An Assessment of Five Small N-nitrosamines as Positive Controls for the Enhanced Ames Test (EAT)

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Abstract

Current guidance for testing of N-nitrosamine Impurities in drugs requires using an Enhanced Ames Test (EAT) to assess the impurity's mutagenic potential. The design calls for use of a 30 min preincubation method, 30% induced rat and hamster liver S9, and requires two to three nitrosamine specific positive controls. The current study was designed to assess several small N-nitrosamine molecules for their use as positive controls in the EAT. These include Nitrosodimetylamine (NDMA), Nitrosodipropylamine (NDPA), Nitrosodibutylamine (NDBA), Nitrosodiisopropylamine (NDIPA), 1-Cyclopentyl-4-nitrosopiperazine (CPNP). All the compounds were tested under standard EAT conditions. None of the compounds were positive with tester strains TA98 or TA1537. NDIPA was considered weakly positive based on a 3.1-fold increase with tester strain TA1535 in the presence of hamster S9 at 500 µg/plate. All other compounds induced mutagenic responses in multiple strains as well with either rat or hamster S9. For instance, NDPA yielded positive results in tester strains TA100, TA1535, and WP2 *uvr*A (pKM101) in the presence of hamster S9 as well as TA1535 and WP2 *uvr*A (pKM101) with rat S9. Similarly, NDMA was positive in the same strains as NDPA in the presence of hamster S9, although only positive in WP2 *uvr*A (pKM101) with rat S9. NDBA was also positive in the same strains with hamster S9, but negative in all strains with rat S9. Lastly CPNP was positive in TA100 and TA1535 with either type of S9 and positive with WP2 *uvr*A (pKM101) in the presence of hamster S9. While none of the nitrosamines tested yielded positive results across the board for tester strains TA100, TA1535, and WP2 *uvr*A (pKM101) in the presence of both rat and hamster S9, an EAT study design incorporating a combination of two to three small n-nitrosamines such as NDMA, NDPA, and CPNP may provide ample evidence that the test system and metabolic activation systems are sensitive and optimized for detecting an unknown mutagenic nitrosamine.

Materials and Methods

NDPA was obtained from Sigma Aldrich (St. Louis, MO). CPNP was obtained from TRC Canada (New York, ON). NDMA, NDBA, and NDIPA were manufactured by ArZa Bioscience (Greater Manchester, UK) and provided by HESI originally for use intended use in the HESI Ames Optimization Ring Trial. The *S. typhimurium* tester strains were from Dr. Bruce Ames, University of California, Berkeley. The *E. coli* tester strain was from the National Collection of Industrial and Marine Bacteria, Aberdeen, Scotland (United Kingdom). Liver homogenate (S9) prepared commercially from either male Sprague-Dawley rats or male Golden Syrian Hamsters that have been injected intraperitoneally with Phenobarbital-5,6 Benzoflavone (PB/BNF) was purchased from Molecular Toxicology, Inc (MolTox; Boone, NC). Minimal selective bottom agar plates, selective top agar, and all positive controls were purchased from Moltox.

Preincubation (PRE) method:

One-half milliliter (0.5 mL) of S9 mix or sham mix were added to pre-heated 13 x 100 mm glass culture tubes. To these tubes, 100 µL of tester strain and 50.0 µL of vehicle, test article dilution or positive control were added. After vortexing, the mixture were allowed to incubate for 30±2 minutes at 37±2°C with shaking. Two milliliters of selective top agar were then added to each tube and the mixture was overlaid onto the surface of a minimal bottom agar plate. After the overlay solidified, the plates were inverted and incubated for approximately 48 to 72 hours at 37±2°C prior to scoring.

Evaluation Criteria for Datasets:

Data sets were judged as positive if the increase in mean revertants at the peak of the dose response was equal to or greater than 2.0 times the mean vehicle control value for tester strains TA98, TA100, and WP2 *uvrA* (pKM101) or equal to or greater than 3.0 times the mean vehicle control value for TA1535 and TA1537.

