# Nitrosamines: Regulatory *In Vivo* Mutagenicity Study Workshop 10-11 July 2024

**Background**

Approaches to the safety assessment of nitrosamines have been developed by the international regulators with the objective of aligning acceptable intakes (AI)s. Initially most of the nitrosamines reported to be present in medicinal products were small nitrosamines. For many of these nitrosamines, sufficient substance specific animal carcinogenicity data were available to support the establishment of the AI. However, Nitrosamine drug substance-related impurities (NDSRIs) were increasingly found and now make up the vast majority of nitrosamines reported in human medicinal products. As robust carcinogenicity data are not available for NDSRIs, the lack of suitable surrogates for the read-across approach posed challenges to the assignment of AIs for these substances. Consequently, in July 2023, international regulators introduced the carcinogenic potency categorisation approach (CPCA) and the enhanced Ames test (EAT) as new approaches for the AI assessment of new nitrosamines.

The CPCA is a structure-activity relationship-based method that allows the rapid assignment of a nitrosamine to 5 categories, each with a corresponding AI limit between 18 and 1500 ng/day, reflecting predicted carcinogenic potency. The EAT defines the conditions which need to be satisfied for acceptance of negative tests to allow control of nitrosamines at a level decided by the Regulatory Authorities (RA).

For most nitrosamines, the AI limit using CPCA and EAT is achievable. If a higher AI is needed and a suitable surrogate for read-across is not available, a negative result in a relevant well-conducted in vivo mutagenicity study can overrule the CPCA and EAT data to allow control of the N-nitrosamine as a non-mutagenic impurity (NMI) for some RAs.

The current gold standard for detecting mutations in nearly all tissue types in vivo is the transgenic rodent (TGR) gene mutation assay performed according to OECD 488 guidelines. However, there are capacity constraints for the conduct of TGR assays due the limited availability of the transgenic animals and test facilities able to competently conduct the studies.

Error-corrected next-generation sequencing (ecNGS) offers the possibility of measuring mutations in any tissue of non-transgenic animals at multiple genomic locations. Some data have been received from TGR studies in which the same tissues were also analysed by ecNGS. Although very few in number, these studies seemed to show much higher sensitivity and lower variability for ecNGS including one study where for the same tissues analysed the TGR analysis was negative but ecNGS analysis was positive. One standalone negative ecNGS study has been received at EMA. While Duplex Sequencing is a promising technology, there are concerns regarding its use for setting control options as it is still being optimized and no OECD guideline is available.

Independent of the question of whether the TGR or ecNGS is the most appropriate study, is the selection of tissues to be sampled and analysed. Nearly all studies received have adopted the proposal in OECD 488 that *In the absence of background information and taking into consideration the site of contact due to route of administration, the liver and at least one rapidly dividing tissue (e.g., glandular stomach or duodenum, or bone marrow) should be evaluated for mutagenicity*. However, bearing in mind the tissue specific occurrence and potency of tumours induced by many nitrosamines, particularly in the

oesophagus and lung, analysis of only liver, stomach/duodenum and bone marrow may not always be appropriate.

Regulators are receiving an increasing number of in vivo mutagenicity studies. Pharma companies are also conducting studies on (mostly) small nitrosamines with robust carcinogenicity data to evaluate concordance between mutagenicity and carcinogenicity with the aim of using this approach to establish AIs for NDSRIs from the in vivo mutagenicity data. At EMA, from the reports received, all nitrosamines which have tested positive in the EAT and subsequently been tested in an in vivo study, have also tested positive in the in vivo study. Based on the data obtained, most companies then propose an AI based on the NOEL or benchmark dose (BMD) and relative potency compared to small nitrosamines such as NMDA and NMEA. However, ICHM7 Q&A 7.2. states that *in vivo gene mutation assays alone are currently not validated to directly assess cancer risk because the endpoint is mutation and not carcinogenicity (i.e., they are used for hazard identification).*

