

Ames Test assay parameters important for the detection of N-Nitrosamine mutagenicity

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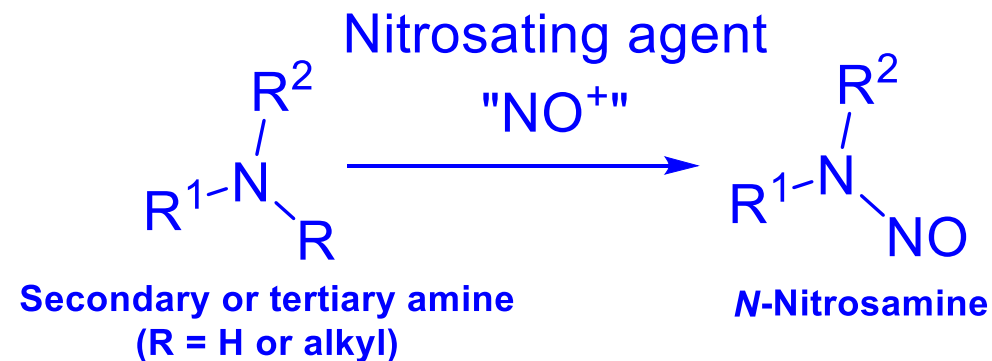
Spring Symposium Sections Pharmaceutical Toxicology and Genetic Toxicology

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Today's Presentation

- Background to *N*-nitrosamines, ICH M7 and the “Request to Industry”.
- Rat and Hamster S9 CYP Characterisation
- GSK Ames method for testing of *N*-nitrosamines - qualitative and quantitative data for NDMA, NDEA and NMEA
- Discordant *N*-nitrosamine assessment
- Using the GSK Ames method on a larger list of industrially significant *N*-nitrosamines



N-nitrosamines a “Cohort of concern” and “Regulatory concern”

7.5 Exceptions and Flexibility in Approaches

- Higher acceptable intakes may be justified when human exposure to the impurity will be much greater from other sources e.g., food, or endogenous metabolism (e.g., formaldehyde).
- Case-by-case exceptions to the use of the appropriate acceptable intake can be justified in cases of severe disease, reduced life expectancy, late onset but chronic disease, or with limited therapeutic alternatives.
- Compounds from some structural classes of mutagens can display extremely high carcinogenic potency (cohort of concern), i.e., aflatoxin-like-, N-nitroso-, and alkyl-azoxy structures. If these compounds are found as impurities in pharmaceuticals, acceptable intakes for these high-potency carcinogens would likely be significantly lower than the acceptable intakes defined in this guideline. Although the principles of this guideline can be used, a case-by-case approach using e.g., carcinogenicity data from closely related structures, if available, should usually be developed to justify acceptable intakes for pharmaceutical development and marketed products.

- Alongside being in a ICH M7 ‘cohort of concern’, regulators have expressed concern with the Ames test and its sensitivity in identifying the mutagenicity of a N-nitrosamine (NA)

- Concern stems from several NA chemical structures have been deemed discordant in terms of Ames Test predictivity of rodent carcinogenicity i.e., Ames test negative but rodent cancer bioassay positive

MUTAGENICITY OF ALIPHATIC NITROSAMINES IN *Salmonella typhimurium*

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Summary

25 aliphatic nitrosamines were examined in the Ames assay for bacterial mutagens, using rat liver “S-9” for activation. Of them, 8 carcinogens were mutagenic and 5 non-carcinogens were not mutagenic. However, 2 compounds not carcinogenic in rats were mutagenic and 9 carcinogens were not mutagenic, including 6 that are liver carcinogens in rats.

The Mutagenicity of 45 Nitrosamines in *Salmonella Typhimurium*

A.W. Andrews and W. Lijinsky

Chemical Carcinogenesis Program, Frederick Cancer Research Center, Frederick, Maryland

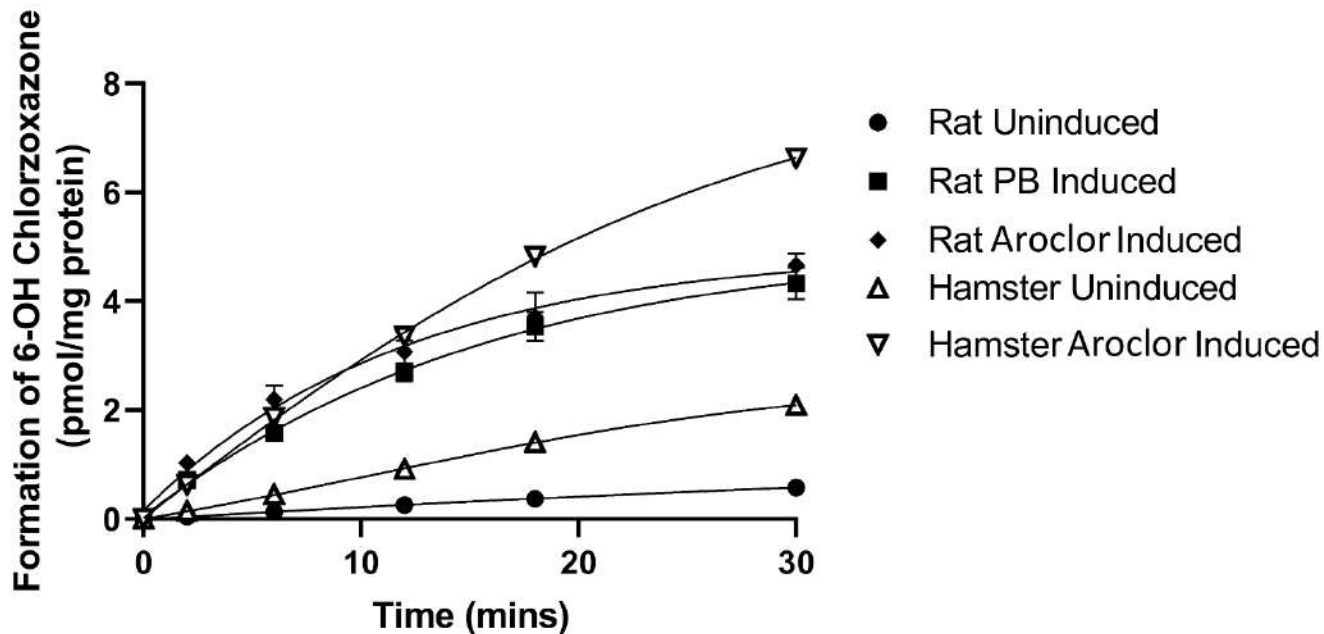
The correlation between mutagenicity in the rat liver microsome-mediated *Salmonella* Mutagenicity Assay of Ames and carcinogenicity in rats was examined with three groups of nitrosamines. Qualitatively the correlation was good, but there was poor correlation between mutagenic potency and carcinogenic potency. Of 23 cyclic nitrosamines, 19 were carcinogenic and mutagenic, and two were carcinogenic but not mutagenic, and the carcinogenicity studies of the remaining two are not complete. Of six symmetrical aliphatic nitrosamines, five were carcinogenic and mutagenic while only one carcinogen was not mutagenic. The greatest discrepancy occurred among 16 asymmetric nitrosamines, where 11 were both carcinogenic and mutagenic, four were carcinogenic but nonmutagenic, and one carcinogenicity study is incomplete.

