Comment

Comment on "Alternative acute oral toxicity assessment under REACH based on sub-acute toxicity values"

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The research article by Gissi et al. (2017) comprises three scientific flaws:

1. A correlation between acute and subacute oral toxicity data does not *per se* serve the 3R principle. Only in rare cases do subacute oral toxicity data but no acute oral toxicity data exist.

We discussed this in Buesen et al. (2016), a publication that Gissi et al. disregarded. Gissi and co-authors from the European Chemical Agency (ECHA) contend that low acute oral toxicity can be predicted from low toxicity in oral subacute toxicity studies, and they highlight:

> *"According to the REACH Regulation, this approach for predicting acute oral toxicity needs to be considered as part of a weight of evidence analysis."*

Gissi et al. estimate that registrants of about 550 substances will be able to omit the *in vivo* acute oral toxicity study by using this adaptation when submitting dossiers for the 2018 REACH registration deadline. They base their estimation on an analysis of the REACH database where they "*found suitable studies on both acute oral and sub-acute oral toxicities for 1,256 substances. 415 of these substances had low toxicity in the subacute toxicity study (i.e., NO(A)EL at or above the limit test threshold of 1,000 mg/kg). For 98% of these substances, low acute oral toxicity was also reported (i.e., LD_{50} above the classification threshold of 2,000 mg/kg)*."

This line of argument is factually flawed. While the estimation summarized above shows that low toxicity in subacute oral toxicity studies can be used retrospectively to correlate low acute toxicity, it does not provide evidence that such a correlation serves the 3Rs principle. To determine whether it is actually useful to reduce acute oral toxicity testing, it is not only relevant to consider the correlation itself (by comparing the study outcomes for substances for which both studies are available), but also to determine the number of substances for which subacute oral toxicity data are available, but acute oral toxicity data are not available.

Gissi et al. did not include this evaluation. Instead, they write:

> *"Concerning the last registration deadline in 2018, […] from a forecast number of 5,200 substances […], approximately 35% will require the generation of information for the acute oral toxicity endpoint... In addition, if the newly registered substances show a distribution of toxicity values comparable to the substances already registered and subject to the analysis presented in this paper, approximately 30% of the 5,200 substances may have a 28-day oral NO(A)EL indicating low acute toxicity, and consequently a predicted acute oral LD_{50} higher than 2,000 mg/kg bw/day."*

The forecast number of 5,200 substances without acute toxicity data is irrelevant because it does not take into account the proportion of these 5,200 substances for which subacute oral toxicity data are already available. In fact, it would be a rare case that subacute, but no acute toxicity data existed. Rather, repeated-dose toxicity studies regularly use the data from the acute study to determine the dose-range finding study for the subacute test. If data on acute toxicity are unavailable, the dose range for the range finding study has to be guessed and will frequently lead to more animals used in the dose-range finding study. This may result in no actual reduction of the total number of animals.

We have further explored this in Buesen et al. (2016):

> *"In practice, only very few substances exist for which a 28-day oral toxicity study, or any other repeated-dose oral toxicity study, is available, but not an acute oral toxicity study. On the contrary, if repeated dose toxicity studies are required in a given regulatory context, data from the acute study are generally useful to determine the dose for the range finding study for the subacute*

test (unless, e.g., physico-chemical substance properties strongly indicate non-bioavailability). If data on acute toxicity are unavailable, the dose range for the range finding study (that is generally conducted as 14-day study in the authors' laboratories) for the regulatorily required repeated-dose study has to be guessed. This implies a risk of overdosing in which case many or all animals submitted to the range finding study would experience unnecessary distress, or even die. Similarly, if neither an acute oral toxicity study, nor a range finding study is available, there is a risk of overdosing in the repeated-dose oral toxicity study which may again result in unnecessary distress or even death of the test animals."

2. The recommendations formulated by Gissi et al. contradict current ECHA Guidance.

The scenario from Buesen et al. (2016), presented above, is confirmed in the ECHA *Guidance on Information Requirements and Chemical Safety Assessment* (ECHA, 2016), which Gissi et al. cite with respect to the evaluation of subacute oral toxicity data. Regarding dose-range finding studies, Section 2.3.1 of the ECHA Guidance states:

> *"Before a novel sub-acute oral toxicity study (OECD TG 407 or OECD TG 422) is conducted, appropriate doses must be identified. For this purpose, the registrant should use existing data (e.g. screening studies,* ***acute toxicity studies****, literature data) and relevant results from validated* in vitro *tests, and only if all those data are insufficient will he need to perform one or more dose-range-finding studie(s)" (emphasis added).*

Hence, while Gissi et al. require that registrants should use data from oral subacute toxicity studies to predict (low) acute oral toxicity, the current ECHA Guidance (ECHA, 2016) requires considering data from acute toxicity studies to determine the appropriate dose for subacute toxicity studies.

3. The additional request of Gissi et al. to use results from the Neutral Red Uptake (NRU) cytotoxicity test to support the evidence for low acute oral toxicity is rebutted by scientific evidence.

