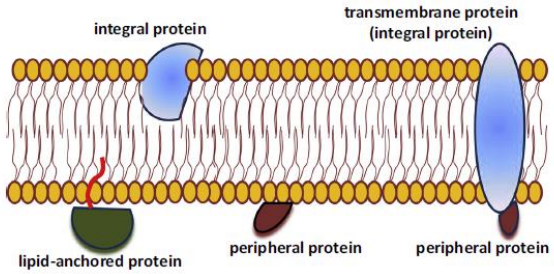
The Toxicity Assessment of Newly Expressed Proteins (NEPs): Intractable Proteins Challenging Current Practices

Ad hoc **meeting EFSA – GMO applicants**

16 November 2022

Intractable Proteins

- **Intractable proteins have properties that make it extremely difficult or impossible to:**
  - Express in heterologous systems
  - **Quantify (due to low expression levels)**
  - Isolate, concentrate, or purify from **either heterologous expression systems or the GM plant**
  - Demonstrate functionality of the isolated protein
  - Prove equivalency of the heterologously produced protein with the plant-expressed protein
- **Taken together, these technical difficulties combine to provide scientific justification for why the 28-day toxicity study at the limit dose is not feasible.**



Membrane proteins are an example of intractable proteins. Bushey *et al.*, 2014

Testing of NEPs (Regulation (EU) No 503/2013)

1.4.1. *Testing of newly expressed proteins*

The applicant shall provide an evaluation of all newly expressed proteins. The studies required to investigate the potential toxicity of a newly expressed protein shall be selected on a case-by-case basis, depending on the knowledge available with respect to the protein's source, function or activity and history of human or animal consumption. As regards proteins expressed in the genetically modified plant, in the case where the history of safe use for consumption as food and/or feed of both the plant and the newly expressed proteins is duly documented, specific toxicity testing as provided for in this Section shall not be required. In such case, the applicant shall provide the necessary information regarding the history of safe use of the proteins.

- **The 28-day toxicity study is triggered in the case where the history of safe use is not "duly documented".**

Weight of Evidence (WOE) Approach

- The WOE approach for safety assessment should include all the available information to support hazard identification and the history of safe use (HOSU) evaluation (*see* next slide).

- "The WOE approach is critical, as in a vast majority of cases no single assay or biochemical characteristic can identify a protein as a hazard. A stepwise approach is recommended to evaluate the safety of [proteins] taking the totality of information into account" (Roper *et al.*, 2021).

WOE approach is particularly important for intractable proteins

Hazard identification

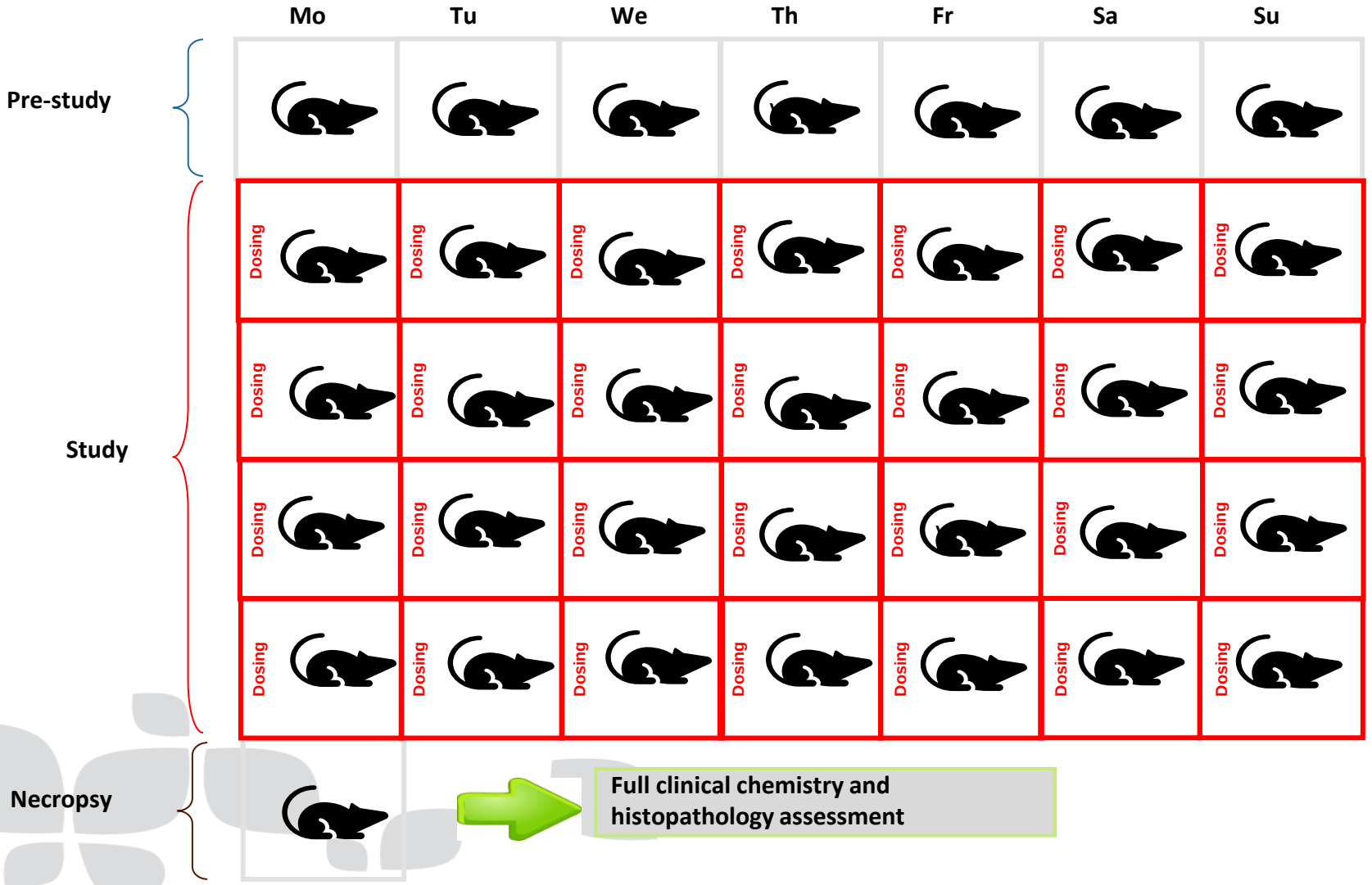
- **Requires little or no protein**
  - Protein's source, function or activity
  - Evidence of Exposure or Consumption
  - Bioinformatics
  - Resistance to Digestion *In Vitro*
  - Stability to Processing Conditions
  - Expression Level and Dietary Intake
- **If no hazard is identified using these *in silico* and *in vitro* studies, then additional hazard characterization studies are not needed to conclude on the safety of the protein or on the HOSU.**

