological principles. When including more cell biological principles, for instance, do (a selection of) agents also influence osteoclast formation; the article could have more impact.

**Answer:** It is a good comment from the reviewer. However, we have chosen to look specifically at acid secretion and bone resorption by mature human osteoclasts. The main reason for this is our general focus on acid secretion by the osteoclasts (Henriksen et al, BBRC, 2009; Henriksen et al, CTI, 2008; Sørensen et al, JBMR, 2007). Secondly, since several of the compounds exhibited toxic effects within the 5 day time-span used in the resorption experiments, we are concerned about false-positives if the cultures were extended to include the full time needed for an osteoclastogenesis experiment (10 days).
**Comment:** If a certain agent does not show an effect in a biological assay, like many inhibitors used in this study, the question arises immediately whether the chemical compound was solubilized. So, very importantly, did the authors do a quality control for the inhibitors which did not show an effect? Quality controls could be a test in a biological assay where it is bound to have an effect or a spectrophotometric/chromatographic quality control of solubility.

**Answer:** We thank the reviewer for this important comment, and it is a comment which should always be taken into account when compounds do not show an inhibitor effect. We have not controlled the compounds in specific assay for kinases. However, we have used freshly prepared solutions each time, and we have bought the compounds from a well-established source of chemicals (BIOMOL International LP). In addition, we controlled dissolution and/or precipitation by visual inspection. In addition, we used low concentrations to avoid too much quenching or auto fluorescence of the compounds and to be sure that the compounds do not precipitate.

**Comment:** p. 4, below the middle: sentence starting with: "In avian osteoclasts indicated the tyrosine kinase" does not make sense.

**Answer:** “indicated” has been removed from the sentence, so it hopefully makes more sense now.

**Comment:** p. 7: Authors should clarify the use of AlamarBlue assay in more detail. What is the principle of the technique, why is it considered to be a good measure for viability?

**Answer:** The AlamarBlue assay was described in more detail in the method paragraph (page 7-8). In addition, the AlamarBlue assay is a well described assay to measure the cell viability because it is non-toxic to the cells and it is easy to use. The AlamarBlue assay is a well used assay to measure cell viability. What is very important is that the result for the AlamarBlue assay correlates with the counted number of cells (Karsdal et al. Am J Pathol, 2005; Sørensen et al. JBMR, 2007).

**Comment:** Legend of Figure 2 is an exact copy of legend of Figure 1! There is bound to be an unfortunate mistake, since the description of Figure 2 does not match with the figures.

**Answer:** This mistake has been corrected and the legend now match figure 2. We apologize for this mistake and the inconvenience it has given reading the manuscript.

**Comment:** In general: Far too much detail is given in the figures. For instance: it suffices when time point A and B are shown in Figure 1. Figures C-F do not give any extra information which cannot be dealt with in the text of Results. It is strongly advisable to use the whole width of the page for panel A. Same for Figure 2: show 1 time point, use width of the page for panel A or C or E.
**Answer:** We thank the reviewer of the comment on our figures. We have taken this into account and Figure 1 has been changed and is now only showing the 45 min incubation data (A and B). The other time points are listed in the text of the Results paragraph. Figure 2 has also been changed and is also showing only the 45 min incubation time.

**Comment:** Figure 3 could be largely improved when the X-axis data are not shown in an angled version. Instead, use only concentration in X-axis and put GF109203X (as an example) in the title of the figure, just above the bars.

**Answer:** We really thank the reviewer for the suggestion putting the name of the inhibitor on top of the figures. We have changed Figure 3 regarding this suggestion. However, we did not find it possible not to angle the concentrations on the figure because of lack of space. We have also added the change to Figure 4 both with the inhibitor name on top of the figure and in this figure we were able not to angle the concentrations tested.

**Comment:** Figure 4 is very much appreciated. It clearly shows that some agents do not affect viability, whereas the Ca2+ release is clearly hampered, whereas others affect both viability and Ca2+ release.

**Answer:** We thank the reviewer for this comment on Figure 4.

**Comment:** I strongly suggest to include Table 1 in the article instead of using it as a supplementary table. Great advantage is that targets and horribly named inhibitors are listed in one table, makes it easier to read. Table could be simplified when leaving out two time points (AO), since columns 3, 4 and 5 are exactly the same. Please introduce AO in the table (it was introduced before, but the readability increases when used here).

**Answer:** It is a good idea to include Table 1 in the text so it is an integrated part of the results and this has been done. Furthermore, the table has been reduced leaving out 4 and 24 hours acridine orange data to simplify the table. In addition, AO has been spelled out to acridine orange as suggested by reviewer. We thank the reviewer for good comments on the table.

