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Use of the bacterial reverse mutation assay to predict carcinogenicity of N-nitrosamines

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ABSTRACT

Under ICH M7, impurities are assessed using the bacterial reverse mutation assay (i.e., Ames test) when predicted positive using *in silico* methodologies followed by expert review. N-Nitrosamines (NAs) have been of recent concern as impurities in pharmaceuticals, mainly because of their potential to be highly potent mutagenic carcinogens in rodent bioassays. The purpose of this analysis was to determine the sensitivity of the Ames assay to predict the carcinogenic outcome with curated proprietary Vitic (n = 131) and Leadscope (n = 70) databases. NAs were selected if they had corresponding rodent carcinogenicity assays. Overall, the sensitivity/specificity of the Ames assay was 93–97% and 55–86%, respectively. The sensitivity of the Ames assay was not significantly impacted by plate incorporation (84–89%) versus preincubation (82–89%). Sensitivity was not significantly different between use of rat and hamster liver induced S9 (80–93% versus 77–96%). The sensitivity of the Ames is high when using DMSO as a solvent (87–88%). Based on the analysis of these databases, the Ames assay conducted under OECD 471 guidelines is highly sensitive for detecting the carcinogenic hazards of NAs.

1. Introduction

The bacterial reverse mutation test (commonly referred to as Ames test) uses strains of *Salmonella typhimurium* (*S. typhimurium*) and *Escherichia coli* (*E. coli*) which require an external source of amino acid for growth (Ames et al., 1975; Gatehouse et al., 1994; Maron and Ames, 1983). Point mutations can be detected in the Ames assay as it restores the bacterial capability (reverts) to synthesize the essential amino acid and allows for sustained growth. The Ames test has been the cornerstone of genetic toxicology as it is the standard assay highlighted in ICH S2 (R1) guidelines (ICH, 2011) for pharmaceutical development as it has been shown to detect relevant mutagenic effects in a majority of rodent and human carcinogens.

The Organization for Economic Development (OECD) 471 guideline harmonizes the process for the conduct of the Ames based on a large database of a wide variety of chemicals. An OECD 471 (OECD, 1997) validated assay includes selection of 5 appropriate strains that detect base or frameshift mutations that occur at the GC and AT base pair primary reversion site. The recommended top dose in the assay is 5 mg/plate. There are two main methodologies used in the Ames assay; plate incorporation and preincubation methods. The plate incorporation

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Abbreviations

AI	Acceptable Intake
API	Active pharmaceutical ingredients
BA	Balance accuracy
COC	Cohort of concern
DMSO	Dimethyl sulfoxide
EMA	European Medicines Agency
FN	False negative
FP	False positive
HA	Health authorities
NAs	Nitrosamines
MAHs	Marketing authorization holders
NDMA	*N*-Nitroso-dimethylamine
NNPV	Normalized negative predictive value
NPPV	Normalized positive predictive value
PDE	Permissible daily exposure
SAR	Structure Activity Relationship
TN	True negative
TP	True positive
QSAR	Quantitative structural activity relationship

method has fresh bacterial culture and sterile buffer or a metabolic activation mixture (typically a cytochrome P450 induced liver fraction and co-factors referred to as S9-mix) mixed with overlay agar which is then poured on the surface of the agar plate. The preincubation method involves incubating the test substance in a sterile buffer or S9-mix usually for 20 min (or more) and then mixed with the overlay agar which is then poured onto the surface of the agar plate. The primary metabolic activation system is rat induced liver S9, but other species can be used based on the chemical class. The output of the Ames is presented as the number of revertant colonies per plate. Different criteria may be employed per strain within a lab or between laboratories to define a 'positive' (mutagenic) response. Criteria for a positive response typically includes a concentration-related, and reproducible response.

