Optimizing the Ames Assay for Nitrosamine Testing: Concordance Results of a HESI Multi-Sector Ring Trial

Tetyana Cheairs[1] HESI GTTC Nitrosamines Research Program Participants[2]

[1]*New York Medical College, Valhalla, NY, USA* [2]*Health and Environmental Sciences Institute (HESI), Washington, DC, USA*

Abstract

Hazard identification and genotoxicity risk assessment of *N*-nitrosamines (NAs) have been the subject of intensive research, due, in part, to their discovery as impurities in some pharmaceuticals. To address this challenge, one of the initiatives led by the Health and Environmental Sciences Institute (HESI) Genetic Toxicology Technical Committee (GTTC) focused on the improvement of the in vitro detection of nitrosamine-associated hazards by optimizing the bacterial reverse mutation (Ames) assay protocol. Specifically, this multi-sector project aimed to investigate various Ames assay parameters related to nitrosamine mutagenicity and carcinogenicity and to identify the most sensitive conditions for assessing their potency. For this purpose, a group of 32 nitrosamines from different structural classes and with different carcinogenic potency were tested using a protocol comparable to that of an Enhanced Ames Test (EAT), with each compound tested in two independent laboratories. Compounds were tested in 5 different strains, using a 30-minute preincubation protocol, with either water or DMSO serving as the vehicle. To enhance the sensitivity and specificity of the Ames assay, testing conditions included the use of 10% or 30% S9 fractions from induced rat or hamster liver. The results of the testing were mainly concordant among the laboratories, with the exception of 3 compounds, including sterically hindered nitrosamines with weak or absent carcinogenicity. Overall, the results indicate that the current enhanced Ames protocol was highly sensitive for the prediction of the carcinogenicity of nitrosamines.

Introduction

N-nitrosamines (NAs) belong to a group of organic chemicals featuring a functional nitroso group bound to deprotonated amine. NAs can form both endogenously and in foods through reactions between nitrite and amine constituents. NAs have been detected in various products such as personal care items, tobacco, air, and water. They are associated with genotoxic and carcinogenic properties, with a significant percentage of tested compounds producing tumors at multiple sites in rodents. The International Agency for Research on Cancer (IARC) has classified numerous nitrosamines as probable or possible human carcinogens. Concerns have emerged in the pharmaceutical industry due to the discovery of NAs in certain drug products, originating from various sources including manufacturing processes and storage conditions. Many of these NAs have no toxicity data since they are nitrosated drug substances.

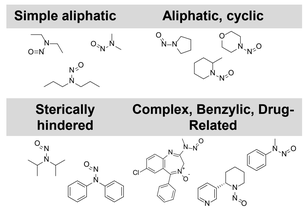
Efforts are being made to improve safety assessment methods for NAs, with a focus on detecting mutagenicity and predicting carcinogenicity using the Ames test and the Enhanced Ames Test (EAT) protocol. The EAT aims to ensure sensitivity in detecting the potential mutagenicity of carcinogenic NAs. The purpose of the HESI Ring Trial was to prospectively test a group of 32 NAs from different structural classes and with varying carcinogenic outcomes in rodents using an Ames protocol similar to that of the EAT. Each NA underwent testing in two independent laboratories under different S9 conditions. The overall goals included generating new data to develop an optimized Ames methodology for sensitive NA hazard detection and characterizing the predictive capability of the Ames test for NAs with known carcinogenicity in rodents.

Methodology

Compound Selection

- 32 NAs
- 11 negative and 21 positive in rodent carcinogenicity bioassays
- Different CPCA compound classes

CPCA Category	# of NAs
1	6
2	6
3	6
4	9
5	2
NA	3



Testing Conditions

Each nitrosamine was tested in two separate laboratories following standardized guidelines, with detailed information about test articles and appropriate testing procedures utilized:

- **Solvent** – H_20 (or DMSO if not H_20 soluble)
- **Preincubation** (30 min)
- **5 strains**
- **S9 from PB/BNF-treated rodents**
  - +10% rat and hamster liver S9
  - +30% rat and hamster liver S9

Statistical Analysis

Concordance metrics such as sensitivity, specificity, and predictive values were calculated using the R 4.3 environment. Bootstrapping was employed to construct 95% confidence intervals for the concordance measurements, ensuring comprehensive assessment of the test outcomes

Results

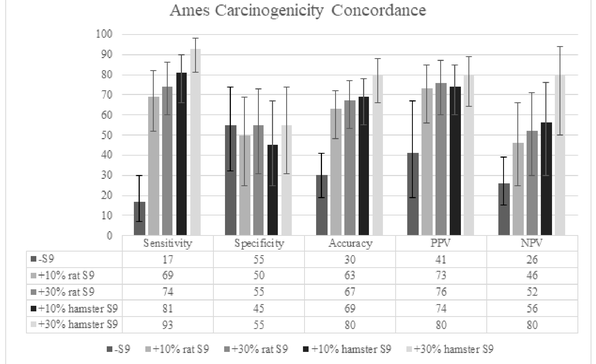


Figure 1. Concordance of Ames with carcinogenicity based on different conditions of liver -induced S9 (equivocal = negative; 64 tests, 32 NAs)

