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An integrated *in vivo* genotoxicity framework for potency assessment of nitrosamines

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Since their first identification as pharmaceutical impurities in 2018, nitrosamines have represented one of the most critical challenges in pharmaceutical quality and regulatory science due to their high mutagenic and carcinogenic potential and the resulting extremely restrictive acceptable intake limits. For nitrosamine drug substance–related impurities, acceptable intake limits are often constrained by the absence of compound-specific carcinogenicity data, leading to the reliance on conservative default values. To address this gap, an integrated *in vivo* genotoxicity assessment framework has been designed to generate sensitive and regulatory-relevant genotoxicity data within a single repeated-dose study design, while also providing mechanistic insight. This approach combines the erythrocyte Pig-a gene mutation assay for the detection of somatic mutations, the alkaline Comet assay for the assessment of primary DNA damage across multiple target tissues, and high-sensitivity error-corrected next-generation sequencing to detect ultra-rare mutations and characterize mutational spectra.

The integrated application of these complementary endpoints within the same cohort of animals enables the generation of high-resolution, tissue-specific mutational profiles while maximizing scientific output in accordance with 3Rs principles. The framework is designed to be readily compatible with regulatory safety assessment workflows and to support benchmark dose modelling approaches aimed at defining compound-specific acceptable intake limits.

Overall, the proposed testing strategy provides a structured approach within the regulatory framework to support the risk assessment of nitrosamine drug substance–related impurities lacking carcinogenicity data, addressing a critical gap in current regulatory practice without the need for dedicated carcinogenicity studies.