EEMGS 2024 | Rovinj, Croatia | 23-27 September 2024 | Poster #08

Investigation of nitrosamines using miniaturized Ames tests

Csaba Boglári[1*], Cécile Koelbert[1]
[1]Xenometrix AG, Gewerbestrasse 25, 4123 Allschwil, Switzerland
* Corresponding author (cbo@xenometrix.ch)

Introduction

Nitrosamines are a class of chemical compounds, many of which are potent carcinogens found in various industrial, pharmaceutical, agricultural, and consumer products. Due to their potential health concern, particularly their ability to induce genetic mutations and cancer, rigorous genotoxicity testing is essential. This testing includes in vitro assays like the Ames test to characterize their mutagenic potential. Regulatory authorities propose the Enhanced Ames Test (EAT) testing protocol with 30% hamster S9 having a higher activity as compared to rat liver S9. In this study we investigated if the EAT protocol can be applied to miniaturized Ames test versions and present herein data with several nitrosamine compounds.

Important highlights

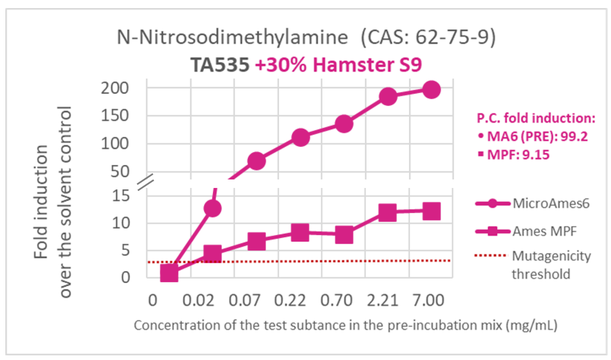
- Miniaturized Ames assays, the pre-incubation 6-well agar plate format and the microplate fluctuation format were used to characterize the mutagenicity of Nitrosamine test substances
- Miniaturized Ames assays can detect mutagenic Nitrosamines at lower concentrations compared to the agar plate tests conducted on Petri dishes.
- Cytotoxicity can be assessed in both miniaturized Ames assays

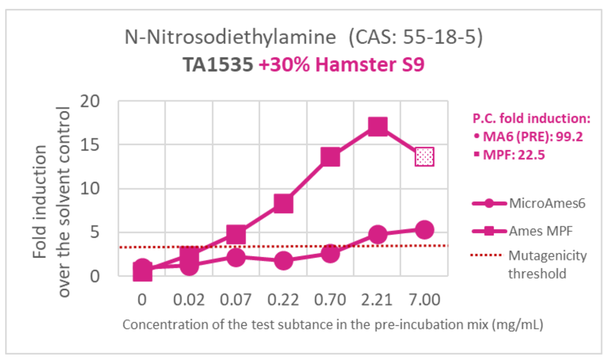
Methods

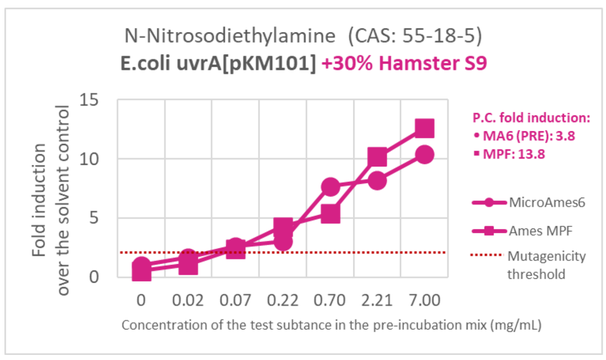
Two **parallel running miniaturized Ames assays** were applied in this study to generate data on the mutagenicity of **Nitrosamine test substances:** the Ames test in microplate fluctuation format, **Ames MPF**, and the Ames test in 6-well agar plate format, **MicroAmes6.** Both miniaturized assay implementations follow the **pre-incubation protocol**. We used overnight cultures of the Salmonella and E.coli Ames tester strains followed by 14 hours of incubation at 37°C. Following the **overnight incubation** the OD600 value was measured and the cell number was determined using a cell counting chamber. The **same overnight culture** served as an input to both **miniaturized Ames assays**, either diluted or undiluted, for the 6-well plate format or for the liquid microplate fluctuation format, respectively. Six different concentrations of the Nitrosamine test substances were tested with concurrent negative and positive controls. For the microplate fluctuation test 25x concentrated stock solution was applied, while 50x concentrated stock solution was used for the 6-well agar plate format. The top dose of the investigated Nitrosamines was adjusted to achieve the same concentration of the test substance during pre-incubation in both miniaturized assays. **Water was used as solvent** for all tested Nitrosamines. Three strains were included in the testing: Salmonella Ames tester strains **TA100**, **TA1535** and **E.coli uvrA[pKM101]**. For the metabolic activation of the Nitrosamines **30% Hamster S9** was applied to harmonize our protocol with the **Enhanced Ames Test (EAT)** recommendations by regulatory authorities [1,2].