Key words: carcinogenicity, mutagenicity, Ames test, *Salmonella* assay, nitrosamines, potency, activation

Assay Criteria – GSK Methodology Testing NDMA and NDEA

- Strains – TA100, TA98, TA1535, TA1537 and WP2uvrA(pKM101)
- Plate Incorporation and Pre-Incubation methods, in the presence of Rat or Hamster S9
- In the absence of S9 (Plate Incorporation method only)
- Multiple solvents: Water, DMSO, Methanol, Acetonitrile, Acetone, n-methyl-2-pyrrolidone (NMP), dimethylformamide (DMF), and dihydrofuran (DHF).
- 10% Rat or Hamster S9
- Pre-incubation – 30 minutes
- Pre-incubation – 50 µL additions (solubility 100 mg/mL)
- Plate Incorporation – 100 µL additions (solubility 50 mg/mL)
- **Positive** criteria – 2-fold (TA100, TA98 and WP2uvrA(pKM101)), 3-fold (TA1535 and TA1537)

CYP assessments

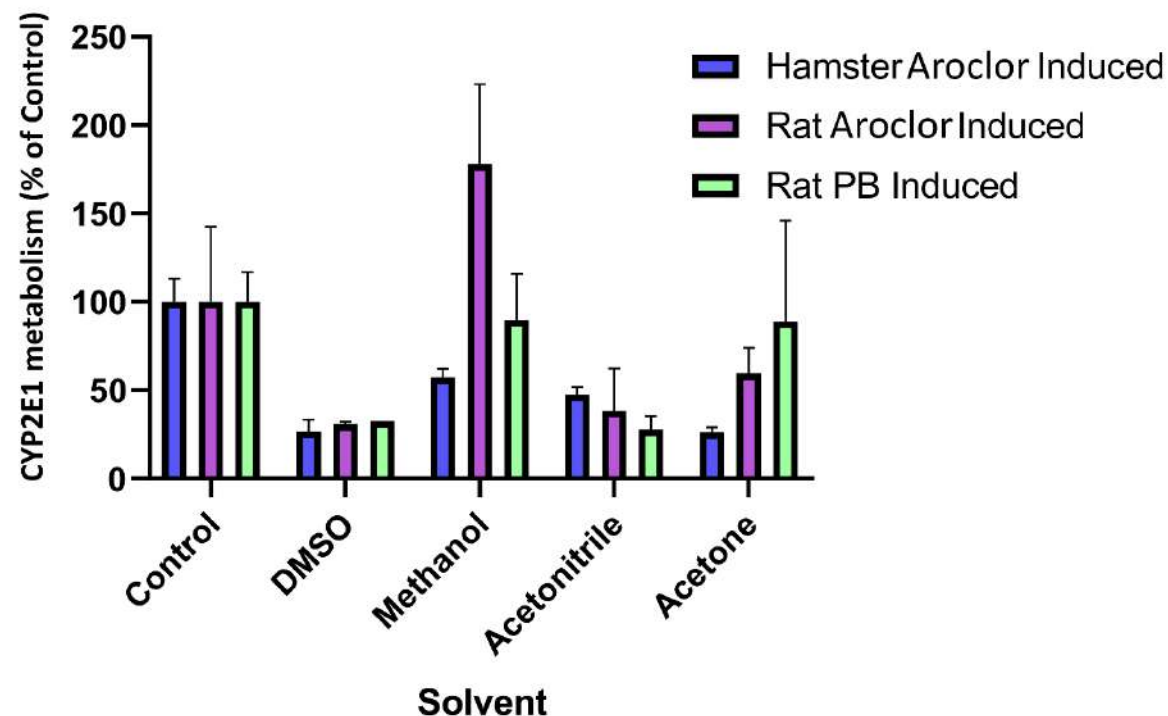


6-hydroxychlorzoxazone was observed in all rat and hamster liver S9 fractions investigated.

The rate of production of metabolite was significantly higher in induced S9 fractions compared with non-induced S9 in both species

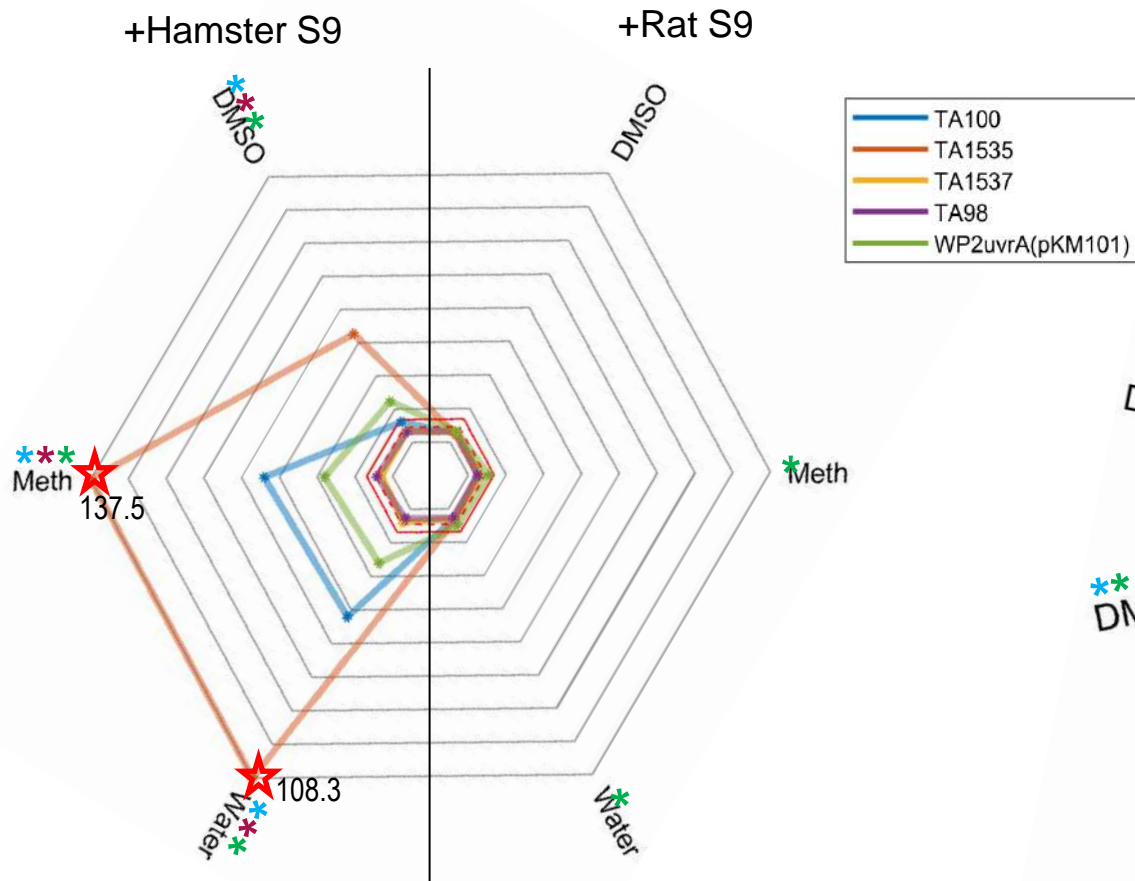
Addition of DMSO and acetonitrile (7.7%) inhibited the formation of 6-hydroxychlorzoxazone in rat and hamster induced liver S9.

Methanol and acetone had negligible effect on PB induced rat liver S9 but reduced 6-hydroxychlorzoxazone formation by ~50% and ~75%, respectively, using Aroclor induced hamster liver S9



