Author’s response to reviews

Title: The antifibrotic drug pirfenidone inhibits spondyloarthritis fibroblast-like synoviocytes and osteoblasts in vitro

Authors:

Julie Stougaard (jkl@biomed.au.dk)

Søren Lomholt (soren.lomholt@gmail.com)

Pernille Ommen (pernille.ommen.andersen@post.au.dk)

Jens Kelsen (jenskels@rm.dk)

Tue Kragstrup (kragstrup@biomed.au.dk)

Version: 1 Date: 08 Aug 2018

Author’s response to reviews:

Editor Comments:

This is a revised version of their previous manuscript submitted to BMC Musculoskeletal Disorders. The manuscript has been revised well. However, the reviewers and myself still have a couple of concerns to be addressed before publication in BMC Rheumatology. In addition, I do see the need for some corrections as I wrote in the comments below.

1. As reviewer 2 repeatedly pointed out, the transmission microscopy images do not allow to conclude that PFD does not cause cell death. The authors added two references to claim that the concentration of PFD they used in this study does not cause cell death. However, the two references do not support the statement. The concentration of PFD to cause cell death could be different if the origin of fibroblasts and/or culture condition are different.

Answer: We deliberately did not do more than light microscopy to observe for cell death during all experiments. This is because pirfenidone has been used extensively in both preclinical and clinical research. We have now added a reference using the exact same experimental conditions and cells as used in our experiments (Kaneko et al, Clin Exp Immunol, 1998). Further, we now state in the “Flow cytometry” section that the percentage of dead cells was below 1% using the Live/Dead fixable viability marker from Life Technologies. It is also seen that pirfenidone did not cause decrease in all the measured secreted and membrane proteins, e.g. membrane ICAM-1 increases and secreted IL-6 and IP-10 are not changed.
2. The authors should state that PFD did “not” decrease the expression of aSMA and HLA-DR, since the differences were not statistically significant. The discussion should also be amended accordingly.

Answer: We acknowledge that the differences are not statistically significant using a t test. Therefore, we have changed the heading of the Results section so that we no longer state that pirfenidone inhibits aSMA and HLA-DR. We have also changed the Discussion to include that the changes were not significant because of large interdonor variation and needs confirmation by others. Additionally, we have found some recent publications supporting the finding and added these references.

3. In mineralization assay, the results of Saos-2 cells looks convincing. However, it looks like the significant decrease of mineralization by PFD was not observed in HOB. It is required to interpret and state the results appropriately based on the results of statistical analysis.

Answer: We agree that the HOB data do not reach significance using a t test. We have stated this in the Results and Discussion sections. We have also added the statistics. We would like to keep the data in the paper because it shows that pirfenidone inhibition of osteoblasts is reversible. However, if reviewer 2 is not satisfied with our current adjustments we will agree to remove the HOB data.

BMC Rheumatology operates a policy of open peer review, which means that you will be able to see the names of the reviewers who provided the reports via the online peer review system. We encourage you to also view the reports there, via the action links on the left-hand side of the page, to see the names of the reviewers.

Reviewer reports:

Angelo Calado (Reviewer 1): In this manuscript, the authors present in vitro data supporting the use of the antifibrotic drug pirfenidone (PFD) for the problem of new bone formation in spondyloarthritis (SpA). Briefly, it is here shown that PFD inhibits the proliferation of fibroblast-like synovial cells (FLS) from SpA patients, either unstimulated or stimulated with TNFa, TGFb and IFNg, as well as their differentiation into myofibroblasts. Furthermore, PFD was shown to alter FLS expression of membrane proteins and several chemokines and cytokines, like DKK-1 and OPG which are critical for bone homeostasis. Finally, PFD was shown to inhibit mineralization in Saos-2 cells or human osteoblasts.

The current manuscript is well written and is presented in a clear manner. Authors have clearly posed an important and well defined scientific question and working hypothesis and consistently, have performed a battery of experiments to tackle them. The obtained data are original and sound and have enabled the authors to withdraw relevant preliminary insights on the potential use of PFD to target new bone formation in SpA.
This manuscript is a substantially revised and improved version of their previous manuscript submitted to BMC Musculoskeletal Disorders. In this novel version, I was pleased to note that the authors have fully and well answered to all my previous comments to this latter manuscript.