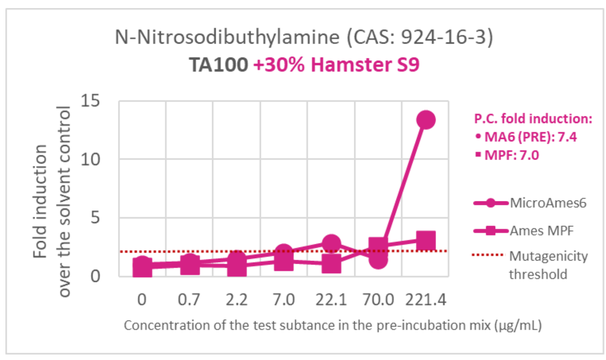
Results

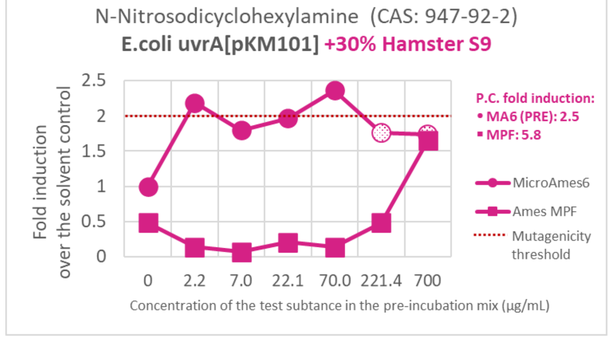
Performance of the miniaturized Ames assays in Nitrosamine testing