In the manufacturing process of pharmaceutical compounds, impurities are controlled based on their innate toxicological hazards. Mutagenic impurities are identified and controlled in accordance with ICH M7 (R1) (ICH 2017). As such, impurities are analyzed using two (quantitative) structural activity relationship ((Q)SAR) models (i.e., *in silico* rules-based and statistics-based models), followed by expert review to determine their potential for mutagenicity. If an impurity is predicted to be positive using (Q)SAR, then the next step is Ames testing, or the impurity should be controlled as if it were mutagenic. If positive in the Ames test, then the impurity is controlled to the appropriate acceptable intake (AI) to reduce the potential excess risk of cancer to 1 in 100,000 or to a permissible daily exposure (PDE) if a threshold mechanism has been identified. Negative results in an OECD 471-compliant Ames test allows the impurity to be managed without concern for mutagenic carcinogenicity (ICH 2006a; ICH 2006b).

N-Nitrosamines (NAs) are organic compounds of the chemical structure $R_2N–N=O$. Nitrosamines are formed by a reaction between a nitrosating agent such as nitrite and secondary amines and can be found in processed foods, drinking water, cosmetics and cigarette smoke (Gushgari and Halden, 2018; Lee, 2019). Many NAs are classified as animal carcinogens (Buist et al., 2015). Out of 228 NAs tested, 81% were considered carcinogenic in animals (Thresher et al., 2020). Most NAs are considered carcinogenic via a mutagenic mechanism of action (Guttenplan, 1987b; Lijinsky, 1987). Out of the 381 NAs tested in the Ames, 81% were also considered mutagenic (Thresher et al., 2020). The metabolism to form the reactive metabolite depends on the type of NA. For example, short-chain aliphatic NAs generally involve metabolic activation via alpha-hydroxylation adjacent to the amine; however there are also many other mechanisms for metabolic activation, especially where the NA does not contain a simple ethyl or methyl group (Guttenplan, 1987a).

NAs have been observed as trace impurities in some pharmaceutical products (Teasdale, 2020). In June 2018, *N*-nitrosodimethylamine (NDMA) was detected as an impurity stemming from the drug substance synthesis of valsartan, an angiotensin-II receptor antagonist used for the treatment of hypertension (Snodin and Elder, 2019). Since then, the control of NA impurities has become a focus for pharmaceutical manufacturers and health authorities (HAs) and led to several product recalls when identified to be above an AI in active pharmaceutical ingredient (API) batches of marketed drugs. In 2019, the European Medicines Agency (EMA) asked marketing authorization holders (MAHs) to take precautionary measures to mitigate the risk of NA formation or presence during the manufacture of all medicines containing chemically synthesized active substances. As part of Article 5 (3) of Regulation (EC) No 726/2004, MAHs were requested to review their manufacturing processes to identify and, if necessary, mitigate the risk of presence of NA impurities (EMA 2020a; EMA 2020b). This three-step review process requires the pharmaceutical industry to conduct a NA risk assessment, to perform analytical testing to confirm the presence and amount of NA, and finally if NA presence is confirmed at above acceptable levels defined based on rodent carcinogenicity data or structure activity relationship (SAR) and/or read-across, to apply changes to the manufacturing process. This NA risk assessment has been adopted by other HAs as well, including Health Canada, United States Food and Drug Administration, and Swissmedic (Health Canada, 2020; Swissmedic, 2020; USFDA 2020). Implementation of HA mitigation and control recommendations need to be managed in a practical, patient-focused and targeted manner to ensure a safe drug supply while avoiding critical drug shortages.

NAs are considered cohort of concern (COC) impurities given that many in the class are highly potent animal carcinogens and may require lower AIs than standard mutagenic impurities (ICH 2017). There was an additional concern raised by HAs about the use of the Ames test to de-risk NAs in accordance with ICH M7. The concern was that the Ames test may not be sensitive enough to detect the carcinogenic potential of NAs and this concern stemmed from older published research that was not conducted under OECD 471 guidelines (Rao et al., 1979). Out of 25 aliphatic NA compounds evaluated for mutagenicity in Rao et al. (1979), 18 were reported carcinogens in the rodent bioassay and 9 of these carcinogenic compounds were determined to be non-mutagenic after testing up to 2 mg/plate with both a plate incorporation and a pre-incubation Ames test procedure.

Current analysis of NAs using the Vitic 2020.1 database indicated that the Ames test is able to predict the carcinogenic hazard from a rodent bioassay (Thresher et al., 2020). When comparing Ames test results with carcinogenicity bioassay outcomes the NA Ames sensitivity was 94% (171 compounds). Interestingly, the sensitivity is much higher compared to an Ames sensitivity of 53% for non-NAs (1862 compounds).