Still, there are a couple of issues that the authors show correct:

- In figure 1, the authors have eliminated the legend of the histograms presented in panels C and D.

Answer: This was clearly a mistake. We greatly appreciate that this was seen by the reviewer and have made corrections.

- The legend of figure 3 does not match exactly the figure presented. Moreover in the figure, the presented panels are not letter labelled.

Answer: Again, we greatly appreciate that this was seen by the reviewer and have made corrections.

Leyre Brizuela (Reviewer 2): This is a paper from Stougaard and colleagues proposing that « the antifibrotic drug pirfenidone inhibits spondyloarthritis fibroblasts and osteoblasts in vitro». Although the message of the article is interesting, the proposal of a new approach for SpA treatment, manuscript needs a revision and

1. What was the technique used to mesure the deposition of hydroxyapatite ? this should be explained.

Answer: We acknowledge that explaining the technique is actually much better than just referring to our previous papers using this technique. We have added the full description to the M&M section.

2. The part « osteblast cultures » has to be rewritten. Culture and mineralization conditions used with osteoblast cell cultures are not explained. The authors have included two references to illustrate the culture conditions for osteoblasts. Nevertheless, mineralization assay has to be detailed and explained because one of the conclusions is that the drug inhibits osteoblast mineralization.

Answer: We have completely rewritten the “Osteoblast culture” section.

3. Results line 29. …without causing visible cell death, debris or detachment of cells … what « visible cell death » means ? authors seem to conclude quite quickly that their drug has no effet on
the cells. The way to confirm the effect of the drug is not sufficient, apoptosis markers can be checked (Clived caspase 3 or PARP) by western blot, flow cytometry or immunocytochemistry.

Answer: We have deleted the sentence “visible cell death”. We deliberately did not do more than light microscopy to observe for cell death during all experiments. This is because pirfenidone has been used extensively in both preclinical and clinical research. The percentage of dead cells was below 1% using the Live/Dead fixable viability marker from Life Technologies. We agree that studying apoptosis could be interesting. PFD has been shown to induce apoptosis in hepatocellular carcinoma cells but prevent apoptosis in lung epithelial cells. In vivo, PFD ameliorated ciclosporine nephrotoxicity by decreasing pro-apoptotic genes. However, we cannot conclude anything about apoptosis from this study. We have now added this to the Discussion.

The stats from figure 1 are not correct. Paired t-test has to be done at least when 4 independent experiments have been realized, in this figure there are only 3. A non-parametric Mann-Whitney test would be more appropriate.

Answer: It is not possible to do non-parametric tests with n<6 (it is not possible to get a significant result at least). Therefore, we use parametric tests. We fully agree that this practice is a matter of discussion. The reason why this is common practice is that larger studies with in vitro data usually shows normality when expressed as ratios and log transformed. We believe that this is so common practice that we have not commented it in the M&M.

4. The most important message of this article for the reviewer is that PFD has an effect in osteoblast mineralization. Authors have presented a new figure with effects of PFD in osteoblast maturation. PFD decreases DKK-1 (and osteoblast inhibitor after treatment of cytokines). This result is interesting but error bars are huge, more experiments have to be done in order to improve the stats because they do not seem correct. OPG (an osteoclast inhibitor) is also decreased. This controversy is well explained in the discussion.

Answer: We agree that the error bars are large for some of the experimental conditions. However, this is not unusual when working with human samples. There are always greater variations between patients compared with rodents. We have added this in the Discussion. If the reviewer prefers, we could exclude the conditions with all the different stimulations. However, we would prefer to include it because it shows how DKK1 and OPG changes with different cytokine stimulants. We have stated that “it is not possible to conclude whether PFD is more effective in preventing MCP-1 or YKL-40 secretion under influence of TGFβ, TNFα, IFNγ or a combination of all three cytokines”.

Nevertheless, mineralization figure for HOB has to be improved. Error bars are huge and protocol has still to be explained. What it means « mineralization ratio » ?

Answer: We have taken care to explain the protocol for the osteoblast mineralization assay. We agree that the HOB data do not reach significance using a ttest. We have stated this in the Results and Discussion sections. We have also added the statistics. We would like to keep the data in the
paper because it shows that pirfenidone inhibition of osteoblasts is partly reversible. However, we are willing to remove the HOB data I the reviewer prefers.