Hazard characterization

- **Requires large quantities of protein and use of animals**
- **Determined on a case-by-case basis and might include one or more of the following:**
  - Acute Oral Toxicity
  - Repeated Dose Toxicity
  - Hypothesis-based Studies

Delaney *et al.*, 2008
Hammond *et al.*, 2013

A 28-day toxicity study requires large quantities of protein and animals



A 28-day toxicity study does not provide additional value to the protein safety assessment

- "If the NEP is related to a family of proteins that has a history of safe use based on bioinformatics and literature review, and is not homologous to known protein toxins, then any supplementary toxicology study is not necessary" (Brune *et al*., 2021).

- To date, 28-day toxicity studies have not provided additional information on the protein safety assessment, but instead have only confirmed existing data.
  - *See* slide regarding 3R legislation

- Defaulting to a toxicology study is, therefore, not science based and is inconsistent with the WOE approach outlined for the safety assessment of NEPs and with scientific assessments by other Regulatory Authorities.

Protein Requirements

- OECD TG 407 requires 5 animals/sex/group for testing of **chemicals.**
  - EFSA requires 10 animals/sex/group for testing **NEPs**.
  - However, to assess all required endpoints in mice, 12-20 animals/sex/group may be needed.
- At the limit dose, 50-70 g of protein (purity corrected) are required.
- For intractable NEPs, production of this quantity of protein may not be technically feasible either from heterologous expressions systems or the GM plant.

Consideration of the 3R Legislation

- **DIRECTIVE 2010/63/EU** (protection of animals used for scientific purposes) requires "an evaluation of the objectives of the project, the predicted scientific benefits or educational value"

- "Defaulting to *in vivo* toxicology studies, as is often required for regulatory approvals, does not reflect ethical use of animals in scientific research and testing as outlined by the 3R's of responsible animal use" (Roper *et al.*, 2021).
  - On average, CLI member companies have performed eleven 28-day toxicity studies for EFSA.
  - On average, 120 animals were used per 28-day toxicity study.

- **None of these studies have identified a hazard for the NEP.**

Factors Impacting Dose Level

- **Humane treatment of test animals requires limits on the dose volume and number of administrations.**
  - Dose level (mg/kg bw) = dose concentration (mg/mL) x dose volume (mL/kg bw)
  - Recommended maximum dose volumes:
    - 20 mL/kg bw (aqueous solutions), OECD TG 407
    - A volume of 0.6 mL to a 30 g mouse is equivalent to 1.4 L to a 70 kg human.
  - Number of administrations:
    - A single dose should be administered (oral gavage), OECD TG 407.
  - Difficulties with repeated administration of large volumes:
    - Stresses animals
    - Increases the chances of spurious findings

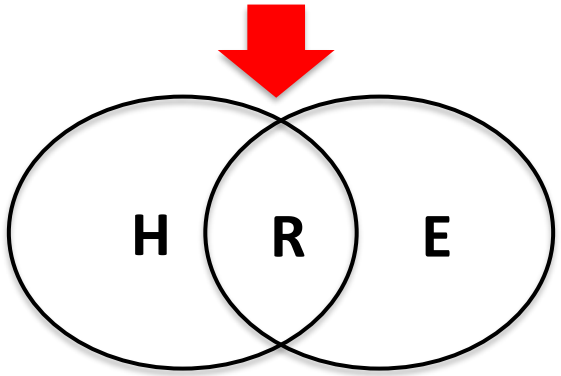
Vs.

