Author's response to reviews

Title: The development of Response Surface Pathway Design to reduce animal numbers in toxicity studies

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Dear Editor,

Enclosed please find our revised manuscript entitled:

The development of Response Surface Pathway Design to reduce animal numbers in toxicity studies

The manuscript has been revised based on comments from the reviewers. We have addressed the reviewers’ comments by adding some new parts in the Results and the Discussion sections.

Our responses addressing the reviewers’ comments are attached.

On January 1st the Norwegian School of Veterinary Science merged with another institution in Norway. We have therefore changed the addresses of several of the authors.

Thank you for your consideration.

On behalf of the authors

With best regards

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Responses to Reviewer I

Major Compulsory Revisions

1. **Comment**: As written, the manuscript retrospectively applies the Response Surface Pathway design to two different LD50 experiments. While the data suggest that this design would prove helpful in reducing animal usage, there is no prospective data to demonstrate this point. Thus, the manuscript remains a proof of concept, but does not give the readers enough information to determine if the method will be useful.

**Response**: The AZA-1 study presented in the manuscript *(Page 14; line 9 – 20)* was performed with prospective 4-level optimized RSP design and has additionally been analysed as a 3-level RSP design *(Page 14; line 13 - 16)*. Additionally, two other studies using optimized 3-level RSP design have recently been performed and the manuscripts are under preparation *(Page 21; line 9 – 10)*.

Responses to Reviewer II

Major Compulsory Revisions

1. **Comment**: While the RSP design is of great interest and seems promising, the study would be greatly improved by inclusion of a prospective element. Without this, it's difficult to determine whether RSP is something that could be implemented in future toxicity studies, or if it is limited to retrospective analysis.

**Response**: The AZA-1 study was a prospective study, which applied optimised 4-level RSP design. At that time, we were not confident enough to conduct the study using the optimised 3-level RSP design. However based on the results obtained, and supported by the simulation
results based on Yessotoxin data, we found that, in the AZA-1 study, the fourth design level of the 9 mice was not needed, and 15 mice would have been sufficient. The data obtained from the AZA-1 study showed that the area of interest was detected on the third design level. If the LD<sub>50</sub> was estimated using only data up to the third design level, omitting the fourth design level, the LD<sub>50</sub> would be estimated to be 76.3 µg/kg BW and 70.7 µg/kg BW using isotonic regression and Sperman-Karber, respectively (Page 14; line 13 – 16). If the AZA-1 study had been performed using optimised 3-level RSP design, the k-adjustment factor would be 1.78 and the estimated LD<sub>50</sub> would not be different from that estimated using the optimised 4-level RSP design (Page 20; line 11 – 23). We have performed a simulation study using the AZA-1 in vivo study to estimate the probability of death at a given dose using logistic regression in a binominal distribution, where a total of 10,000 simulated mice samples were generated for each dose. The simulation study estimated the LD<sub>50</sub> of AZA-1 to be 76.3 (95% CI: 38.5 - 101.6) and 75.7 µg/kg BW (95% CI: 49.4 – 116.2) using isotonic regression and Spearman Karber, respectively. When all the three mice died at the first design level, the assigned dose at design level 2 would be 88µg/kg BW, where the simulated result on this dose resulted in the death of 3 of 5 mice. The assigned dose at the third design level would be 68, where at this dose 3 of 7 mice died in the simulation. The optimised 4-level RSP design has been applied to the AZA-1 LD<sub>50</sub> study. The results of the simulation indicated that the optimised 3-level RSP design estimated the LD<sub>50</sub> as well as all the optimised 4-level RSP design. The optimised 3-level RSP design has recently been applied in two other studies (Page 21; line 9 – 10). The manuscript presenting these results is under preparation and cannot been used as a reference in this paper.
The objective with this manuscript was to describe the development of RSP design, based mainly on simulations. This is also discussed as one of the limitations. Further studies and research are needed to confirm the performance of the RSP design (Page 21; line 10 – 11)