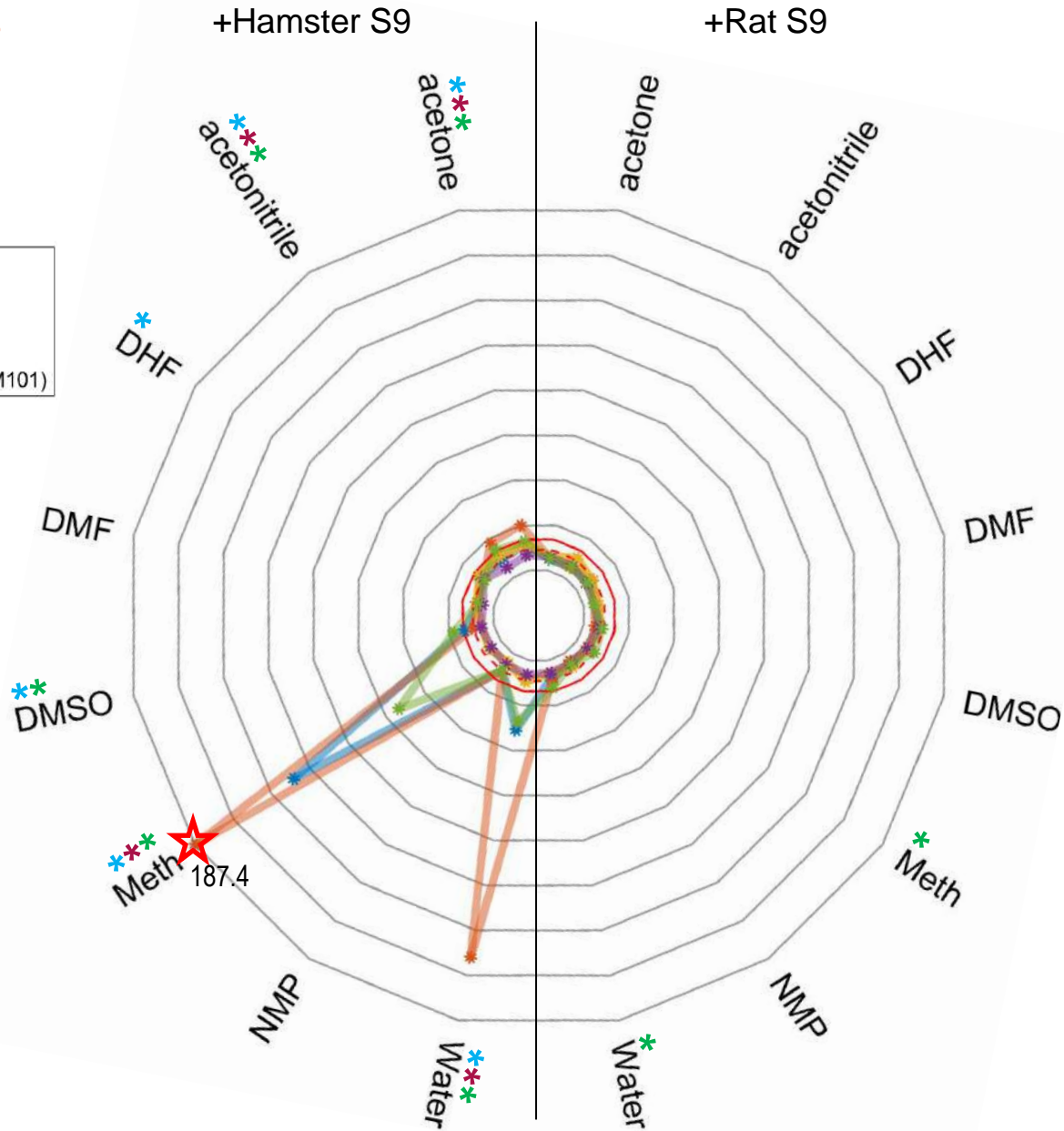
NDMA Ames data – Spider Plots

Plate Incorporation

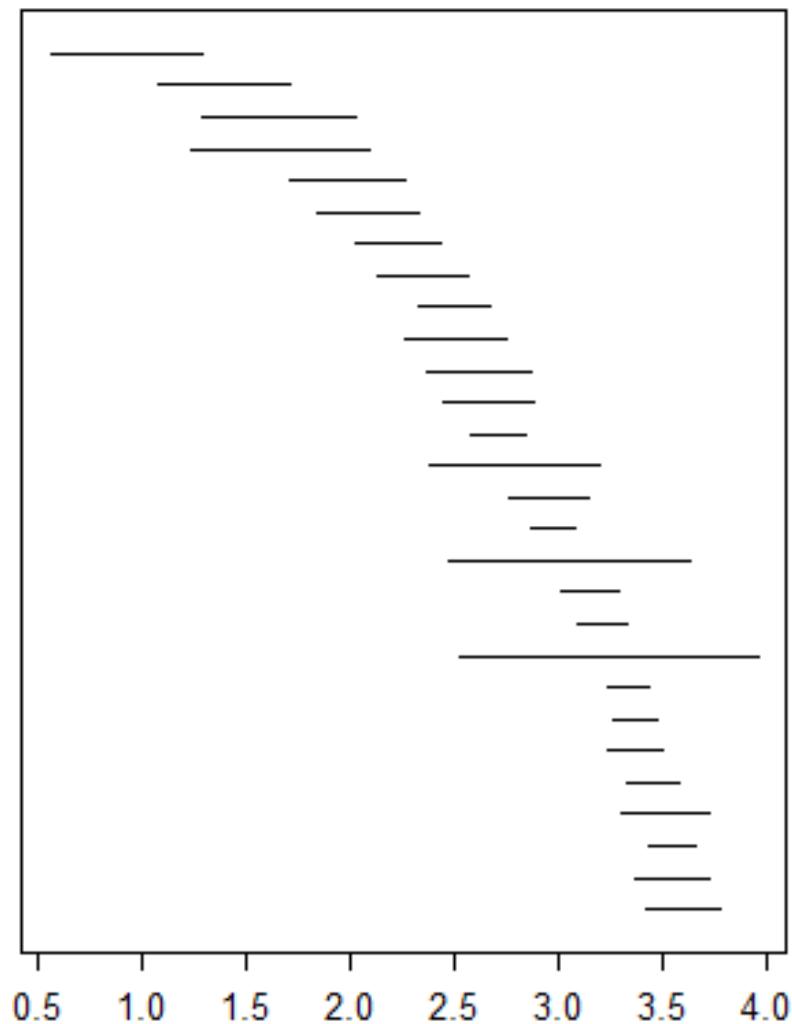


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Pre-incubation



NDMA Ames data – Quantitative Benchmark dose Analysis



log₁₀- CED-1

← Increasing sensitivity

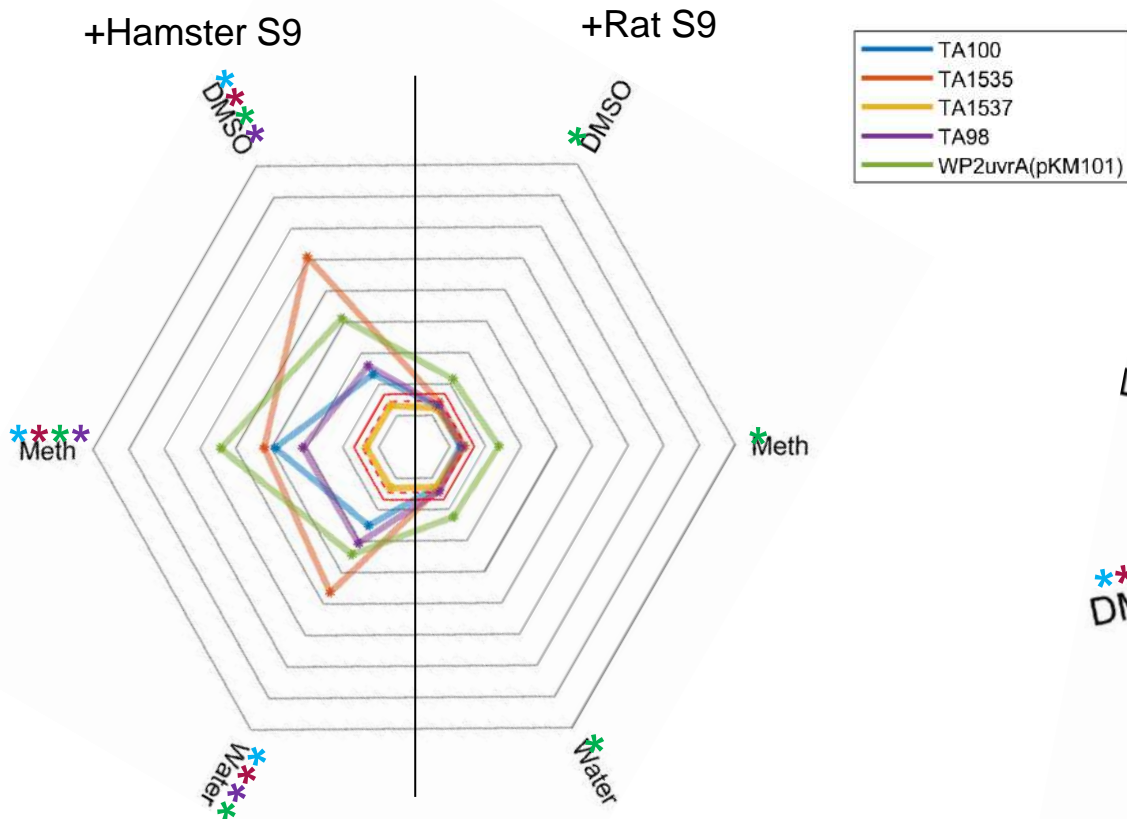
Methanol	Plate	TA1535	+H
Methanol	Pre	TA1535	+H
Water	Plate	TA1535	+H
Water	Pre	TA1535	+H
Methanol	Pre	TA100	+H
Methanol	Plate	TA100	+H
Methanol	Pre	WP2uvrA(pKM101)	+H
Water	Plate	TA100	+H
Methanol	Plate	WP2uvrA(pKM101)	+H
Water	Plate	WP2uvrA(pKM101)	+H
Water	Pre	TA100	+H
Water	Pre	WP2uvrA(pKM101)	+H
DMSO	Plate	WP2uvrA(pKM101)	+H
DMSO	Plate	TA1535	+H
Acetone	Pre	TA1535	+H
DMSO	Pre	WP2uvrA(pKM101)	+H
Acetonitrile	Pre	TA1535	+H
Acetone	Pre	WP2uvrA(pKM101)	+H
DMSO	Pre	TA100	+H
Acetonitrile	Pre	TA100	+H
Water	Pre	WP2uvrA(pKM101)	+R
DMSO	Plate	TA100	+H
Acetone	Pre	TA100	+H
Methanol	Plate	WP2uvrA(pKM101)	+R
DHF	Pre	TA100	+H
Methanol	Plate	WP2uvrA(pKM101)	+R
Methanol	Pre	WP2uvrA(pKM101)	+R
Water	Plate	WP2uvrA(pKM101)	+R

Sensitivity ranking using 90% confidence intervals of the BMD₁₀₀ (*i.e.*, 90% confidence interval of the dose estimated to cause a two-fold increase in response relative to vehicle control.). For each confidence interval, test conditions (*i.e.*, solvent, incubation method, strain and S9 source) are indicated in the Table on the right-side of the plot.

