Review of: "Targeting Alzheimer's disease hallmarks with the Nrf2 activator Isoeugenol"

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Potential competing interests: No potential competing interests to declare.

This is a very comprehensive study that looks at the potential beneficial effects of the phenylpropanoid, isoeugenol (ISO) in models of Alzheimer's disease (AD). The authors use a combination of both cell and animal models to provide evidence that ISO might have potential as an AD therapeutic. In addition, the authors characterize the pharmacokinetics of ISO, perform some preliminary toxicology studies and look at its ability to cross the blood brain barrier. Moreover, they use an intranasal approach to administration which is relatively novel for AD treatments. However, there are a number of points that need to be clarified and these are listed below. In addition, the manuscript is too long. The Introduction should be shortened and the Results section contains quite a bit of information that is not only more appropriate for the Discussion but is also found there.

1. Isoeugenol has been designated as "possibly carcinogenic" by the IARC (PMID37454664). At the least, the authors need to mention this in the Discussion and indicate how it could impact potential long term use of ISO in AD patients.

2. The structure of ISO should be included in one of the figures.

3. section 2.2.2.: If this is a kit and the authors followed the manufacturer's protocol then a detailed description is not needed.

4. pg. 7, section 2.2.3, 4th paragraph, 3rd line: I think "e" should be "and"

5. Animal studies: How was the initial dose of 50 mg/kg of ISO chosen? Why were 5 mo old females used but 10 mo old males used for the ISO-treatment studies? At the least, the authors need to justify changing sex for the ISO treatment studies with the older mice. Also, the order of the analyses is not clear. Was the GGT done after the behavioral tests?

6. section 2.2.4.2: It is not clear that the animals were perfused with PBS prior to removing the tissues for analysis of ISO levels. If the animals were not perfused, then it is hard to conclude anything about the tissue levels of ISO as it could be in the blood vessels rather than the tissue itself. The authors need to address this potential problem.

7. pg. 12, 1st paragraph, line 10: I assume this should be "antibodies to the loading control proteins"?

8. Figure Legends for Figures 2-5: Please indicate the concentration of ISO used in each of these experiments.

9. In Figure 3B, why are total Akt levels so low following ISO treatment? Is this a consistent observation? While phosphorylation of Akt may go up, if the levels are much lower following ISO treatment, then there might not be any

functional increase in activity. At the least, the authors need to address this point.

10. In Figure 4C, no HMOX1 bands are visible. Thus, how were the authors able to quantify the results to get the data shown in the graph?

11. pg. 19, 1st paragraph, line 3: “reversed” should be “prevented” since ISO was added before the LPS treatment.

12. Figures 6-8: Why is there no data with WT + ISO for these studies? Also, the legends need to include the number of mice per group.

13. What adipose tissue was analyzed in this and the 11 month mouse study?

14. Figures 10 and 11 show results for 11 month old female mice while Figures 12-17 show results for 11 month old male mice. Why weren’t all of the studies done with both female and male mice? This is especially a problem because the 6 month data is only for female mice making it very hard to compare the 6 and 11 month data. At the least, these choices need to be justified by the authors. Also, the Figure legends for Figure 10, 11 and 12 should include the numbers of animals per group.

15. pg. 27, 1st paragraph, lines 4-5: What do the authors mean by “increased cell activation in the brain”? What specifically indicates this?

16. pg. 27, 2nd paragraph: Please carefully check the Figure numbers cited here.

17. pg. 31, line 8: The authors state in the text that Prkcg expression was induced by ISO but Fig. 13E shows the opposite. Please clarify?

18. In the experiment shown in Fig. 8 with 6 month old female mice, a graph showing latency to first entry (not clear into what) is the only data set from the Open Field Test that demonstrates a difference between WT and APP/PS1 mice. Why was that analysis not included in Fig. 14 with the 11 month old male mice? It should be.

19. In the experiments shown in Fig. 16, the legend says that the data is from 4-7 animals and in Fig. 17 the legend says 5-6 animals while the data for presumably the same animals tested in different assays and shown in Fig. 14 and 15 says that 6-8 animals were used. Why the difference in the number of animals used for the memory tests shown in Fig. 16 and 17. The authors need to explain this.

20. pg. 34, 1st paragraph, lines 2 and 5: The authors cannot conclude that these changes were reverted because they don’t know when they began. Prevented would be a better word choice.

21. Discussion, pg. 35-36: The evidence that ISO works in vivo by activating Nrf2 is not particularly strong, especially as the authors only look at one indicator of Nrf2 activation (HMOX1) in vivo which is also the target of other transcription factors. Thus, they need to tone down and trim the section on Nrf2 activation playing a key role in the effects of ISO in vivo. Until they test ISO in Nrf2 knockout mice, they cannot say for sure how important Nrf2 is in the in vivo actions of ISO.

22. pg. 36, 3rd paragraph, line 12-13: Here it is stated that ISO increased the weight of APP/PS1 mice but that is not

consistent with the data shown in Fig. 6 A and B and is also not consistent with the statement in the first paragraph of pg. 37. Please clarify.

23. pg. 38, 1st paragraph, lines 11-15: The authors first state here that lungs from ISO-treated as compared to PBS-treated mice show bronchiolar hyperplasia but then they say that the bronchiolar hyperplasia was also seen in the mice treated with PBS. Which is correct?

24. pg. 38, 2nd paragraph, lines 12-14: The authors cannot state that differences between WT and APP/PS1 mice are not gender-related as they did not directly compare the two genders of either the WT or APP/PS1 mice.

25. pg. 39, 2nd paragraph: This paragraph on the potential effects of ISO on BACE is much too speculative and not adequately supported by the data. Do the authors really think that 27% inhibition of BACE activity at 50 µM (Table 1) would be physiologically relevant in vivo?

26. pg. 40, last paragraph: This was already stated. There is no need to repeat this information .