**Issues to address:**

- What is the preferred methodology to assess mutagenicity: either the current OECD 488 TGR or ecNGS.?
    - Conduct of the in vivo mutagenicity study [methodology: TGR (OECD guideline available) versus ecNGS (no OECD guideline available)],
    - Is incorporation of ecNGS into an OECD guideline needed before it can be used to derive regulatory limits for pharmaceutical impurities?
    - Selection of tissues for analysis
- How can we get to the point where we can use in vivo mutagenicity data to set a regulatory limit for pharmaceutical impurities
    - Mutagenicity as an endpoint versus carcinogenicity (HESI data)
    - Quantitative assessment of the in vivo mutagenicity data (e.g., Point of Departure?, BMD – which software, application of uncertainty factors [which factors]).
    - What are the data gaps precluding the use of in vivo mutagenicity studies in setting control limits?

**Final Agenda**

**D1 10 July 2024: 4h (Regulators + Academia)**
**06:00-10:00 EDT**
**07:00-11:00 Brazil**
**12:00-16:00 CEST**
**18:00-22:00 Singapore**
**19:00-23:00 JST**
**20:00-24:00 AEDT**
**Chair Rhys Whomsley (EMA) D1 and Leon van Aerts (MEB) D2**

| Agenda D1 In Vivo Mutagenicity Workshop | | |
|---|---|---|
| 12:00-12:15 | Welcome and introduction: | Rhys Whomsley/Leon van Aerts: |
| Nitrosamines In vivo mutagenicity and carcinogenicity: | | |
| 12:15-12:35 | Comparison of in vivo mutagenicity and carcinogenicity for nitrosamines: | Tim McGovern |
| 12:35-12:50 | Organ specific carcinogenicity: | Rhys Whomsley |
| In vivo mutagenicity studies: | | |
| 12:50-13:10 | Principles and use in regulatory decision making with focus on TGR: | Roland Froetschl |
| 13:10-13:40 | ecNGS: | Carole Yauk |
| 13:40-14:10 | Advantages/disadvantages of TGR/ecNGS: | Francesco Marchetti |
| 14:10-14:20 Break | | |
| 14:20-15:00 | Recommendations for conduct of TGR/ecNGS studies:<br>• Study design<br>• Organ sampling<br>• Internal Positive controls<br>• Toxicokinetics<br>• Statistical/biological significance<br>• Acceptability criteria | Moderator Roland Froetschl |
| Positive studies; Quantitative framework in establishing AI values | | |
| 15:00-15:25 | Benchmark dose-based approach: | Paul White |
| 15:25-15:50 | Nitrosamine Comparators from external studies: | George Johnson |
| 15:50-16:00 | Wrap-up D1: | Rhys Whomsley |

**D2 11 July 2024: 3h (Pharmaceutical regulators only)**
**06:00-09:00 EDT**
**07:00-10:00 Brazil**
**12:00-15:00 CEST**
**18:00-21:00 Singapore**
**19:00-22:00 JST**
**20:00-23:00 AEDT**

| Agenda D2 In Vivo Mutagenicity Workshop | | |
|---|---|---|
| 12:00-13:00 | Harmonisation of control options<br>• Negative studies<br>• Positive studies<br>• Read across for similar substances e.g. quinapril and lisinopril | Leon van Aerts: |
| 13:00-13:20 | Ongoing activities:<br>• In vivo Ames follow up HESI group<br>• Comparison of in vitro/in vivo mutagenicity testing for NDSRIs for hazard identification<br>• Less-than-lifetime literature analysis and in vivo mutagenicity analysis<br>• Comet prediction to carcinogenicity outcomes for hazard identification | Tim McGovern |
| 13:20-13:50 | Data Gaps<br>• What further data do regulators require?<br>• Nitrosamines prioritised for in vivo testing | Rhys Whomsley |
| Break 13:50-14:00 | | |
| 14:00-14:50 | Data sharing:<br>• Nitrosamines for which In vivo mutagenicity studies received<br>• What data can be shared if reports cannot be shared (template for minimum information required) | Ciska van Doesum |
| 14:40-15:00 | Wrap up | Rhys Whomsley/Leon van Aerts |