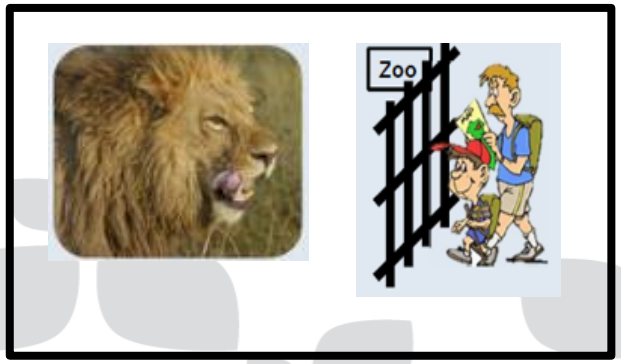
Limit Dose is Not Scientifically Justified for Proteins

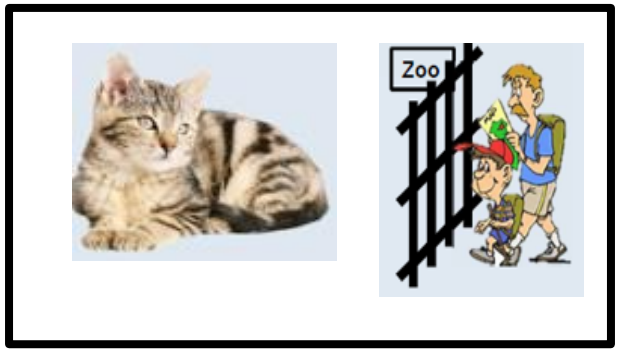
- EFSA has required in previously evaluated applications that the test should be conducted at the limit dose of 1000 mg/kg/day to comply with the OECD TG 407 for testing of **chemicals** when “no effects would be expected”.
- Results in testing doses that are many thousands to hundreds of thousands of times in excess of realistic human and animal exposure
  - Equivalent to corn consumption of 2 000 kg to 1 300 000 kg for 70 kg human, assuming a range of high to low protein concentration values
- Unnecessary, irrelevant to risk assessment, and unjustifiable based on scientific or animal welfare considerations

Risk = Hazard x Exposure

For most intractable proteins, a very low level of expression (exposure) is combined with the lack of an identified hazard.







Summary

- **Without an identified hazard to test, the conduct of a 28-day toxicity study cannot be scientifically justified.**
  - Such a study would only produce additional confirmatory safety data that would add to the dataset already available, which is not a valid justification for the use of animals.

- **In the case that toxicity testing is needed (if a hazard has been identified), testing at the limit dose is not scientifically valid.**
  - For most intractable proteins, a 28-day toxicity study at the limit dose will not be technically feasible.

How do we demonstrate the safety of proteins, particularly for intractable proteins where the conventional safety assessment is difficult or impossible to perform?

Options for Consideration (1)

Consider WOE approach

- The WOE approach for safety assessment should include **all** the available information to support the hazard identification and HOSU evaluation.

- If no hazard is identified using these *in silico* and *in vitro* studies, then additional hazard characterization studies are not needed to conclude on the safety of the protein.

Options for Consideration (2)

Consider additional *in silico* tools and predictions

- No protein required
- For example, bioinformatic framework to assess protein toxicity

Consider *in vitro* toxicity testing

- Still requires some amount of protein
- “Should continue to be investigated and remains of considerable interest” (Bushey *et al.*, 2014)
- Proof of concept publications (2016-2019) using human intestinal epithelial cell line monolayers
  - Respond differently to hazardous and non-hazardous proteins
  - Role of digestive enzymes can be useful
  - No obvious advantage to using primary monolayers
  - May be useful for intractable proteins
  - Single exposure sufficient
- **Would require further development, validation, and acceptance by Regulatory Authorities (long-term solution)**

Options for Consideration (3)

If the need for a toxicity study is determined (when a hazard is identified using *in silico* and *in vitro* studies), Consider an exposure-based approach to dose selection instead of the limit dose

- Use GM protein concentration and human and/or animal dietary consumption levels to establish relevant doses to be tested

Intractable proteins play a role in sustainability

"The potential benefits of intractable proteins include a broad range of valuable traits such as disease resistance, drought tolerance, nitrogen use efficiency, and enhanced nutrient value" (Bushey *et al*., 2014).

"Many newer GM crops will express different types of intractable proteins to withstand drought, to enhance utilization of nitrogen, or to alter their composition for nutritional purposes" (Delaney, 2017).

Thank you

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References

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