Author's response to reviews

Title: The differential anti-tumour effects of zoledronic acid in breast cancer - evidence for a role of the activin signaling pathway

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Version: 3
Date: 14 January 2015

Author's response to reviews: see over
Dear Editors,

Thank you for giving us the opportunity to submit a revised version of our manuscript entitled ‘The differential anti-tumour effects of zoledronic acid in breast cancer – evidence for a role of the activin signaling pathway’. Following the constructive comments made by the reviewers, we have made changes to the manuscript. We have gone to great lengths to address reviewers concerns and have detailed our responses, changes to the manuscript and addition of experimental findings below.

**Responses to reviewer 1**

1. What is still unclear is why neither of the adjuvant BIS studies including ER-ve patients comprised a significant DFS or OS benefit? This has not been discussed in the manuscript, but would be clinically of great interest and should be included into the manuscript. I would be interested how the authors would explain this disparity.

*Our response*

This is an excellent suggestion. We have focused the manuscript primarily on the direct affects of ZOL on breast cancer cells which is more representative of the neo-adjuvant clinical setting where there is a direct effect of ZOL on primary breast cancer cells. In the adjuvant clinical trials the bisphosphonates have been initiated after removal of the primary tumour and it is likely in this situation that the residual tumour cells are sat in niches outside of the primary site, possibly bone. The indirect effect of ZOL on the bone microenvironment and subsequent effect on breast cancer cells in this niche is likely to be more important in this setting rather than a direct tumour cell effect of ZOL and is part of ongoing work within our group. We have added a discussion point regarding this to the conclusion lines 402-406.

2. The outcome of ZOL not altering proliferation of ER+ve MCF7 and T47D cell lines was somewhat unexpected. Is there any explanation for this and what is the implication with regard to the results of the clinical adjuvant BIS study reports?

*Our response*

We agree that there is controversy regarding the differential effects of zoledronic acid on proliferation of ER-ve and ER+ve cell lines. Rachner et al confirmed the differential effect of zoledronic acid on proliferation according to ER status of cell lines showing that MCF7 cells did not demonstrate a significant change in proliferation in response to increasing doses of zoledronic acid whereas MDA-MB-231 cells showed a dose-dependent inhibition of proliferation and increase in apoptosis via activation of caspase 3 and 7 (Rachner, Singh et al. 2010). These results were supported by Fehm et al who showed the antitumour effect of zoledronic acid on primary breast cancer samples removed at surgery (n=116), was greatest in ER-ve tumours using an ATP-tumour chemosensitivity assay (Fehm, Zwirner et al. 2012). Some studies have reported ER+ve cells to be more responsive to zoledronic acid (Fromigue, Lagneaux et al. 2000), or no differential effect of zoledronic acid according to ER status (Senaratne, Pirianov et al. 2000; Jagdev, Coleman et al. 2001).

A possible explanation for the differential effects of zoledronic acid on proliferation according to ER status may be due to differences in uptake of the drug. Previously published data have
shown that the maximal anti-tumour effects of zoledronic acid are obtained in vivo when unprenylated Rap1a is detected in tumour lysates (Ottewell, Woodward et al. 2009; Ottewell, Lefley et al. 2010). I detected unprenylated Rap1a in both MDA-MB-231 and MCF7 cells after treatment with the same dose of zoledronic acid (figure 6B). However, addition of the mevalonate intermediate GGOH partly reversed the accumulation of Rap1a in MCF7 cells only, suggesting the ER-ve MDA-MB-231 cells either take up more zoledronic acid, or do not take up the GGOH as effectively as MCF7 cells. This is discussed in lines 354-69 of the manuscript.

The clinical adjuvant BP studies have shown that ER status of the primary tumour does not influence the beneficial effects of ZOL on PFS or OS. This is likely due to the fact that in the adjuvant setting the anti-tumour effects of ZOL are primarily through modification of the bone microenvironment rather than a direct affect on the tumour cells. Further work on this is ongoing in our lab and therefore we have not included this within this manuscript.

3. The same applies to the results on page 7, line 237 were the authors state that Activin A secretion was unaffected by ZOL in MCF7 and MDA-MB-436 cell lines. Was this expected and if not please try to explain your findings.

Our response
This is an excellent point and we refer to work we have previously published on changes in serum levels of both activin and follistatin in neo-adjuvant ER+ve and ER-ve breast cancer patients receiving chemotherapy+- ZOL [Winter MC, Wilson C, Coleman RE et al. Neoadjuvant chemotherapy with or without zoledronic acid in Early breast cancer-A randomized biomarker pilot study. 2013. Clinical Cancer Research 15;19:2755-65]. We found that addition of zoledronic acid to chemotherapy differentially altered follistatin levels according to menopausal status of patients, but did not affect serum activin levels and so we did not anticipate that ZOL would have a direct affect on secretion of activin from breast cancer cells.

Responses to reviewer 2

1. The authors have not demonstrated that the pSmad2/3 signaling occurs in an activin-dependent manner (for which soluble activin receptor 2 could be used to inhibit the activin signaling pathway in ZOL treated cells). The authors also have not tested whether the transcriptional targets of pSmad2/3, such as p21, are regulated upon treatment with ZOL. Finally, it is also important to understand whether ZOL treatment would still sensitize the ER-ve cells in the presence of the activin signaling inhibitor molecule.

Our response
We agree with the reviewer that we needed to demonstrate that ZOL would still sensitise ER-ve cells to activin and affect proliferation. To address this concern we have added a section describing the results of further experiments we had performed that demonstrated the changes in proliferation of ER-ve MDA-MB-231 cells were activin dependent by use of an ALK 4/5 inhibitor (line 197-204). The graph is included in this response for information (below), however we have not added it to the manuscript in the interest of space but we will add to the figures if the editor believes it is necessary.
As this is the first paper to describe the potential effect of ZOL on the activin signaling pathway we also agree that future work on the transcriptional targets of Smads would be interesting and is an area of ongoing work within our group, but feel this is outside the scope of this current manuscript.

