Author's response to reviews

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

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Author's response to reviews: see over
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 expanded the study by adding more simulations to assess the performance and effectiveness of the RSP design on its stage development in estimation of LD50. In details, the changes in the manuscript are as follows:

1. The abstract has been changed in order to accommodate the changes in the manuscript

2. The aims of the study were restated, in which one more aim was inserted. The new aims of the paper were to introduce and describe the development in optimization of the RSP design concept, to assess its performances and effectiveness in estimation of LD50 and to compare RSP with UDPs and RW design using simulations performed on data from an in vivo experiment (page x, line y)

3. The simulation procedure has been added as a new section, and the procedure of the simulation in the studies has been described further in details. (page x, line y)
4. The new results from the new simulations have been added in the manuscript. We inserted one sub-section in the result section to describe our obtained result. (page x, line y). One table was added, as part of the new results. We also improved figure 1, 2, and 3. We added 1 figure and figure 4 in the previous manuscript was changed become figure 5

5. The discussion in the manuscript has also been expanded, based on our new results.

6. We have also added statements addressing: Competing interest, Author’s contributions

Our responses addressing the reviewers’ comments are attached.

Thank you for your consideration.

On behalf of authors

With best regards

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Responses to Reviewer I Comments

Major Compulsory Revisions

1. **Comment**: The simulation study is not described in any detail. In order for a reader of the article to assess the validity of the simulations, within the context of the manuscript, there must be a clear and thorough description of the simulation.

   **Response**: The simulation procedures are now described in detail in a new section (page 10, line 16 – 24 , and page 11 line 1 - 10)

2. **Comment**: The first in vivo study used 36 male ICR mice with Yessotoxin, while the second in vivo study used 24 female NMRI mice, of unknown age and reproductive history, with Azaspiracid-1. In both cases, death within 24 hours was the study endpoint. There is no explanation, within the manuscript, of why such divergent populations were chosen. Further, using death as a study endpoint is inherently unjustified, as there are many endpoints the authors could have used rather than allowing the mice to unnecessarily suffer.

   **Response**: The two in-vivo studies in this paper are used to show the development of the RSP design in estimation of LD50.

   - The *in-vivo* study of Yessotoxin (YTX) in this paper used a data from published paper (ref. 19). The study aim was to compare the i.p. toxicity of YTX in three strains of mice (ICR (CD-1), Swiss (CFW-1) and NMRI ) of both genders, in order to elucidate whether these factors may explain the great variation in the reported acute toxicity of YTX. At that time the ‘‘index’’ toxin for the YTX group, is uncertain, and it is impossible to establish reliable TEFs for other YTX analogues. The YTX study
(ref.19) was performed using basic RSP design. A set of data with 36 male ICR mice was used to generate the simulation, and develop RSP design.

- The second *in-vivo* study in the manuscript is used to show the application of the RSP design with multinomial outcome in the real study. The aim of the AZA study was to validate the LD50 of AZA1 which was obtained on Japanese strain, where this strain is not commonly used in Europe. The NMRI mice are the standard strain which is used in alga toxins study at the NVH, and it has been used for more than 20 years.

When performing experimental study in our institution it is very common to order mice by weight rather than age. We assumed that the mice provided by the supplier had similar age. All the mice were ordered from the vendor Taconic in Denmark, as 14-16 grams in weight, with the age of about between 3-4 weeks; and the female mice are nulliparous.

Based on the OECD guideline; LD50 studies use death mice as an endpoint. These kinds of studies cause the animal die slowly with moribund pain. As we described in the introduction, the LD50 study is still needed in order to establish TEF and for this reason we proposed the RSP design for reducing the number of mice.

3. **Comment**: The authors have used only a single LD50 study to describe their new design.

   While a single study would be acceptable for a proof-of-concept manuscript, it does not give the reader sufficient data to determine the validity of the method.

   **Response**: The study has been expanded by assessing the performance of all development of RSP designs. The possible pathways were evaluating by performing some scenarios based on the mean of dead mice at assigned dose for 10,000 simulations. Three pathways are considered for simulation for 4-level RSP design for fixed sample size and incorporating k-
adjustment factor and 4-level RSP design with k-adjustment factor and optimal of number animals used. For the optimized 4-level RSP design with multinomial outcome, k-adjustment factor and increasing numbers of mice with increasing design level, eighteen pathways were created but only four is of substantial interest. When assessing the performance of optimised 3-level RSP design with multinomial outcome, k-adjustment factor and increasing numbers of mice with increasing design level, eight pathways were created. The new results are added section Performance of the RSP design (page 13, line 6 – 23 and page 14, line 1-2), and 1 new table (table 3) and figure (figure 4) are also added to show the obtained results. These results are also discussed in the discussion section: (page 16, line 3 – 8; page 18 line, 17 – 19; page 19, line 8 – 12)

Minor Essential Revisions

1. **Comment:** Throughout the manuscript, a number of statements leave the reader wanting a reference or a better description of how the authors came to the decisions that they did. For example, the second paragraph of the background describes the LD50 as needing a large number of animals, with a design that has been criticized for ethical and scientific reasons. There is no reference or context given. Reference and context give the reader a sense of scientific integrity to a statement versus hyperbole. This is but one of many such examples throughout the manuscript which need to be addressed.