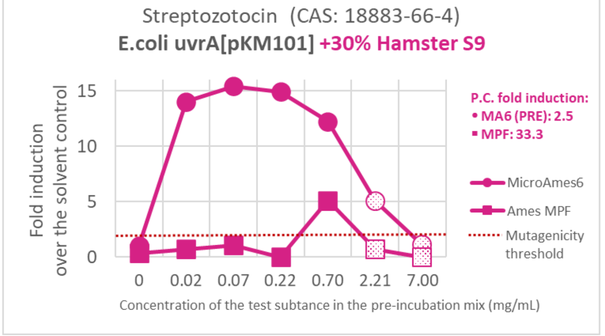


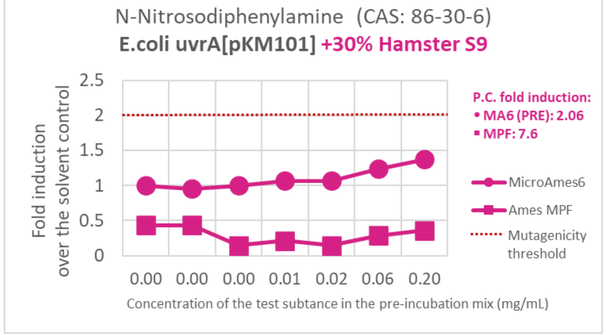


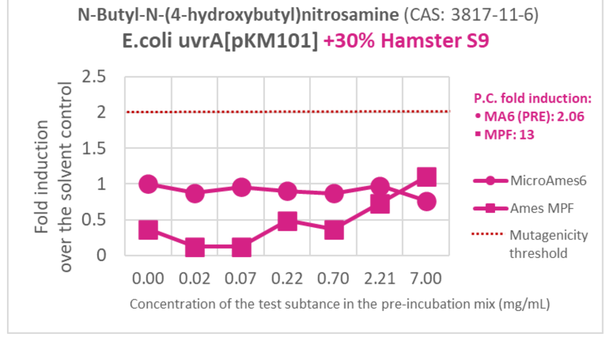












All experiments were performed in the presence of 30% hamster liver S9. Test substance concentration in the pre-incubation mix is presented, which is the concentration of the test substance in the total volume of the bacterial suspension / S9 mix / test substance stock solution mixture in the case of the 6-well agar plate test. For microplate fluctuation format the compound concentration presented on the graphs is the effective concentration of the test substance during exposure i.e. during the 90 minutes of pre-incubation of bacteria exposed to the test substance in the strain-specific exposure medium. The test substance concentration is therefore given in µg/mL or mg/mL units. The concentration of the Nitrosamine test substances were adjusted to have exactly the same effective concentration in the reaction mix during pre-incubation in both miniaturized Ames assays. Fold induction over the solvent control in the number of revertant wells or revertant colonies is presented as the function of the varying concentration of the Nitrosamine test substances, for Ames MPF and for MicroAmes6, respectively. Circles represent the fold induction values of the MicroAmes6 experiment, while squares represent the Ames MPF fold induction values. Dashed red line is the threshold for positivity (2-fold induction for TA100 and E.coli uvrA[pKM101], and 3-fold for TA1535). Circles and squares with dotted pattern represent cytotoxic concentrations. Concurrent positive control fold induction values are indicated next to the graphs. MA6 = MicroAmes6, Ames assay in 6-well agar plate format; MPF: Ames test in microplate fluctuation format; PRE: pre-incubation protocol. The summary table shows the results of the miniaturized assays in comparison with Petri dish-based agar plate data from the NTP database or scientific literature. nLEC = normalized Lowest Effective Concentration, which is the lowest concentration at which the fold induction over the solvent control exceeds the pre-defined threshold for mutagenicity (threshold is set to 2-fold for TA100 and E.coli uvrA[pKM101], and 3-fold for TA1535), the normalized LEC values take into account the differences in the effective concentration during exposure in the pre-incubation mix between the liquid microplate fluctuation format (Ames MPF) and the pre-incubation agar-based Ames test systems (6-well agar plate format and the Ames test in Petri dishes); NEG = negative; POS = positive; EQ = equivocal; N.A. = not applicable.

Summary Table

Compound	CAS Nr.	Strain	Metabolic activation	MicroAmes6		Ames MPF		Petri dish-based Agar Plate Test [NTP, Literature]	
				Result	nLEC [µg/mL]	Result	nLEC [µg/mL]	Result	nLEC [µg/mL]
N-Nitrosodimethylamine	62-75-9	TA1535	30% Hamster S9	POS	20	POS	20	POS	700
N-Nitrosodiethylamine	55-18-5	TA1535	30% Hamster S9	POS	2210	POS	70	POS	2212
N-Nitrosodiethylamine	55-18-5	E.coli uvrA[pKM101]	30% Hamster S9	POS	70	POS	70	N.A.	N.A.
N-Nitrosodibuthylamine	924-16-3	TA100	30% Hamster S9	POS	7	POS	70	POS	70
N-Nitrosodicyclohexylamine	947-92-2	E.coli uvrA[pKM101]	30% Hamster S9	EQ	2.2	NEG	N.A.	N.A.	N.A.
Streptozotocin	18883-66-4	E.coli uvrA[pKM101]	30% Hamster S9	POS	20	EQ	700	N.A.	N.A.
N-Nitrosodiphenylamine	86-30-6	E.coli uvrA[pKM101]	30% Hamster S9	NEG	N.A.	NEG	N.A.	N.A.	N.A.
N-Butyl-N(4-hydroxybutyl)nitrosamine	3817-11-6	E.coli uvrA[pKM101]	30% Hamster S9	NEG	N.A.	NEG	N.A.	N.A.	N.A.

