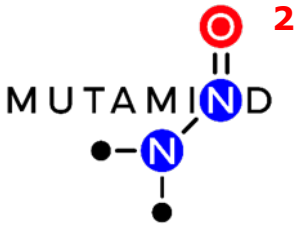
Refinement of enhanced Ames test conditions for nitrosamines



Executive Summary:

The project addressed key protocol uncertainties of the Enhanced Ames Test (EAT) for assessing the mutagenicity of small-molecule N-nitrosamines and nitrosamine drug substance-related impurities (NDSRIs): the choice between induced and non-induced hamster S9, the most appropriate bacterial tester strains, the influence of DMSO solvent levels, the performance of plate incorporation vs. preincubation formats, and the selection of NDSRI-specific positive controls. The metabolic activation of selected NDSRIs by CYP450 enzymes was also investigated.

Approach

- A literature review compiled Ames test data from 100 studies covering 27 NDSRIs. After applying defined positive-response thresholds, 19 data-rich NDSRIs with positive results under hamster S9 conditions were identified as candidate positive controls.
- Six NDSRIs were selected for detailed evaluation: N-nitroso-rasagiline, N-nitroso-duloxetine, N-nitroso-varenicline, N-nitroso-trimetazidine, N-nitroso-desmethyl-diphenhydramine, and N-nitroso-propranolol.
- CYP450-mediated metabolism was studied using induced hamster S9 fractions and recombinant human CYP450 enzymes, with LC-MS/MS-based metabolite identification and confirmatory inhibition experiments.
- Ames tests were performed with the standard strain panel, comparing induced vs. non-induced hamster S9, plate incorporation vs. preincubation, and different DMSO concentrations. BMD modelling was used for quantitative sensitivity ranking and to account for unspecific toxicity.

Key Findings:

NDSRI metabolic activation is CYP450-dependent but compound-specific. Several human CYP isoforms are involved, including CYP1A1, 2A6, 2B6, 2C8, 2C9, 2C19, 2E1 and 3A4. N-nitroso-varenicline was not proficiently metabolised by the tested enzymes, indicating that some NDSRIs may require other metabolic pathways.

The three tester strains TA1535, TA100 and E. coli WP2 uvrA were the most broadly responsive strains for detecting NDSRI mutagenicity. TA98 and TA1537 may add value for certain NDSRIs and can help interpret equivocal results.

DMSO concentration influenced both NDSRI metabolism and assay sensitivity; solvent levels therefore need to be carefully controlled and standardised.

The comparison of induced vs. non-induced hamster S9, and of plate incorporation vs. preincubation, provided the basis for defining the recommended EAT conditions.

Strain-specific NDSRI positive controls are proposed, based on reproducible positive responses and adequate fold changes under hamster S9 activating conditions.

Recommendations:

- S9 Mix: Induced hamster S9 is the most sensitive condition for Ames testing of NDSRIs
- Strains: TA1535, TA100 and E. coli WP2 are the most sensitive strains
- DMSO: DMSO levels should be kept below 5%. Although concentrations up to 14.29 % did not show consistent effects on positive control response, this cannot be extrapolated to all nitrosamines, and the use of high DMSO levels has resulted in negative tests for known carcinogenic small nitrosamines
- Both plate incorporation and preincubation protocols can be used and provide similar data, although the plate incorporation may be preferable for highly aqueous insoluble nitrosamines
- Positive control(s): N-nitrosotrimetazidine and N-nitrosorasagilene may be suitable positive controls for the three most sensitive tester strains and TA98 (N-nitrosorasagilene only).