The current OECD 471 guideline highlights some considerations when testing NAs in the Ames assay. First, the preincubation method may be more suitable for short-chain aliphatic NAs. This is because these NAs are more efficiently detected using the preincubation method (Araki, 1984; Bartsch, 1976; Gatehouse et al., 1994). Solvents should not be suspected to chemically react with the test substance and be compatible with the survival of the bacteria and S9 metabolic activity. While many of the short chain aliphatics are soluble in water, larger, more complex nitrosamines are likely hydrophobic and only dissolved in more polar solvents such as DMSO. DMSO has been known to inhibit the mutagenic activity of NDMA and *N*-nitrosodiethylamine (NDEA), two highly potent carcinogens (Maron, 1981; Yahagi et al., 1977). This is likely due to the inhibition of P450 isozyme CYP2E1 (Yamazaki, 1992). However, other P450 isozymes play a critical role with metabolism of

NAs such as CYP2A6, CYP2C19, CYP2D6, and CYP3A4, especially for larger molecular weight NAs (Cross and Ponting, 2021). Finally, while rat induced liver S9 is the most common metabolic activation preparation for the Ames assay conducted under OECD 471 guidelines, the selection of species for liver S9 preparations also can play a role in mutagenic outcome for NAs. Early literature suggests that hamster induced S9 may be more efficient for metabolizing some NAs resulting in a stronger mutagenic dose-response in the Ames test (Araki, 1984; Lijinsky and Andrews 1983).

One aim of this manuscript was to perform an analysis using well-curated proprietary databases, Leadscope and Vitic, for NAs with both Ames test mutagenicity and rodent carcinogenicity data to determine the sensitivity of the Ames test for identification of the carcinogenic potential of NAs when using standard OECD 471 methodology. Another aim was to assess various conditions used in the Ames test to determine the effect on the sensitivity of the Ames test to predict rodent carcinogenicity outcomes. Among the conditions examined were the use of OECD recommended Ames tester strains, species of induced liver S9 fraction used (i.e., rat versus hamster), pre-incubation versus plate incorporation methodology, and use of solvent (i.e., DMSO versus water) since each aspect has been reported to play a role in the ability of the Ames test to correctly identify potentially carcinogenic NAs. The outcome of this study will help guide future testing strategies for the Ames, maximizing its ability to correctly predict the carcinogenic outcome in the rodent bioassay.

2. Methods

2.1. Lhasa Vitic database analysis

Studies related to alkyl, cyclic, heteroaromatic and phenyl NAs (Table 1), excluding nitrosocarbamates, nitrosoureas and nitrosoamides, were extracted from the Lhasa Vitic database version 2020.1. Data related to the Ames test and rodent carcinogenicity studies were subsequently filtered and analyzed. Data extraction and analysis were conducted using the KNIME Analytics Platform version 4.2. An overall Ames test activity call was generated for each compound within each subgroup using a previously defined workflow (Thresher et al., 2020). This workflow was also used to generate an overall carcinogenicity call for each compound to enable a comparison of mutagenic and carcinogenic activity within each subgroup.

2.2. Leadscope database analysis

The Ames test data for alkyl, cyclic, heteroaromatic and phenyl NAs (Table 1) (excluding nitrosocarbamates, nitrosoureas and nitrosoamides) in the Leadscope Genetox database (2020) were analyzed for comparison of outcomes given different experimental conditions and against rodent carcinogenicity data. Initially all compound records containing any Ames test results were extracted and tabulated for correlation with known NA carcinogenicity data. The carcinogenicity data for comparison was extracted from the Leadscope Carcinogenicity database (2020) as well as the training sets for the Leadscope FDA Rodent Carcinogenicity (Q)SAR models (version 2). The Ames test results for the initial tabulation did not have to include OECD 471 recommended bacterial strains or comply with test guidelines concerning the number and types of strains to test for a negative result, etc. Next, only the Ames test data for NAs that were graded by Leadscope in the Genetox database were analyzed for comparison. These data were from Ames test studies only tested with OECD 471 recommended bacterial strains and judged to be sufficiently complete to either use an author assigned study call or, in the case where one did not exist, to calculate a study call based upon the Leadscope grading algorithm for bacterial mutation studies. In cases with multiple studies per compound, the Leadscope grading algorithm considered each study and calculated an overall Ames test result for the compound. The carcinogenicity studies for comparison met the Leadscope and FDA criteria set by the FDA Cancer Assessment Committee for determining a reliable carcinogenicity study call (USFDA, 2007).