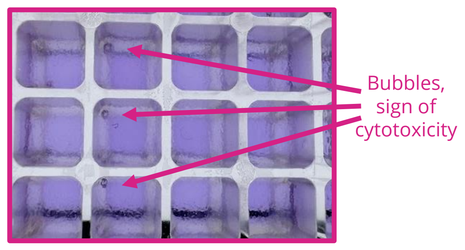
Petri dish-based Agar Plate test data from NTP Database [3] and Scientific Literature: Bringezu & Simon, 2022 [4]

A case study on cytotoxicity

The assessment of **cytotoxicity** is essential in the Ames assay, as it can obscure the mutagenicity of the tested sample, potentially causing **false negative results**. This example demonstrates that higher concentrations of Streptozotocin (CAS: 18883-66-4) are cytotoxic to the bacteria. **Signs of cytotoxicity** appeared in both miniaturized Ames test systems at higher doses. A prominent hallmark of cytotoxic concentrations is the dose-dependent decrease in the number of revertants after an initial increase in the revertant number. This characteristic pattern can be observed in both miniaturized systems. The cytotoxicity of the compound can be suspected in the Ames MPF system by the presence of **bubbles** in the wells of the 384-well plates – arrows are pointing to bubble formation on the images with higher magnification below. In the Ames test in 6-well agar plates, the cytotoxic concentrations of a test sample often result in **microcolonies** – which are also visible on the images with higher magnification in the background of the wells of the 6-well agar plates – arrows point to microcolonies.

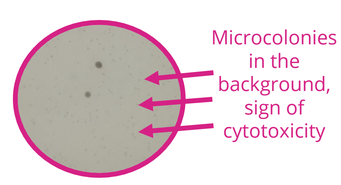
Ames MPF: 384-well plate after 48 hours at 37°C

Magnified image of the wells on the 384-well plate



MicroAmes6: 6-well agar plates after 72 hours at 37°C

Magnified image of the well on the 6-well plate



Conclusion

Our data suggests that the miniaturized Ames assays are applicable to reliably assess the mutagenicity of nitrosamines. The **evaluation of cytotoxicity** is possible in both miniaturized Ames test systems, the microplate fluctuation format and the pre-incubation 6-well agar plate format. There is a good concordance between the two miniaturized Ames assays in predicting the mutagenicity of Nitrosamines, i.e. the **same assessment outcome** was gained with both miniaturized Ames assays for **6 out of 8 test substances**. There were only one case, where MicroAmes6 gave an equivocal result, while Ames MPF was negative: N-Nitrosodicyclohexylamine tested with E.coli uvrA[pKM101]. In the case of Streptozotocin MicroAmes6 was positive, and Ames MPF gave an equivocal result. There is an **excellent concordance with the Petri dish-based Ames test data**: **all positive Nitrosamines in the Petri dish-based Ames test assessed in the scope of this study were also positive in the miniaturized Ames tests**. Furthermore, the **miniaturized Ames assay formats can detect mutagenic Nitrosamines at lower concentrations** compared to the Agar Plate test in Petri dishes, for instance, NDMA with TA1535 strain is detected positive already at 20 µg/mL with both miniaturized assays, while the Petri dish-based Ames test first detects NDMA positive at 700 µg/mL. Another example is NDEA with TA1535, which is detected positive already at 70 µg/mL in the Ames MPF, while the agar-based Ames assays, 6-well agar plate test and the Petri dish-based test require 2210 µg/mL NDEA concentration in the pre-incubation mix to detect it positive. Finally, the 6-well plate agar plate test provided a positive result for NDBA with TA100 already at 7 µg/mL compared to the normalized lowest effective concentration of 70 µg/mL, which is required for Ames MPF and for the Agar Plate test in Petri dishes. We conclude that the miniaturized Ames assays are an **environment- and resource-friendly alternative** to the Petri dish-based Agar plate test, while providing **high performance** and **excellent predictive power** for the **assessment of mutagenicity of Nitrosamines and other genotoxic impurities**.

References:
[1] European Medicines Agency. (2023, July). Questions and answers for marketing authorization holders/applicants on the CHMP Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products
[2] Food & Drug Administration. (2023, August). Control of Nitrosamine Impurities in Human Drugs. Guidance for Industry
[3] National Toxicology Program (NTP) coordinated by United States Department of Health and Human Services
[4] Bringezu F, Simon S. Salmonella typhimurium TA100 and TA1535 and E. coli WP2 uvrA are highly sensitive to detect the mutagenicity of short Alkyl-N-Nitrosamines in the Bacterial Reverse Mutation Test. Toxicol Rep. 2022 Feb 8;9:250-255. doi: 10.1016/j.toxrep.2022.02.005. PMID: 35198408; PMCID: PMC8850549.

The **Ames MPF** and **MicroAmes6** are proprietary miniaturised Ames assays developed by Xenometrix AG.
Check out our homepage **xenometrix.ch** for further information, and follow us on social media.