Gissi et al. write that data from the NRU cytotoxicity test should primarily be used to correctly identify and classify substances of low toxicity (i.e., those which are not to be classified for acute toxicity). The grounds for this request are rebutted by Schrage et al. (2011), a study that Gissi et al. disregarded: Evaluating a total of 203 substances, only 8% of the 79 substances with an LD_{50} > 2000 mg/kg body weight were correctly predicted for "non-classification" applying NRU cytotoxicity test data (Schrage et al., 2011). To the best of our knowledge, Schrage et al. (2011) is the most comprehensive published study addressing the applicability of the NRU cytotoxicity test during practical, routine toxicity testing for regulatory purposes.

Moreover, Section R.7.4.4.1.2 of the current ECHA Guidance (ECHA, 2016) gives the impression that data from the NRU cytotoxicity test could be used to determine starting doses for *in vivo* studies:

> *"The NRU cytotoxicity assay […] may provide supplementary information, which may be used e.g. to determine starting doses for* in vivo *studies (OECD, 2010; Schrage et al., 2011), and to assist in the evaluation of data from animal studies."*

This citation of Schrage et al. (2011) is distorted. Schrage et al. showed that it is detrimental to the 3Rs principle to use data from the NRU cytotoxicity test to determine starting doses for *in vivo* studies. As recently summarized by Buesen et al. (2016), Schrage et al. (2011) revealed a low overall concordance of 35% when comparing the starting doses predicted in the NRU cytotoxicity test to data from rat acute oral toxicity studies:

> *"Of the [87] substances for which Good Laboratory Practice (GLP)-compliant studies were available, a default starting dose of 2000 mg/kg bw would have resulted in the lowest possible animal numbers per test (average 6.9). Selecting the starting dose by expert judgement would have resulted in slightly higher animal use (average 7.3), whereas a prediction of the starting dose based upon the* in vitro *data would have resulted in a considerably higher average of 9.1 animals per test. Hence,* in vitro *data-based starting dose estimations resulted in an approx. 32% increase of animal numbers as compared to a default starting dose of 2000 mg/kg bw."*

Similarly, regarding the statement from the ECHA Guidance (2016) that "*relevant results from validated* in vitro *tests*" should be used to determine appropriate doses for subacute oral toxicity studies, we are unaware of scientific evidence indicating that this could be done.

Finally, irrespective of their scientific meaningfulness, proposals to apply either *in vitro* data or data from repeated-dose toxicity studies for regulatory classification or non-classification cannot be put into practice without a defined and validated testing strategy combined with a predefined data interpretation procedure. Even in the unlikely event that 28-day oral toxicity data are available for a given substance (and further indicate low toxicity) and the acute oral toxicity data are unavailable, the repeated dose data-based prediction can still not be applied in a regulatory setting: We are unaware of definite guidance from the ECHA that specifies how correlations between the *in vitro*, acute toxicity, and repeated dose toxicity data should be established or which specific correlations would be acceptable for regulatory classification or non-classification. Moreover, just

like for any other 3R approach, acceptance in the European Union will not preclude full animal testing from being requested in other regions of the world.

In conclusion, while we agree that 3Rs methods are needed for regulatory toxicity testing, it does not serve the cause to publish studies that give the impression to serve to replace, reduce and refine animal testing, but that do not stand up to scrutiny. Instead, scientific evidence and practicability should govern 3Rs activities.

References

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Reply to Comment on "Alternative acute oral toxicity assessment under REACH based on sub-acute toxicity values"

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First, we would like to point out that the purpose of our article (Gissi et al., 2017) is to clarify how a regulatory requirement can be adapted. In the REACH Regulation, there are many situations where the legal text formally mentions that an existing long-term test makes a short-term test unnecessary (e.g., subchronic studies can replace sub-acute studies, long-term aquatic toxicity can also cover short-term requirements), but nothing is mentioned for acute toxicity. With the analysis we presented, we have formalized how this could also be the case for acute toxicity. However, since the update of REACH legal text is a long process, the way to make such adaptations available to registrants before the 2018 REACH deadline was the update of ECHA's REACH Guidance text (ECHA, 2016) based on the results described in this paper.

1. The estimates of the number of cases for which an acute toxicity study may not be available for the incoming REACH registrations are given in our article and are based on statistics derived from all registration data hosted by ECHA. We agree that the cases for which an acute toxicity study is missing are not very common. However, because of the high number of new registrations in 2018, even a relatively small proportion of those (~10%, as explained in our article) would matter. Hence, to omit about 550 unnecessary animal tests with no scientific and regulatory value is worth exploring and communicating to the registrants. Furthermore, we ran a new analysis and found that about 24% of the substances that have a reliable and relevant sub-acute study provided, do not have an equivalently reliable and relevant acute oral toxicity study. For those substances, registrants have used weight of evidence (WoE) or other adaptations to cover the information requirement. With the update of ECHA's Guidance based on the results described in the article, registrants have one more possibility to use this adaptation.

2. It seems that Buesen et al. may have misunderstood the text in the Guidance and the article with regard to the testing strategy/sequence. ECHA Guidance does not suggest that an acute toxicity study has to be available before a sub-acute toxicity study