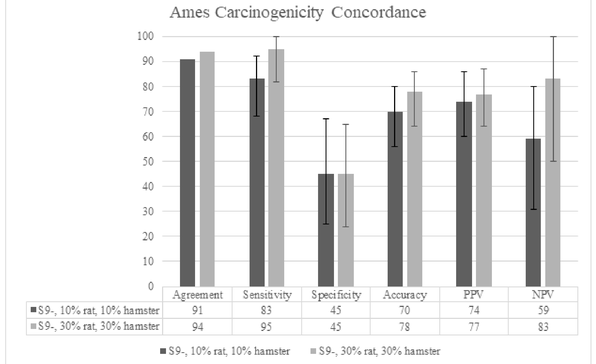


Figure 2. Concordance of Ames with carcinogenicity comparing the combination of -S9 with 10% rat and hamster liver-induced S9 versus -S9 with 30% liver-induced S9 (equivocal = negative; 64 tests, 32 NAs)

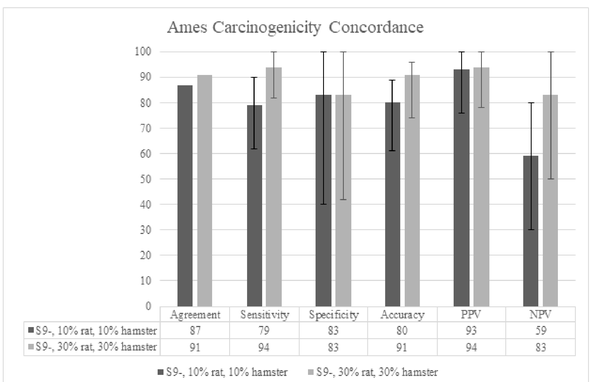


Figure 3. Concordance of Ames with carcinogenicity for the combinations of -S9 with 10% rat and hamster liver-induced S9, and -S9 with 30% liver-induced S9 following removal of NAs positive with -S9 (equivocal = negative; 46 tests, 23 NAs)

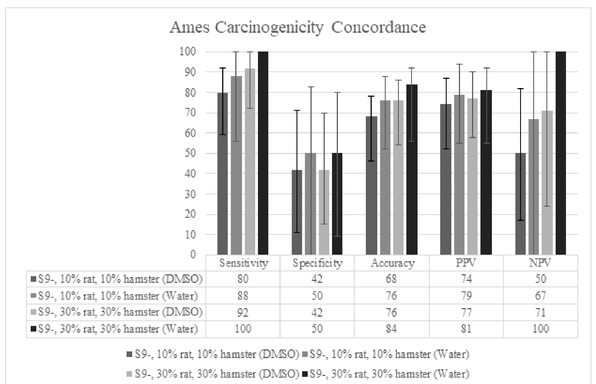


Figure 4. Concordance of Ames with carcinogenicity for the combinations of -S9 with 10% rat and hamster liver-induced S9, and -S9 with 30% liver-induced S9 when DMSO or Water is used as the vehicle (equivocal = negative; DMSO:37 tests, 22 NAs; Water: 25 tests, 16 NAs)

Discussion

The study found that pre-incubation using 30% hamster liver S9 had the highest sensitivity at 93%, while the lowest sensitivity was observed when no metabolizing enzymes were used (-S9) at 17%. When analyzing the combinations of rat and hamster metabolic enzymes, high levels of agreement in the positive or negative call were observed (91-94%). Additionally, the study demonstrated that specificity significantly improved when removing NAs positive without metabolic activation (-S9). Sensitivity remained consistent, while parameters such as accuracy, positive predictive value (PPV), and negative predictive value (NPV) were significantly improved. The study also observed very good sensitivity with both DMSO and water, with up to 92% and 100%, respectively, when considering specific metabolic enzyme combinations.

Conclusions

- The EAT can be considered a conservative method for detecting the mutagenic carcinogenicity of NAs
- Sensitivity was highest using 30% hamster S9. However, sensitivity was also high with other metabolic activation systems
- Solvent comparisons of DMSO versus water indicated both were suitable for detection of NA carcinogenicity. However, caution should be exercised when using DMSO for testing small molecule NAs due to the inhibition of CYP2E1
- Removing NAs positive in the absence of S9 activation improved specificity, suggesting the utility of follow-on testing for these NAs

Acknowledgments

Participating Labs include AbbVie, AstraZeneca, Bayer, Boehringer-Ingelheim, Bristol-Myers Squibb, Charles River Laboratories, Eli Lilly, Gilead, GSK, Inotiv, Merck, Merck kGA, Pfizer, Roche, Sanofi, US FDA/NCTR.

Resourcing: This is a multi-arm program supported by both the HESI GTTC and the Food and Drug Administration (FDA) of the U.S. Department of Health and Human Services (HHS). FDA/HHS support is part of a financial assistance award [FAIN] totaling $347,618 (U01FD006676-03S2), with ~75 percentage funding by FDA/HHS to support the experimental work, statistical analysis and coordination described in this presentation. The HESI GTTC budget is $371,250 and includes funding from multiple industry partners (www.hesiglobal.org) to support multiple projects, one of which is described in this presentation. Additional in-kind contributions from HESI GTTC's academic, industry and government partners are described in this presentation. The contents are those of the author(s) and do not necessarily represent the official views of, nor an endorsement, by FDA/HHS, or the U.S. Government.

HESI Genetic Toxicology Technical Committee

Nitrosamines Research Program