**Reviewer:** Koichi Matsuo

**Level of interest:** An article of importance in its field

**Major Compulsory Revisions:**

**Comment:** As the authors are aware, there is no evidence that “PKC inhibitors” elicit the effects on acid secretion specifically via PKC. The involvement of PKC delta is suggestive based on the panels of PKC inhibitors. However, the authors should experimentally demonstrate that PKC regulates acid secretion in osteoclasts either by RNAi-mediated gene silencing or by gene-knockout experiments. Alternatively, the authors could use constitutively active forms of PKC-delta or other PKC isoforms to reinforce their conclusions.
Answer: We thank the reviewer for this comment. However, it will be very difficult to make siRNA experiments on mature human osteoclasts. This will be a whole new study for a separate article if it was a success. We believe that it is beyond the scope for this article to do siRNA experiments. However, the suggestion is of high importance in general and a sentence has been added in the end of the discussion about the usefulness of extending this study by a follow-up using siRNA (page 18, line 21-24).

Minor Essential Revisions:

1. **Comment:** Table 1 needs a legend.

   **Answer:** Table 1 has been given a legend in the text.

2. **Comment:** Something is wrong with the text of Figure 2 legend, which is identical to that of Figure 1.

   **Answer:** This has been corrected and we apologize for the inconvenience it has made.

3. **Comment:** The role of Bafilomycin in Figures 1 and 2 should be explained in the text.

   **Answer:** Bafilomycin is used as a positive control in the different assays. Bafilomycin is a known V-ATPase inhibitor and therefore it inhibits the acidification and bone resorption using the human osteoclast system robustly (Henriksen et al, CTI, 2008; Sørensen et al, JBMR, 2007). We have added the role of bafilomycin in figure 1 and 2 in a paragraph in the Result section (Page 12, line 13-15).

Discretionary Revisions

**Comment:** Although the most of the methods used in this manuscript have been published (ref. 27), the principle of experiments using acridine orange should be described for readers.

**Answer:** We thank the reviewer for the comment, and we have described the acridine orange assay in more details in the Method section in the manuscript (page 7, line 17-21). In short we wash away the dye and take pictures of the cells, and alternatively we measure fluorescence using a SpectraMax M5 at excitation 492 nm and emission 535 nm.

**Reviewer: Jean-Pierre David**

**Level of interest:** An article of limited interest

**Comment:** The manuscript is difficult to follow due to major editing mistakes such as the lack of clear legend for the figure 2 (the provided legend is a copy of figure1)
Answer: We apologize for the editing mistakes in the manuscript especially the one with the legend for figure 2. This has been corrected in the manuscript so figure 2 now has the correct legend.

**Major Compulsory Revisions:**

1) *Comment:* The choice of 10 #M as ‘standard’ dose to compare the inhibitors is quite artificial and certainly not based on their dose effect on kinase activation.

*Answer:* We thank the reviewer for this important comment. In addition, to the test done at 10µM, we have performed a series of analyses at 50µM; however, since these tests only provided more compounds showing significant toxicity and quenching effects, we did not mention them in the previous version of the manuscript. We have now included a paragraph describing these findings and the rationale for not going into further detail with this. Although the doses tested do correlate directly to the effect on kinase activation, a direct comparison between the assays used here and the kinase activation assays is very difficult due to the varying compositions of the assays, and thus we chose to go for high concentrations to see whether any effect could be detected.

2) *Comment:* Nowhere the efficiency of the treatment in term of kinase inhibition is demonstrated. That may explain the lack of effect of general tyrosine kinase inhibitors that should certainly be inhibiting CSF1 receptor activation by M-CSF, as well as c-src activation.

*Answer:* The comment by the reviewer is correct and we thank him for this important comment. However, in our system using mature human osteoclasts we do not see any effect of the general tyrosine kinases. An effect could probably have been seen if we have studied osteoclastogenesis, and not done the experiments on mature human osteoclasts as we did. Furthermore, increasing the time of the cultures might also expose effects of these broad spectrum inhibitors; however, we have focused specifically on the relation to acid secretion and for this the time-span studied is sufficient as seen in other studies (Henriksen et al, CTI, 2008; Henriksen et al, BBRC, 2009; Sørensen et al, JBMR, 2007). We have now included a section in the Discussion with a discussion on why the tyrosine kinase inhibitors do not work on resorption (page 15, from line 21).

3) *Comment:* It is difficult to extrapolate on the role of PKCs or more specifically PKC# (Rottlerin effect) without demonstrating the presence of the kinase in the extract used for the assay.

*Answer:* We thank the reviewer for this important question. We have done Western blotting both on osteoclast lysate and on the membranes from the osteoclasts using a PKC antibody, and an antibody against the V-ATPase B2 as a control. The Western blot shows that PKC is indeed in the membranes of the human osteoclasts and also in the lysate. In addition, V-ATPase B2 is also present in both preparations as expected showing that the Western blot was done properly. The Western blot has been added as Figure 5 (figure legend: page 28-29), in Methods (page 9, line 4-19), in Results (page 14, line 6-12) and in the Discussion (page 18, line 16-20).