Thomas et al, Mutagenesis, 2024

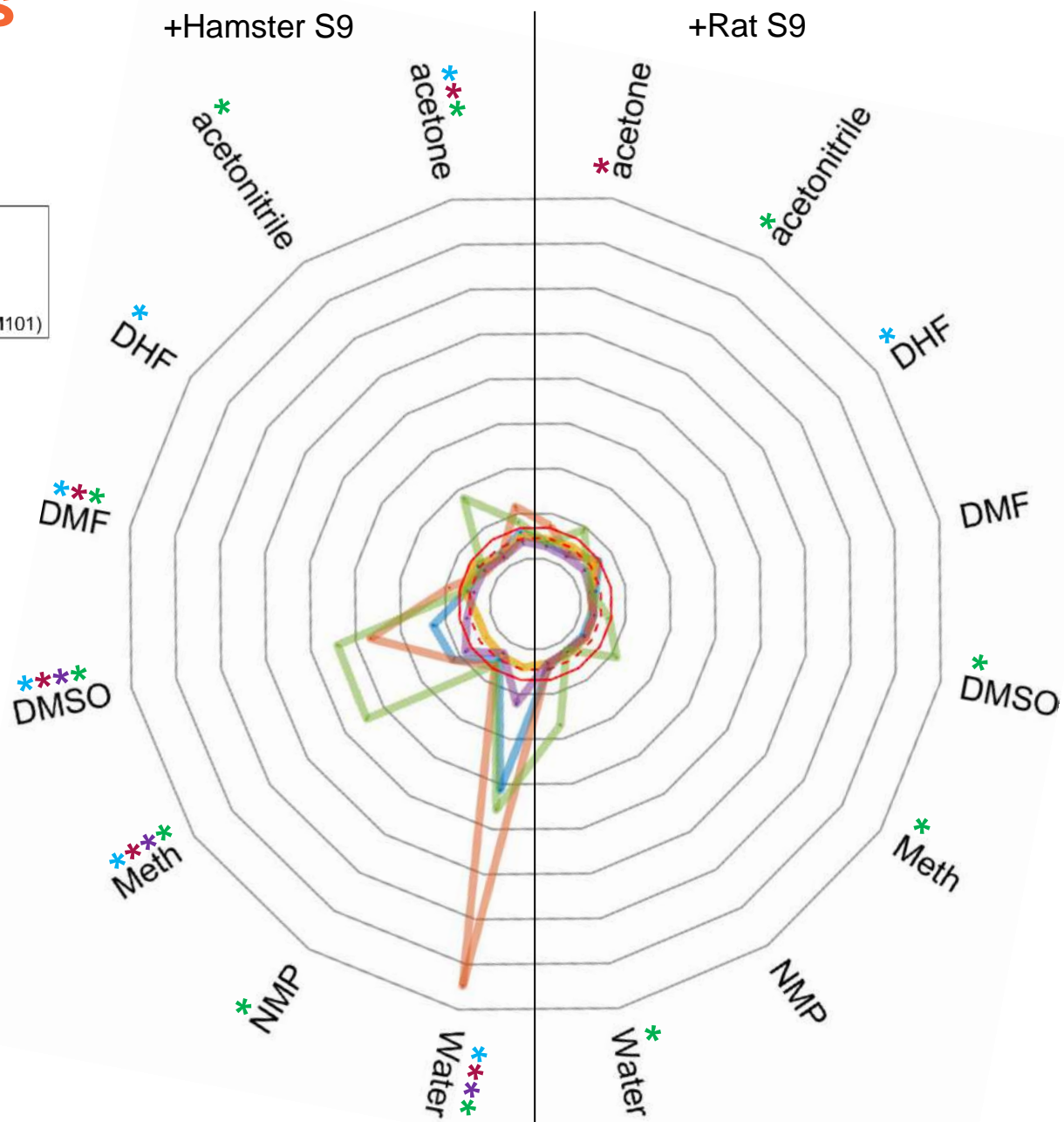
NDEA Ames data – Spider Plots

NDEA (plate incorporation)



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NDEA (pre-incubation)



NDEA Ames data – Quantitative Benchmark dose Analysis



Log₁₀ of BMD₁₀₀ (µg.plate)

← Increasing sensitivity

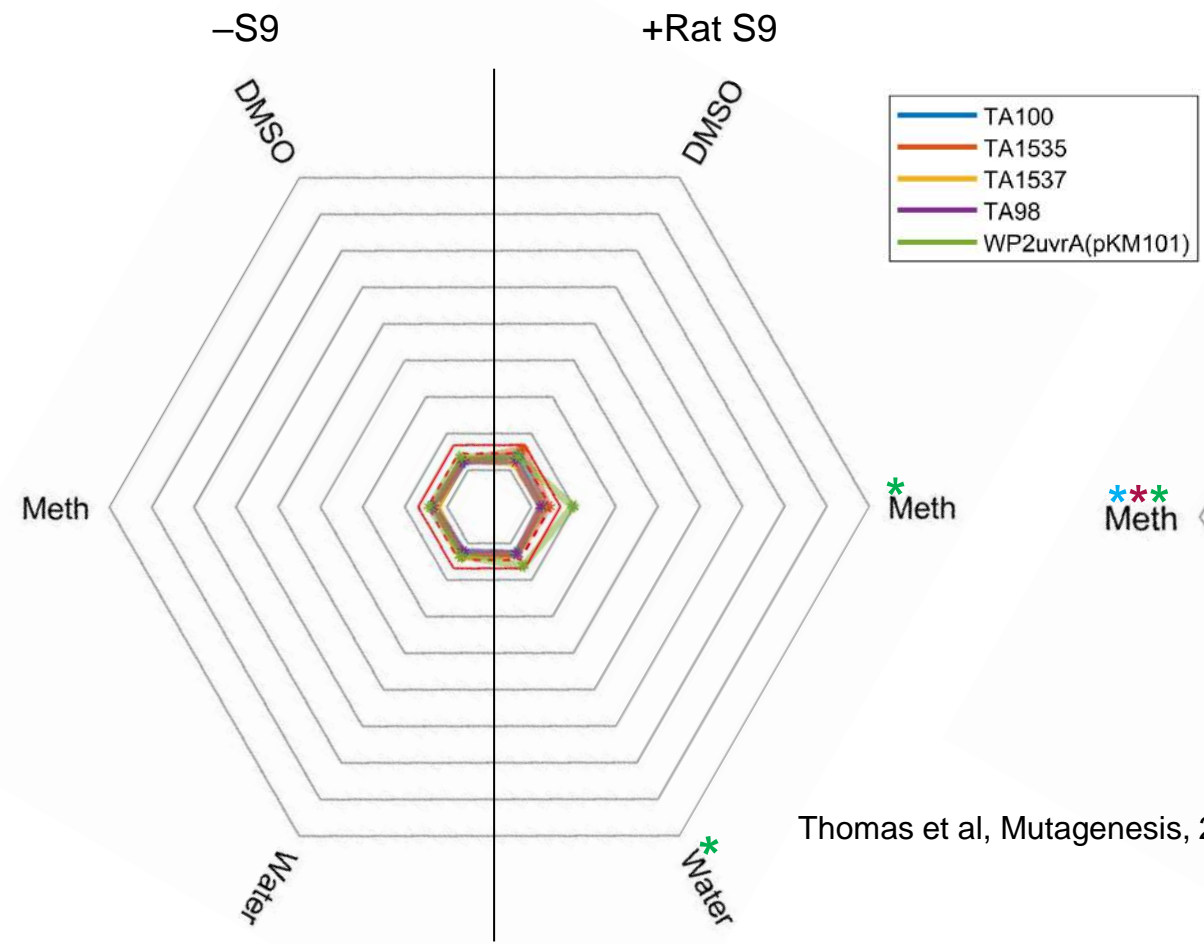
Water	Pre	WP2uvrA(pKM101)	+H
Methanol	Pre	WP2uvrA(pKM101)	+H
Acetonitrile	Pre	WP2uvrA(pKM101)	+H
Water	Pre	TA100	+H
Methanol	Plate	WP2uvrA(pKM101)	+H
Water	Pre	TA1535	+H
DMSO	Pre	WP2uvrA(pKM101)	+H
Water	Plate	WP2uvrA(pKM101)	+H
DMSO	Plate	WP2uvrA(pKM101)	+H
DMSO	Pre	TA1535	+H
Methanol	Plate	TA1535	+H
Water	Plate	TA1535	+H
DMSO	Plate	TA1535	+H
Acetone	Pre	TA1535	+H
Methanol	Plate	TA100	+H
Methanol	Plate	TA98	+H
Water	Pre	WP2uvrA(pKM101)	+R
Acetonitrile	Pre	WP2uvrA(pKM101)	+R
Methanol	Pre	TA100	+H
Water	Plate	TA100	+H
DMSO	Plate	TA98	+H
Methanol	Plate	WP2uvrA(pKM101)	+R
DMSO	Plate	TA100	+H
Water	Pre	TA98	+H
Water	Plate	WP2uvrA(pKM101)	+R
Water	Plate	TA98	+H
DMSO	Pre	TA100	+H
DMSO	Plate	WP2uvrA(pKM101)	+R
Methanol	Pre	TA1535	+H
Methanol	Pre	WP2uvrA(pKM101)	+R
DMF	Pre	TA1535	+H
Acetone	Pre	WP2uvrA(pKM101)	+H
NMP	Pre	TA1535	+H
DHF	Pre	TA100	+R
Acetone	Pre	TA1535	+R
DMSO	Pre	WP2uvrA(pKM101)	+R
Acetone	Pre	TA100	+H
Methanol	Pre	TA98	+H
NMP	Pre	WP2uvrA(pKM101)	+H
DMF	Pre	TA100	+H
DMF	Pre	WP2uvrA(pKM101)	+H
DMSO	Pre	TA98	+H