Table 1
Count of NAs in Lhasa Vitic and Leadscope Genetox Databases with bacterial mutation and carcinogenicity data.

Chemical Description of NA	Lhasa Vitic (#)	Leadscope Genetox (#)
Alkyl	23	17
Aliphatic with alcohol- keto- carboxy and/or ester	31	23
Alkyl chain with other polar groups	11	4
Aliphatic cyclic	35	15
Cyclic alkyl with alcohol-keto-carboxy and/or ester	12	4
Heteroaromatic substituted	7	4
Phenyl	12	3
Total	131	70

2.3. Analysis and statistics

The following data and subsets of data were analyzed to understand how different conditions of the Ames test influences the predictivity of the assay for NAs carcinogenicity.

- All Ames test data (as baseline to compare against OECD recommended parameters)
- Using OECD recommended bacterial strains only
- Using OECD recommended strains with rat S9 as metabolic activation
- Using OECD recommended strains with hamster S9 as metabolic activation
- Using OECD recommended strains according to the plate incorporation protocol
- Using OECD recommended strains according to the preincubation protocol
- Using OECD recommended strains under plate incorporation protocol with rat S9 metabolic activation
- Using OECD recommended strains under preincubation protocol with rat S9 metabolic activation
- Using OECD recommended strains with dimethyl sulfoxide (DMSO) as solvent
- Using OECD recommended strains with water as solvent

Data from the literature search of Vitic, and Leadscope were then analyzed using Cooper statistics (Cooper and Herskovits, 1992). Outcomes included normalized positive predictive value (NPPV) and normalized negative predictive value (NNPV), specificity, sensitivity, and balanced accuracy (BA). BA is the average of sensitivity and specificity. Although the current datasets show a high prevalence of carcinogenic compounds (136 of 174 (78.2%) for the Vitic dataset and 122 out of 141 (86.5%) for the Leadscope dataset), the chemical space for which data is available is biased towards small aliphatic NAs that are more likely to be carcinogenic. For this reason, we present the positive and negative predictivities against a balanced background rate of 50% carcinogenicity (normalized PPV/NPV) which may better represent the wider chemical space for NAs. For additional analysis of predictivity values against different background rates for the Vitic dataset, see Supplement 1.

The equations for normalized positive and negative predictivity follow, where TP = true positives count, FP = false positives count, TN = true negatives count, and FN = false negatives count.

$$\text{Normalized Positive Predictivity (NPPV)} = \frac{TP/\%\ \text{Actives}}{TP/\%\ \text{Actives} + FP\ /(1 - \%\ \text{Actives})}$$

$$\text{Normalized Negative Predictivity (NNPV)} = \frac{TN\ /(1 - \%\ \text{Actives})}{(TN\ /(1 - \%\ \text{Actives}) + FN/\%\ \text{Actives})}$$

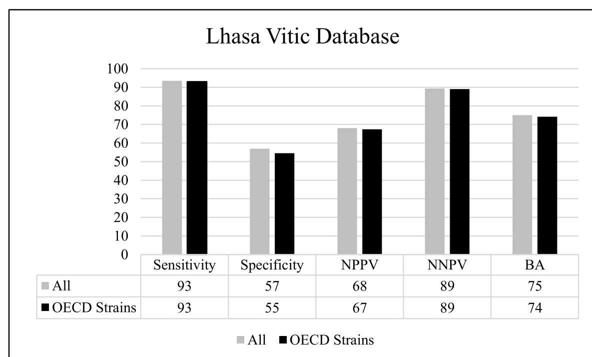
Sensitivity indicates how well a model will find true positives in a data set, and the NNPV indicates the probability of a negative result being correct. These are the statistics of greatest importance when assessing and comparing predictive models of toxicity that are used for regulatory decision-making (Landry et al., 2019).