Figure 1: Chemical structures of small N-nitrosamine molecules tested

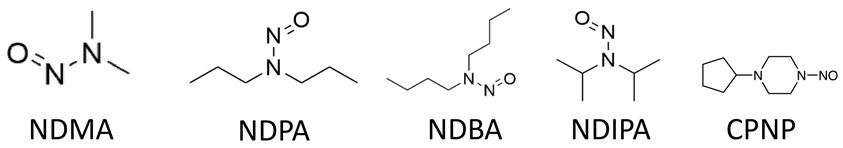
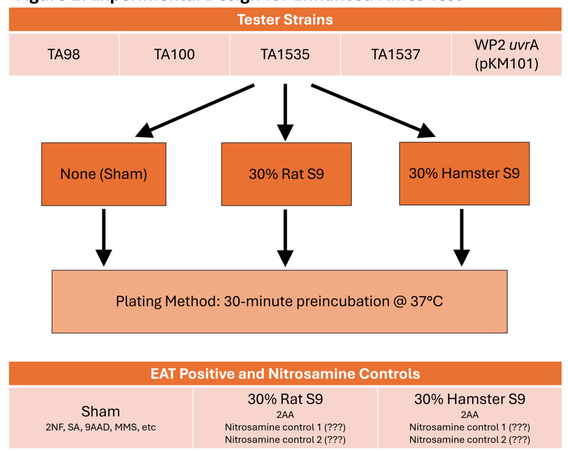


Figure 2: Experimental Design for Enhanced Ames Test



Results

- NDMA, NDPA, and NDBA induced positive responses in TA100, TA1535, and WP2 *uvr*A (pKM101) in the presence of 30% hamster liver S9
- In the presence of 30% rat liver S9, NDPA was positive in TA1535 and WP2 *uvr*A (pKM101), NDMA was positive in WP2 *uvr*A (pKM101) and NDBA was negative in all strains.
- NDIPA induced either equivocal or weak positive results with TA1535 in the presence of hamster liver S9
- CPNP induced consistent positive results in TA100, TA1535, in the presence of either type of S9, and WP2 *uvr*A (pKM101) in the presence of hamster S9

Table 1: Ames mutagenicity results for five small N-nitrosamines.

	TA100		TA1535		WP2 *uvr*A (pKM101)	
Nitrosamine	30% Rat S9	30% Hamster S9	30% Rat S9	30% Hamster S9	30% Rat S9	30% Hamster S9
NDMA	Negative	Positive; ≥500 µg/plate	Negative	Positive; ≥1500µg/plate	Positive; ≥1500µg/plate	Positive; ≥50µg/plate
NDPA	Negative	Positive; ≥1500 µg/plate	Positive; ≥1500 µg/plate	Positive; ≥1500 µg/plate	Positive; ≥1500 µg/plate	Positive; ≥1500 µg/plate
NDBA	Negative	Positive; ≥150 µg/plate	Negative	Positive; ≥15.0 µg/plate	Negative	Positive; ≥150 µg/plate
CPNP	Positive; ≥2000 µg/plate	Positive; ≥2000 µg/plate	Positive; ≥2000 µg/plate	Positive; ≥2000 µg/plate	Negative	Positive, ; ≥2000 µg/plate
NDIPA	Negative	Negative	Negative	Positive; ≥500 µg/plate	Negative	Negative

Conclusions

- None of the small nitrosamines tested induced positive results in the three critical tester strains of TA100, TA1535, and WP2 *uvr*A (pKM101) in the presence of both rat and hamster liver S9, though several were positive in multiple assay conditions.
- Incorporating a combination of 2-3 small nitrosamines such as NDPA, NDMA, and CPNP based on robustness of increases in individual assay conditions is necessary to meet criteria for a valid EAT study.
- Further testing with a variety of Nitrosamine compounds, including NDSRIs, could be helpful to standardize EAT study designs and strain specific nitrosamine control recommendations.

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