**Response:** We inserted the references to support our statement.

- TEF studies of marine algal toxins are based on LD_{50} studies in mice (ref. no : 3)
• The classical LD50 design introduced by Trevan in 1927 requires the use of a large number of animals (ref no: 9) with a design has been criticised for ethical and scientific reasons (ref. no10 & 11: )

• Single animals are dosed until one of three criteria to stop the trial is met. (ref. no: 13)

2. **Comment:** On page 16, the term "waste of power" is used without any context. Please provide context or change the term.

   **Response:** This term was already changed

**Discretionary Revisions**

1. **Comment:** Please contemplate different formats for the tables and figures, which would allow them to be more readily understood by a reader.

   **Response:** Figure 1-3 shows how each design allocated dose for design level and how the pathways are formed using 3 different 4-level RSP designs. We refined the figure 1 and 2 and as the reviewer suggested we have added figure legends to get the figure more understandable.
Responses to Reviewer II Comments

Major Compulsory Revisions

1. **Comment**: The simulation procedure, which represents the crux of the study, is described in very little detail. It is impossible to determine the validity of this method or the data obtained from it based on the information provided in the manuscript.

**Response**: The study has been expanded by assessing the performance of all development of RSP designs. The possible pathways were evaluating by performing some scenarios based on the mean of dead mice at assigned dose for 10,000 simulations. Three pathways are considered for simulation for 4-level RSP design for fixed sample size and incorporating k-adjustment factor and 4-level RSP design with k-adjustment factor and optimal of number animals used. For the optimized 4-level RSP design with multinomial outcome, k-adjustment factor and increasing numbers of mice with increasing design level, eighteen pathways were created but only four is of substantial interest. When assessing the performance of optimised 3-level RSP design with multinomial outcome, k-adjustment factor and increasing numbers of mice with increasing design level, eight pathways were created. The new results are added section Performance of the RSP design (page 13, line 6 – 23 and page 14, line 1-2), and 1 new table (table 3) and figure (figure 4) are also added to show the obtained results. These results are also discussed in the discussion section: (page 16, line 3 – 8; page 18 line, 17 – 19; page 19, line 8 – 12)
2. **Comment:** Yessotoxin and Azaspiracid-1 in vivo studies were performed in mice of different genotypes and genders. Moreover, the two studies used different numbers of animals, and each study divided animals into groups different. No explanation was offered for these differences, or how they might affect study outcome. There is no indication as to the age of the animals or whether animals in each study were approximately the same age.

**Response:** These two in-vivo studies were performed in different settings to show the development of the RSP design in estimation of LD50 in real situation.

- The in-vivo study of Yessotoxin (YTX) in this paper used a data from published paper (ref. 19). The study aim was to compare the i.p. toxicity of YTX in three strains of mice (ICR (CD-1), Swiss (CFW-1) and NMRI ) of both genders, in order to elucidate whether these factors may explain the great variation in the reported acute toxicity of YTX. At that time the ‘‘index’’ toxin for the YTX group, is uncertain, and it is impossible to establish reliable TEFs for other YTX analogues. The YTX study (ref.21) was performed using basic RSP design. A set of data with 36 male ICR mice was used to generate the simulation, and develop RSP design.

- The second in-vivo study in the manuscript is used to show the application of the RSP design with multinomial outcome in the real study. The aim of the AZA study was to validate the LD50 of AZA1 which was obtained on Japanese strain, where this strain is not commonly used in Europe. The NMRI mice are the standard strain which is used in alga toxins study at the NVH, and it has been used for more than 20 years. The in-vivo YTX study was performed using 4-level Basic RSP design with 36 mice. The design did not describe in detail the rationale behind the dose steps and did not consider the dose window which is already known within 100 and 700 µg/kg BW. Based on this study,
we refined the design by establishing the k-adjustment factor, reducing the number of animals used and incorporating the multinomial outcome as a decision variable. From the simulation study that aims to assess the performance of the RSP design on its stage development using YTX in-vivo data, it is shown that the optimised RSP design (applying k-adjustment factor and multinomial outcome as a decision variable) can estimate LD 50 as good as the basic RSP with lesser number of animals.

The AZA-1 study showed that the optimised RSP design can estimate LD50 in the real study.

When performing experimental study in our institution it is very common to order mice by weight rather than age. We assumed that the mice provided by the supplier had similar age. All the mice were ordered from the vendor Taconic in Denmark, as 14-16 grams in weight, with the age of about between 3-4 weeks; and the female mice are nulliparous

**Minor Essential Revisions**

1. **Comment:** Revise figures to make them more readily understandable and easy to follow, and provide more detail in the figure legends.

   **Response:** Figure 1-3 shows how each design allocated dose for design level and how the pathways are formed using 3 different 4-level RSP designs. We refined the figure 1 and 2 and as the reviewer suggested we have added figure legends to get the figure more understandable.

2. **Comment:** There are a number of spelling and grammatical mistakes throughout the manuscript.

   **Response:** We have corrected some spelling and grammatical errors.
3. **Comment:** In the Background, paragraph 3, it is stated that the UDP is halted when one of three criteria is met. Authors should elaborate on what these criteria are (at least provide a reference), since they later state that their test was halted when one criterion was met.

**Response:** The reference for this statement has been added.