2) In figure 1, the error bars for the controls are missing. If the variations in the control sample were to be considered, would the results from the statistical test for significance still stand true? Would the depletion of follistatin (by siRNA) in the ER- cells cause an increased sensitivity to activin?

Our response
I believe this refers to figure 2. The graph shows percentage change in mean absorbance from control not median values of raw data. Using the Wilcoxon Signed-Rank test the treatment groups are compared against a hypothetical control which is assumed to be 100% absorbance in untreated control cells and therefore there are no error bars on the hypothetical ‘control’. A significant p value therefore refutes the null hypothesis that the percentage change in median absorbance from the hypothetical control (100% absorbance) is due to chance alone and is considered significant at p<0.05. Depletion of follistatin by either removal of endogenous secreted follistatin or use of siRNA in ER-ve cells has been shown by others [reference 12 in the manuscript] to result in an increase in pSmad2c and activin induced inhibition of proliferation in ER-ve MDA-MB-436 cells which were previously weakly affected by exogenous activin. In our experiments activin significantly inhibited proliferation of the ER-ve cell line MDA-MB-231 provided we removed endogenous secreted follistatin by washing cells every 24 hours. We demonstrate in figure 3B that endogenous secretion of follistatin in MDA-MB-231 cells increases at 24 hours and is significantly greater than cell culture medium levels at 48 hours. Using follistatin siRNA is likely to yield the same results as removal of endogenous secreted follistatin by washing cells and is unlikely to change the findings or conclusions.

3) In figure 2, treatment of the cells with follistatin does not rescue the cells from activin mediated growth repression. However, the authors mention otherwise in the text. This needs to be clarified. And a possible explanation why follistatin fails to rescue the growth of the cells needs be included.
Our response

Figure 2C&D show that exogenous activin induces a statistically significant reduction in percentage change of absorbance in the MTS assays compared to control. This significance is lost when both MDA-MB-231 and MCF7 cells are treated with endogenous activin+follistatin, therefore showing the significant fall in percentage change in absorbance from control with activin is rescued in the presence of follistatin.

4) In figure 7, the method of quantification for the nuclear localization by immunofluorescence assay has not been described. If software was used, please mention.

Our response

Thank you for highlighting this. We have added the method to line 163.

5) In figure 7, while ELISA assays have been used for evaluating the signaling components, additional validation using western analysis would be highly informative and would strengthen their conclusions.

Our response

We believe that ELISA represents a more accurate methodology for quantification of signaling components and was therefore chosen over western blotting for the quantification of total cellular psmad2C/total smad2c in figure 7d. No such ELISA is available for pSmad2L and thus western blotting was used for quantification of total cellular quantity in figure 7h. It is unlikely that western blotting would change the findings or conclusions to figure 7d and therefore these were not performed.

6) In figure 8, the authors performed a xenograft assay and show only the IHC data. Tumor volume/size data could be included.

Our response

The tumour volume/size data is within the reference quoted in the methodology line 168 [reference 13 of the manuscript]. In the interest of space within the manuscript we have not added the results of this previously published data.

For discussion;

7) In figure 7, the authors observed no change in total levels of pSmad2L upon ZOL treatment, however, in the xenograft assays, the authors observe a significant reduction in cells having pSmad2L upon ZOL treatment. An explanation for the discrepancy between in vivo and in vitro data could be included.

Our response

We do not feel that we can directly compare the in vitro and in vivo results due to differences in experimental design and overall exposure of tumour cells to ZOL. In vitro the cells were exposed to continuous ZOL for 24 or 48 hours. This is not possible in vivo, and as such the animals were treated with a single IV ZOL treatment weekly for 6 weeks. The overall findings and conclusions however are the same that ZOL decreased nuclear localization of pSmad2L in
ER-ve breast cancer cells in vitro and the number of cells staining positive in vivo.

8) In figure 5B, the authors find that ZOL treatment reduced the ratio of follistatin to activin to be 4:1. However in the present study, this appears untrue.

**Our response**

*We have shown that ZOL decreases the molar ratio of secreted follistatin:activin from MDA-MB-231 cells from 14:1 to 4:1 in figure 5B however this is not seen in MCF7 cells and we discuss that “ZOL has a more noticeable effect on the follistatin:activin ratio in ER-ve cell lines.”* (line 257) which we believe we have shown in the figure.

9) Figure 6B could be replaced with a better quality image

**Our response**

Many thanks. We have improved the image quality within the figure.

10) Figure 1-3 seem unessential. The focus of the manuscript could be narrowed to solving the mechanism of differential response of ZOL in ER+ and ER- breast cancers. Figures 2, 3 do not appear to add more information than what is already known in the literature or contribute to the conclusions made in this research paper.

**Our response**

*We feel figure 1 adds reference for the reader if they are not entirely familiar with the activin signaling pathway, but we will remove it if the editor agrees. We believe Figure 2 is necessary to show that activin and follistatin affected the proliferation of both ER-ve and ER+ve cell lines in our hands. This is especially important given the controversy in the published literature from different research groups regarding the sensitivity of ER-ve cell lines to growth inhibition from activin as discussed in the manuscript. Figure 3 is required to show that the endogenous secretion of both activin and follistatin differ according to cell line. This information is needed for the reader to understand the impact of changes in secretion of these soluble factors from the cell lines in response to ZOL treatment.*

As detailed above we have addressed all the reviewers’ comments and we hope you will find the revised manuscript acceptable for publication in BMC Cancer,

Yours truly,

Dr Caroline Wilson