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In vitro mutagenicity methodology for nitrosamines

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Abstract

The SC02 project "In vitro mutagenicity methodology for nitrosamines" explored and compared different test conditions for genotoxicity assessment of NAs, using the Ames and the *in vitro* alkaline comet assays, in order to develop an optimized testing strategy to reliably detect the mutagenic properties of nitrosamines (NAs).

Central to the project was the optimization of the Ames test, the testing of structurally diverse NAs under the optimized conditions and the evaluation of a selected subset (10) of NAs in the *in vitro* alkaline comet assay. It was also important to investigate the metabolic capacity of different *in vitro* hepatocyte models, as metabolic activation of NAs by specific Cytochrome P450 (CYP) enzymes is a key event in their mode of action. Ten structurally diverse NAs (including small NAs and nitrosamine drug-substance-related impurities (NDSRIs), differing in their known metabolic activation and thus in their mutagenic and carcinogenic properties, were selected for comparison in the optimized Ames test, the Ames fluctuation assay and the *in vitro* comet assay with liver models.

The impact of solvents and of different types (rat and hamster) and concentrations of S9 homogenates on the mutagenic response in a panel of three tester strains of the Ames test was analyzed. Furthermore, the induction of DNA strand breaks was investigated in four different liver cell models, including precision cut human liver slices (PCLiS), primary human and rat hepatocytes as well as human HepG2 cells using the *in vitro* alkaline comet assay.

Testing the metabolic competence of the human and rodent cell types used as well as of the S9 homogenates from rat and hamster liver at low DMSO concentrations revealed good metabolic conversion rates for the tested NA-relevant CYP enzymes in all models, except HepG2 cells. Low *CYP* expression levels were confirmed in HepG2 cells by quantitative real-time PCR. These results indicated that, as anticipated, metabolic activation of NAs by rat or hamster S9-mixture is required in the comet assay with HepG2 cells.

Ames testing initially focused on defining the optimal test conditions regarding the exposure scenario, the metabolic activation system and applied solvents using three tester strains, namely, TA 1535, TA 100 and *E. coli* WP2 *uvrA*. A clear impact of the solvents on the responsiveness in the assay using 10% induced rat S9-liver homogenates was visible for only a few NAs like *N*-Nitrosodibutylamine or *N*-Nitrosodipropylamine. A decrease in activity with higher solvent concentrations of DMSO and acetone, but not methanol, was observed. In line with the OECD TG471 guideline, the results indicate that water is the preferred solvent for testing of NAs and organic solvents (like DMSO and acetone) are compatible with the mutagenicity testing in bacteria but should be used at low concentrations.

The comparison of the metabolic activation of NAs by 30% non-induced hamster S9-mix versus 10% induced rat S9-mix in the Ames preincubation test showed an increased sensitivity for hamster liver homogenate, which was also confirmed in the Ames fluctuation test. Four NAs showed mutagenic activity with the hamster S9-mix while only two were found mutagenic using the rat S9-mix. The remaining three compounds were negative in both activation systems. The results presented in this study indicate that the sensitivity of the Ames preincubation test was strongly increased by using 30% hamster S9-mix versus the use of any variation of rat S9-mix.

BMD analysis of the *in vitro* comet data provided insight into the suitability of the assay for assessing genotoxicity of NAs. Using the BMD approach, PHHs (and PCLiS) assays provided the most robust concentration-response data and hence the best assessment of the selected NAs. Sensitivity and specificity analyses, calculated for all comet assays (beside PCLiS) and the Ames preincubation test, indicate that the comet assay with HepG2 and hamster S9-mix was able to distinguish positive and negative NAs with highest accuracy. The Ames preincubation test had an overall high sensitivity and specificity as well. However, the assessment of the specificity (and thus false positives) per assay was

hampered, as only two known-negative, carcinogenic NAs were tested. Specificity could be more accurately calculated with the assessment of additional known-negative, carcinogenic NAs.

In vitro comet assay potency BMD analysis of the NAs was performed to assess each cell line performance in differentiating NAs based on potency. Results obtained from PHH testing provided a clear distinction of positive tested NAs regarding their ranked potency values. However, it was often not possible to statistically distinguish between the different potencies of the compounds, due to large BMD confidence intervals (CIs). One reason contributing to large BMD CIs is a high level of within group variation. Follow up analysis could include a larger number of cells and events scored per replicate and should cover low concentrations near the point of departure of response. Ideally, five or more concentrations are recommended for follow up analyses.

There was some indication that the potency ranking reflected known *in vivo* mutation and cancer potency for the model NAs under specific criteria (e.g., 100% and median TI).

A critical finding is that the *in vitro* alkaline comet assay could be used to assess the genotoxicity of NAs. Also, BMD modelling might be a useful tool for potency ranking but requires thorough selection of the proper concentration range and a larger number of cells scored per replicate. The *in vitro* comet assay with liver cell models already looks promising for a standard, hazard-based assessment to define a positive or negative NA based on options of pairwise testing, trend analysis and use of historical controls to make the call. It might yet be useful in special cases to complement the Ames assay as a further *in vitro* test with a different endpoint. However, further work is needed before the *in vitro* comet assay, preferably with models exhibiting adequate metabolic competence, like primary human liver cells or HepG2 cells with hamster S9-mix, can be implemented into decision making.

The performance of the Ames preincubation test was finally compared with the performance of the *in vitro* alkaline comet assay. It is, however, at this point difficult to finally assess the added value of combining the results of both assays for assessment of the genotoxic potential of NAs, as only 10 structurally diverse NAs had been tested in both assays. Overall, both assays agreed on the classification of 8 out of 10 compounds as positive or negative. In the case of the Ames false negative compound NDELA (negative in the Ames pre-incubation test, but positive in TA 1535 in the Ames fluctuation assay), the best performing comet assays (PHHs and HepG2 with hamster S9-mix) both revealed that this substance can induce DNA strand breaks in human cells upon metabolic activation, indicating that the comet assays could, therefore, be useful to support the assessment of mutagenicity of NAs.

The optimized Ames preincubation test was applied to investigate a larger set of structurally diverse NAs, including many NDSRIs. The obtained results will be useful in improving the precision and accuracy of the potency categories identified in the Carcinogenic Potency Categorisation Approach (CPCA) by supporting NA Structure Activity Relationships.