Sensitivity ranking using 90% confidence intervals of the BMD₁₀₀ (*i.e.*, 90% confidence interval of the dose estimated to cause a two-fold increase in response relative to vehicle control.). For each confidence interval, test conditions (*i.e.*, solvent, incubation method, strain and S9 source) are indicated in the Table on the right-side of the plot.

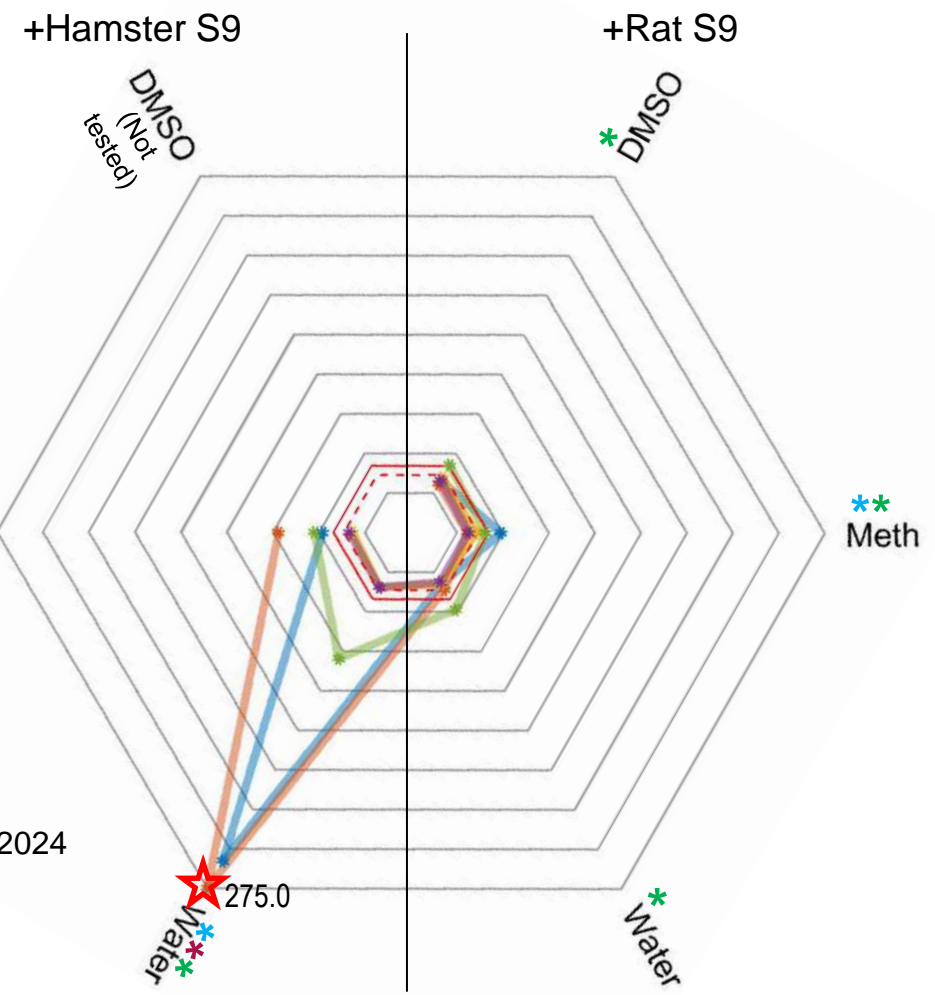
Thomas et al, Mutagenesis, 2024

NMEA Ames data – Spider Plots

NMEA (plate incorporation)



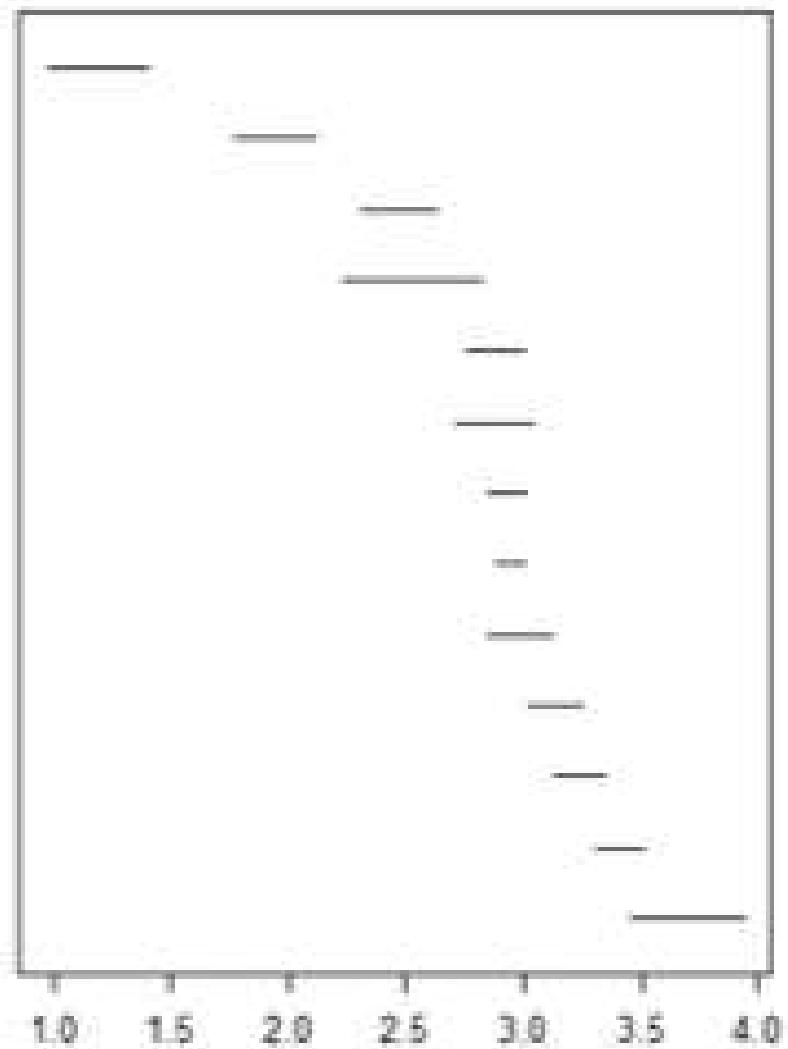
NMEA (pre-incubation)



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To confirm performance of the Ames test using the most sensitive assay parameters identified NMEA was also looked at

