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Dear Editor,

RE: ijms-1856897 Reviewer Comments

Article
CB1 ligand AM251 induces weight loss and fat reduction in addition to increased systemic inflammation in diet-induced obesity.
Lannie O'Keefe , Teresa Vu , Anna C Simcocks , Kayte A Jenkin , Michael L. Mathai , Deanne Helena Hryciw , Dana S Hutchinson * , Andrew J McAinch *

Please see below responses to the reviewer comments and the changes implemented into the manuscript.

Reviewer 1 Comment	**Author Comment**
1. One advantage of using animal models is that, in addition to allowing molecular level studies, it also allows tissue/cell level studies/observations. I wonder why the authors did not consider providing pictures of tissues that show significant weight reduction. Also, some pictures at the histological level of all types of tissues examined could be included in the manuscript. Sometimes, as we know, 'a picture is worth a thousand words'.	Thank you for your comment and yes while we agree that a "picture is worth a thousand words" we were unable to collect pictures of the organs and tissues at the time of the study as well as process them all for further histological analysis. Thus this is something that we are unable to include in this manuscript.
2. The authors examined expression level of several genes by PCR, many of which show significant change in	Thank you for your comment, G protein-coupled receptors can be problematic using Western blot detection. The authors measured citrate synthase on whole muscle to detect changes in activity of the protein caused by the AM251 effects. While

	expression level. No protein-level experiments supporting PCR-level results have been carried out by the authors. I would consider IHC-type of experiments for at least a few of those genes in the relevant tissues between the groups of animals.	completing additional experiments always can add more information to the findings of a study the capacity to do additional Western blot / IHC experiments on the number of tissues presented in this study is currently not possible and would take a number of months to complete. The authors believe that the data we present in this paper which includes whole organ weights, a large number of inflammatory markers in the blood, adipose tissue mRNA expression in three different fat depots, enzyme activity in two different skeletal muscle types and an ex-vivo organ bath treatment in two different skeletal muscle types with mRNA expression of multiple genes adds significantly to the literature regarding the role of CB1 and the effects of one of its ligands AM251.
3.	A picture of the various blood cells at various times (and at various resolutions) with and without AM251 could also be informative. Consider evaluating relative abundance of various blood cells at different times of treatment.	Thank you for your comment, due to the number of experimental treatments and tests that was conducted on these animals (as detailed in the current study but also in Jenkin et al 2015) the collection of additional blood from the animals was not possible at different time points was not possible. As we did not collect and analyse this at the time we are unable to add these pictures to the manuscript.
4.	I would also advise creating a plot of body weight vs time between the two groups of rats.	Thank you, for this suggestion, however this data has previously been published in our previous work in these animals which focused on albuminuria and renal tubular structure (Figure 2 **https://joe.bioscientifica.com/view/journals/joe/225/2/113.xml).** Thus as this data has already been published and the results were in line with the expected effects of AM251 on body weight, we do not believe duplicating this data in the current publication would add significant value. We have referenced this previous data appropriately within the manuscript to help ensure that the readers can appreciate the impact of AM251 on body weight in these animals which we then further investigated. To provide the basic information for the reader however we have included the starting body weights and the body weights at the end of the treatment period into the manuscript lines 83 to 87

5. Also consider creating a plot for food consumption vs time between the two groups.	Thank you for your comment, however as per our response to Comment 4, this data has previously been published and is part of the same figure in this publication (**https://joe.bioscientifica.com/view/journals/joe/225/2/113.xml). Thus like our response to comment 4, the transient reduction in food intake is in line with the expected results with AM251 and we have mentioned this data through the manuscript to help ensure that the readers understand the impact of this compound on food intake in these animals.**
Reviewer 2 Comment	**Author Comment**
1. The authors discuss that tissue remodeling may underly the inflammatory response. Please give the body weights at start of the treatment period! Is there a weight loss or just reduced weight gain, which may not be regarded as tissue remodeling?	Thank you for your comment. The body weights have previously been reported (**https://joe.bioscientifica.com/view/journals/joe/225/2/113.xml)** as we indicate in the manuscript. To improve clarity we have added the starting body weights and the body weights at the end of the treatment period into the manuscript lines 83 to 87.
2. To get hints on the source of inflammation, inflammatory gene expression in the fat depots should be analyzed.	We agree with the reviewer that these experiments would add additional information for the readers, specifically mRNA analysis for IL6, IL1beta and TNFalpha in the fat depots. However, the critical data in this study concerning inflammation is the significant increase in circulating inflammatory cytokines. Thus though interesting, we believe that the novelty and impact of this study is still significant.
3. Is there any data on CB1 knockout (or CB1-ligand treated) animals on systemic inflammatory markers in obesity models?	This is the first study to our knowledge that has investigated such an array of systemic inflammatory markers in an obesity model following treatment with CB1 antagonist/inverse agonists or in CB1 knockout animals. The majority of studies have focused on tissue specific inflammatory responses, not in an obese model and/or very limited number of inflammatory markers.
4. What are the differences between Rimonabant and AM251? Is there a possible explanation for the different results by these differences?	The differences between the actions of AM251 and rimonabant are not clear, as both are potent and selective CB1 receptor antagonists, and can both act as inverse agonists at higher concentrations. However there are differences in their selectivity profiles, with AM251 being ~1000 fold more selective for the mouse CB1 vs the mouse CB2, as opposed to rimonabant

	being only 25 fold more selective (Soethoudt et al., Nat Commun, 2017, 8:13958). There are also reported differences in their off-target activities, with Soethoudt et al., (Nat Commun, 2017, 8:13958) showing that AM251 had activity at 16 off-target receptors including GPR55, whereas rimonabant only had 8 off-target activities. An additional level of complexity may arise from ligand biased signalling, which is the ability of different ligands to differentially activate different receptor signalling effectors/responses. Ligand biased signalling at the CB1 receptor has been reported (Leo & Abood, Molecules, 2021 26(17):5413), although to our knowledge there are no studies comparing bias of AM251 vs rimonabant. It would be beneficial to elucidate the differences observed between AM251 and rimonabant in vivo, but this is outside the scope of the present study.
5. I suggest to present the data of the tables as diagrams to facilitate reading.	Thank you, for the suggestion. We have included the mRNA data from Tables 4-6 as Figures 3-5. We agree that the presentation of this data in figures will facilitate reading. The data from Table 2 which contains 22 different cytokines we believe if converted to figures would not enhance reading due to the number of images required and the impact on the overall flow of the paper. The data from Table 3 which contains 8 different mRNA expression from 2 different tissues and 4 different conditions, with no changes in the data we believe is best presented as a table rather than a number of complex images.

We believe that these changes have significantly enhanced the manuscript.

Sincerely,



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