NMEA Ames data – Quantitative Benchmark dose Analysis



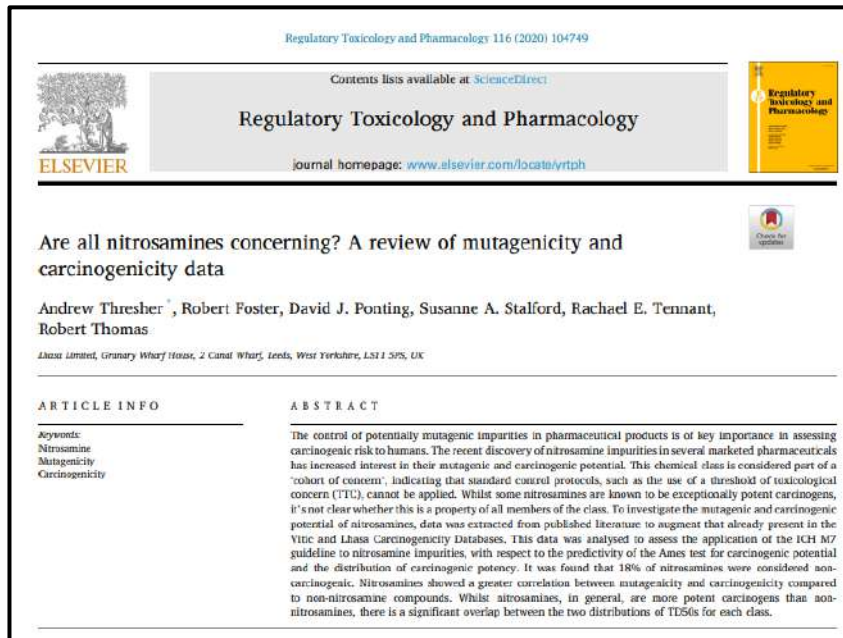
Water	Pre	TA1535	+H
Water	Pre	TA100	+H
Water	Pre	WP2uvrA(pKM101)	+H
Methanol	Pre	TA1535	+H
*Methanol	Plate	WP2uvrA(pKM101)	+R
Methanol	Pre	WP2uvrA(pKM101)	+H
Methanol	Pre	TA100	+R
Water	Pre	WP2uvrA(pKM101)	+R
Methanol	Pre	TA100	+H
Water	Plate	WP2uvrA(pKM101)	+R
DMSO	Pre	WP2uvrA(pKM101)	+R
Methanol	Pre	WP2uvrA(pKM101)	+R
Methanol	Plate	WP2uvrA(pKM101)	+R

Sensitivity ranking using 90% confidence intervals of the BMD_{100} (*i.e.*, 90% confidence interval of the dose estimated to cause a two-fold increase in response relative to vehicle control.). For each confidence interval, test conditions (*i.e.*, solvent, incubation method, strain and S9 source) are indicated in the Table on the right-side of the plot.

Log₁₀ of BMD₁₀₀ (µg.plate)

← Increasing sensitivity

Historic discordant “non-mutagenic” but carcinogenic nitrosamines



Nitrosamine
N-Methyl-N-neopentyl nitrous amide (NMNA; CAS 31820-22-1)
N-Isopropyl-N-methyl nitrous amide (NMIPA; CAS 30533-08-5)
N,N-Diisopropyl nitrous amide (NDIPA; CAS 601-77-4)
N,N-Bis(2-methoxyethyl) nitrous amide (BMNE; CAS 67856-65-9)
N-(4-Fluorophenyl)-N-methyl nitrous amide (MFNA; CAS 937-25-7)
N,N'-(Ethane-1,2-diyl)bis(N-(1-hydroxybutan-2-yl) nitrous amide) (NEHNA; CAS 52322-22-2)

Proposal – GSK “Ames Test” protocol used for NDMA and NDEA (and NMEA) deployed to test historically discordant *N*-nitrosamines.¹

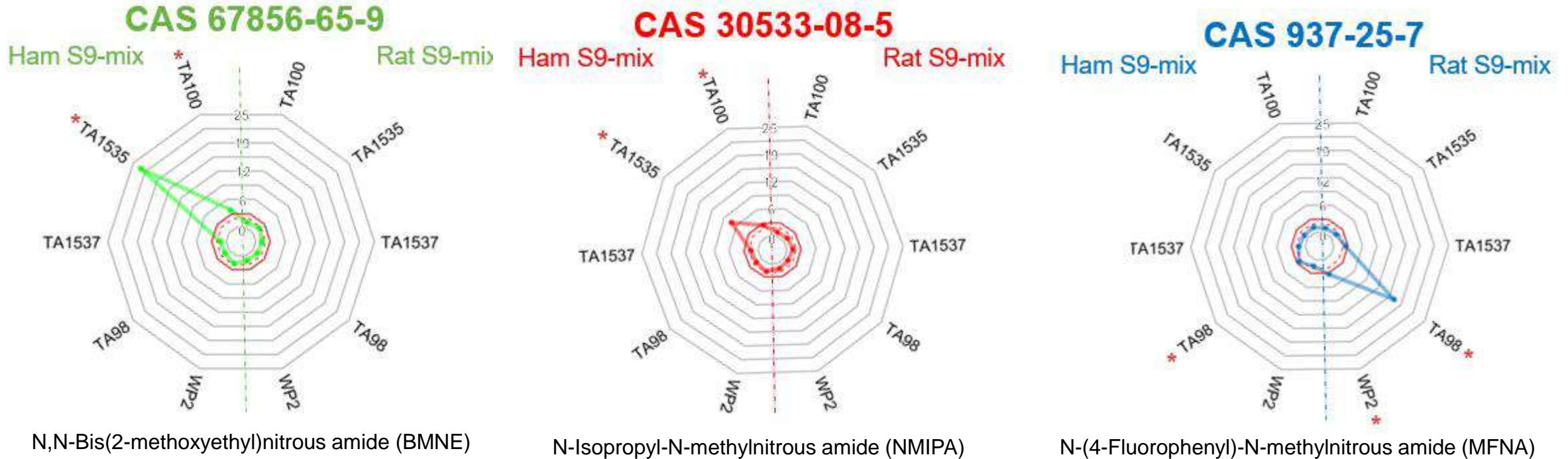
i.e. Ames mutagenicity negative, but rodent carcinogenicity positive

Historic discordant “non-mutagenic” *N*-nitrosamine data

Status – All the historic “non-mutagenic” *N*-nitrosamines are positive (i.e. mutagenic) in the GSK Ames Test.

Therefore, these *N*-nitrosamines **should not be considered as discordant non-mutagenic carcinogens.**

Key discriminating test parameters were pre-incubation using hamster S9 and strains TA1535 and TA100.



Data for 3 discordant *N*-nitrosamines (other in draft manuscript)

*	Positive response
---	2-Fold increase
—	3-Fold increase
—	Test article increase

Further Ames testing of *N*-nitrosamines of various CPCA category

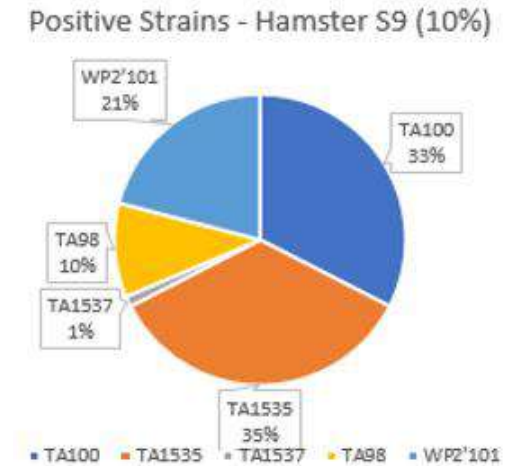
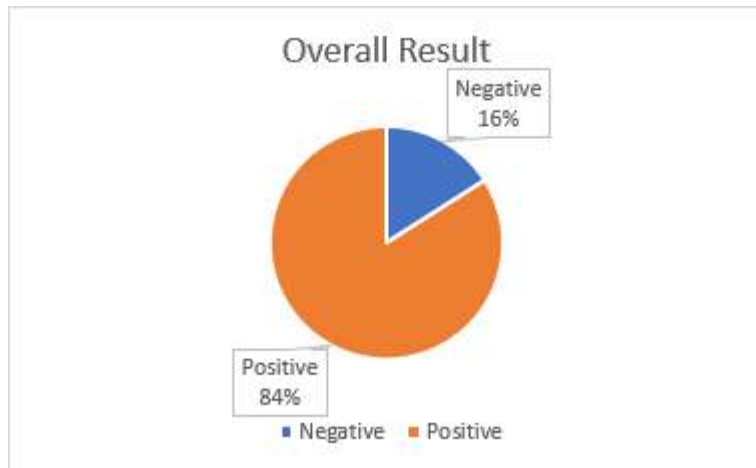
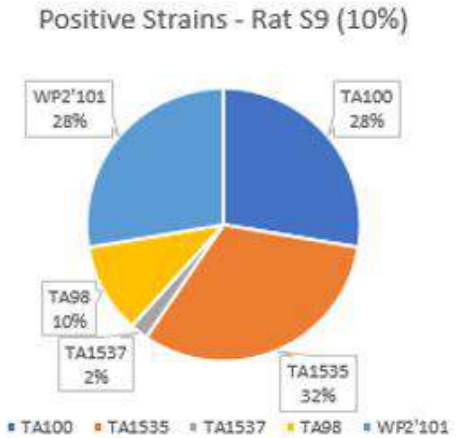
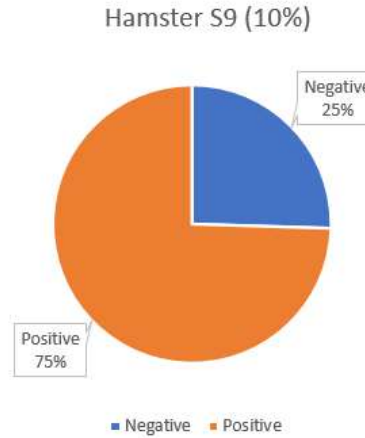
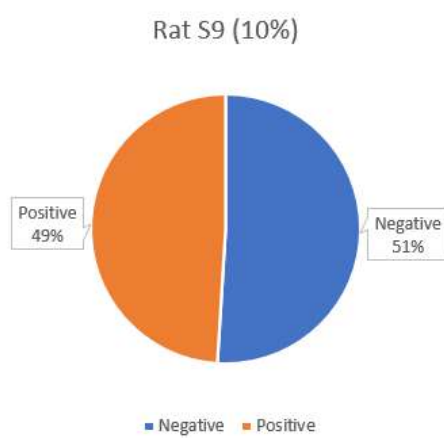
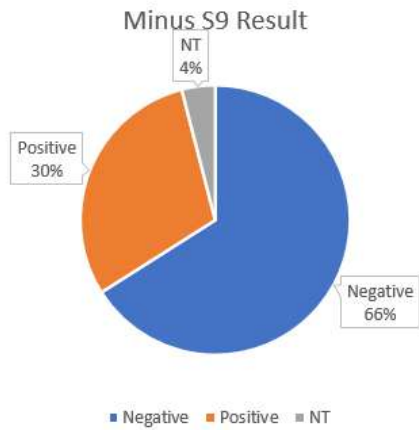
Proposal – Test a range of *N*-nitrosamines which are of significance scientifically and industrially, in our OECD compliant *in vitro* bacterial mutagenicity (Ames) assay, appropriately designed for assessing *N*-nitrosamines.

CAS	CPCA Potency Category
664985-71-1	2
124485-85-4	1
16219-99-1	2
102624-97-5	4
20689-96-7	1
70377-77-4	4
73742-54-8	3
13256-22-9	4
612-64-6	2
18907-82-9	4
25413-61-0	1
16339-04-1	3
65504-33-8	3
2613300-07-3	4
2624140-70-9	3
2624123-51-7	3
2624139-24-6	4
2624134-23-0	4
6415-68-5	4
40675-45-4	3
7633-57-0	4
61379-66-6	3
2624108-82-1	4
55556-85-9	4
68292-94-4	4

CAS	CPCA Potency Category
2418708-78-6	1
59665-02-0	1
2639422-25-4	3
69658-91-9	2
36702-44-0	4
21928-82-5	2
55557-03-4	4
81795-07-5	5
1314916-69-2	4
17721-95-8	5
6652-04-6	3
91180-79-9	4
1-nitroso-3-phenylpiperidine	3
89911-78-4	3
31820-22-1	1
67856-65-9	1
937-25-7	2
52322-22-2	4
61034-40-0	2
5632-47-3	3
53609-64-6	3
10595-95-6	1
924-46-9	1
1116-54-7	3
2375271-12-6	1

Further Ames testing of industrially significant *N*-nitrosamines

Status – GSK tested 50 *N*-nitrosamines of varying structure and CPCA category using the GSK enhanced Ames test method



Conclusions

Summary

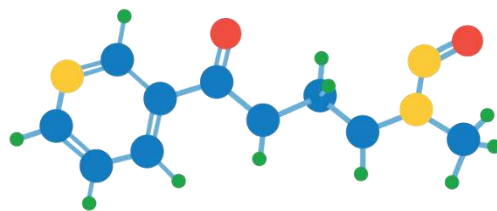
- Data confirms the mutagenicity of *N*-nitrosamines can be readily detected in the bacterial reverse mutation test (Ames Test), when an appropriate design is employed. ✓
- Assay parameters most sensitive for this class of compound include the pre-incubation method (with a 30-min incubation period) compared with plate-incorporation. ✓
- Water or methanol are good vehicle solvents for NAs, although a range of other solvents inc. DMSO are appropriate to assess NA mutagenicity. ✓
- Hamster induced liver S9 is the superior exogenous metabolic system, although rat liver S9 also elicits a positive response. ✓
- TA1535, TA100 and WP2uvrA (pKM101) are the most sensitive bacterial strains, and should be included as part of the 5-strain Ames Test when evaluating the mutagenicity of nitrosamines. ✓

Acknowledgements

Impurities	Jim Harvey	
Genetox	Jon Howe	Dean Thomas
	Abbie Williams	Mark Burman
	Ruby Buckley	Dannii Harte
DMPK	Helen Tracy	Sandy Baldwin
Modelling	John Wills	



MGRA Nitrosamines =

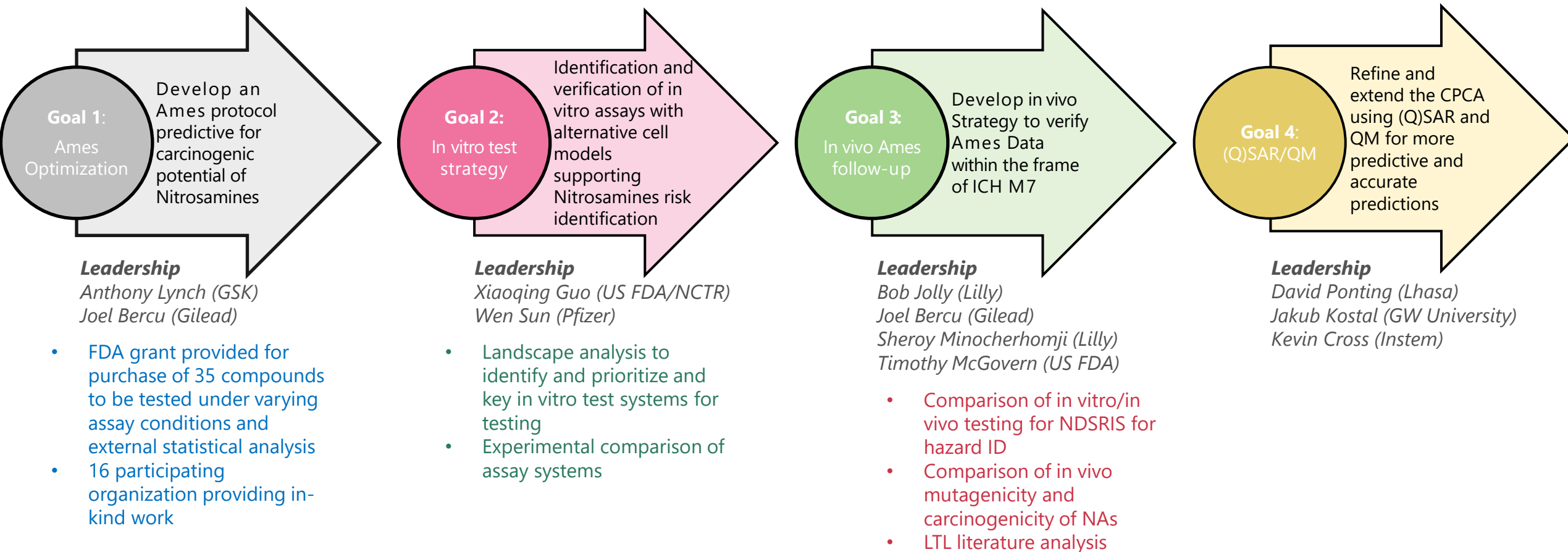


Nitrosamines
Research
Program

HESI
Genetic
Toxicology
Technical
Committee

Leadership

- Tetyana Cheairs (New York Medical College)
- Andreas Czich (Sanofi)



Goal 1:
Ames
Optimization

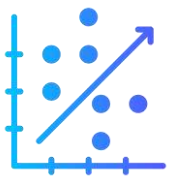
Develop an Ames protocol predictive for carcinogenic potential of Nitrosamines



Generate novel data that can be used to develop an Ames methodology (study method and data interpretation) that optimizes **sensitivity** for nitrosamine hazard detection.



Generate novel data to characterize the Ames' test predictivity of nitrosamines with known carcinogenicity (**specificity**).



Evaluate dose-response data relationships (e.g., BMD-derived potency rankings) to support optimization

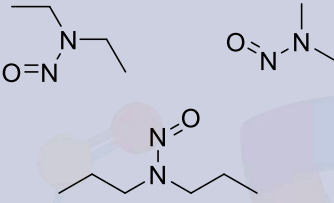
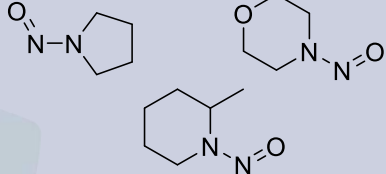
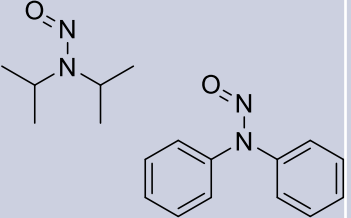
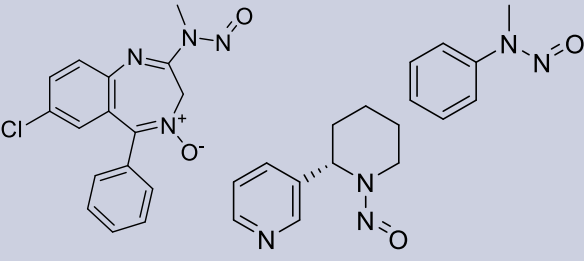
List of Nitrosamines for In Vitro Testing

Different Compound Classes

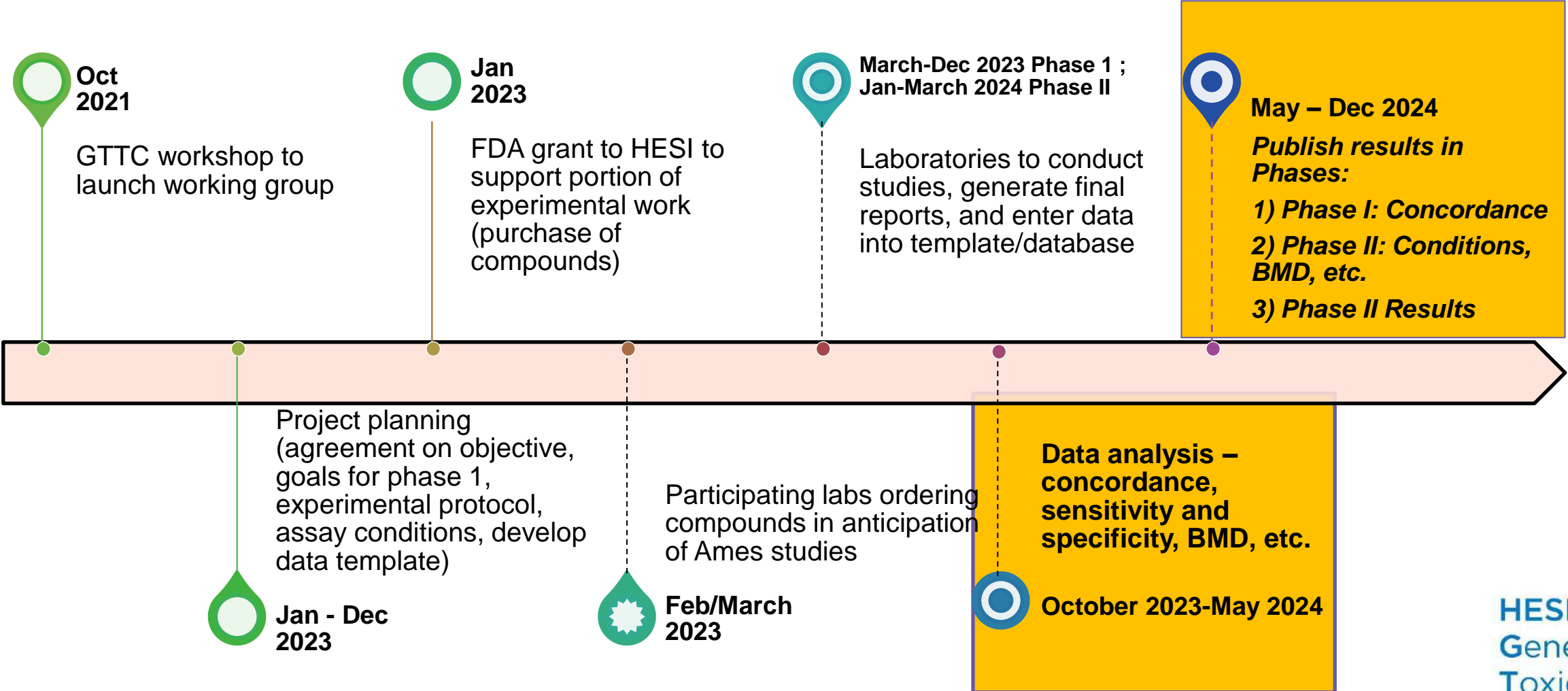
Mixture of carcinogenic and non-carcinogenic

- **32 Nitrosamines (2 labs per compounds)**
- 11 negatives and 21 positives for carcinogenicity

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

Simple aliphatic	Aliphatic, cyclic
	
Sterically hindered	Complex, Benzylic, Drug-Related
	

Milestones and Next Steps



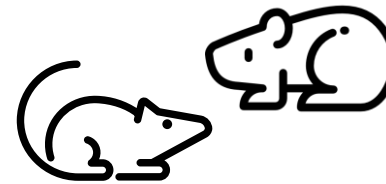
Preliminary conclusions (March 2024)



The **current Enhanced Ames Test (EAT) protocol is highly sensitive** for the prediction of the carcinogenicity for nitrosamines



Lab concordance was high. Exceptions included sterically hindered nitrosamines which are weakly or non-carcinogenic



Higher sensitivity was observed with the hamster compared rat induced liver S9 metabolic enzymes



DMSO as a solvent can be used for nitrosamines. NDMA seems to be an outlier based on specific metabolism of CYP2E1.

EAT – Enhanced Ames Test, NDMA – N-Nitrosodimethylamine

Abstract

Ames Test assay parameters important for the detection of N-Nitrosamine mutagenicity

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The evaluation of *N*-Nitrosamines (NAs) mutagenicity is an important subject for the pharmaceutical industry because certain NAs have been detected as impurities in some marketed drugs and, as a chemical class, they are considered potent mutagenic rodent carcinogens and part of the cohort of concern. Furthermore, the Ames Test has been under the spotlight because of concerns regarding its ability to accurately predict the carcinogenic potential of NAs. Historically several NAs were deemed discordant in terms of the rodent carcinogenicity i.e., they were Ames Test negative but rodent cancer bioassay positive. Several aspects of Ames Test study design were postulated as reasons to explain these discordant results: e.g., solvent choice, liver S9 species selection and other assay parameters (e.g., bacterial strain, compound dose and the use of the pre-incubation versus plate incorporation methods, etc.). We tested nitrosodimethylamine (NDMA) and nitrosodiethylamine (NDEA) in an extended Ames Test design and used qualitative and quantitative modelling approaches to compare various assay parameters to identify those specific elements deemed to correlate with mutagenic potency. We then used an optimised study design to re-evaluate several exemplar discordant NAs i.e. 2,2'-(1,2-Ethanediybis(nitrosoimino))di(1-butanol) (EDNA), Methyl (neopentyl) nitrosamine (MNNA), Methyl(4-fluorophenyl)nitrosamine (MFNA) and 2-methoxy-N-(2-methoxyethyl)-N-nitrosoethanamine (MENA). These chemicals were tested in a plate incubation test in the absence of induced liver S9-mix and pre-incubation tests in the presence of rat or hamster S9-mix, respectively. Our studies show that an OECD complaint Ames Test can detect the mutagenic activity of NDMA, NDEA, EDNA, MNNA, MFNA and MENA and the data will be discussed in terms of comparative assay parameters and mutagenic potency i.e., plate v pre-incubation, bacterial strain sensitivity and liver S9-species. In addition, we conclude EDNA, MNNA, MFNA and MENA should no longer be considered as “Ames Test” discordant. Based on these data an Ames Test study design is recommended for the evaluation of the potential mutagenicity of NAs that we believe will command confidence in the study outcome, and therefore support the regulatory control of NA impurities.