3. Results

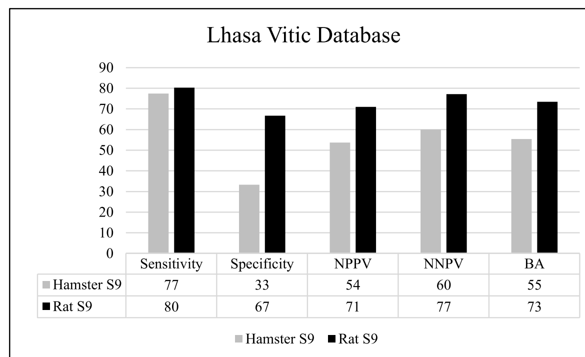
3.1. Lhasa Vitic database

The Vitic database contained Ames test data for 279 NA compounds. However, only 131 of these include an activity call for both the Ames test and rodent carcinogenicity bioassays after removing those without a call or with either equivocal or conflicted calls. There was a high level of correlation between the Ames test calls and carcinogenic activity, with 85% of compounds showing matching calls (Fig. 1A). When the data set was restricted to include only those Ames tests that used bacterial strains recommended in the current OECD guideline 471, there remained a total of 114 compounds with both robust Ames test and rodent carcinogenicity calls. The classification was used to increase confidence in the Ames test data used for the comparison and although the number of compounds in the data set was reduced by this restriction, none of the Ames test activity calls were altered by the removal of the studies that used the non-OECD-recommended bacterial strain data. Therefore, the

A. Strains (All Data (n = 131) vs OECD Strains (n = 114))

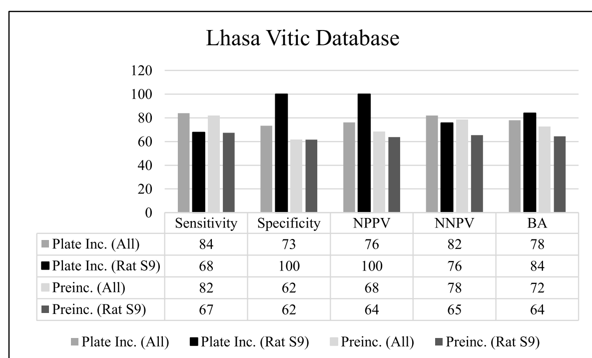


C. Metabolic activation (Hamster S9 (n = 34) vs Rat S9 (n = 89))



B. Methods (Plate Incorporation All (n = 88) /Rat S9 (n = 69)) vs

(Preincubation All (n = 64) /Rat S9 (n = 56))



D. Solvent (DMSO (n = 72) vs Water (n = 21))

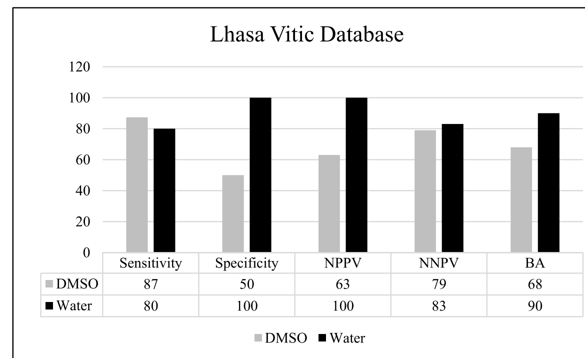


Fig. 1. Lhasa Vitic Performance Metrics of Ames calls vs Carcinogenicity
A. Strains (All Data (n = 131) vs OECD Strains (n = 114))
B. Methods (Plate Incorporation All (n = 88)/Rat S9 (n = 69)) vs (Preincubation All (n = 64)/Rat S9 (n = 56))
C. Metabolic activation (Hamster S9 (n = 34) vs Rat S9 (n = 89))
D. Solvent (DMSO (n = 72) vs Water (n = 21)).

predictivity of the Ames data for carcinogenic activity remains unchanged. Also noted was that the sensitivity was extremely high (93%) in both all and OECD strain only for NAs.

In order to compare the predictivity of different Ames test protocols for carcinogenicity, Ames test summary calls were generated using only studies performed according to either plate incorporation or preincubation methods. The positive/negative designation was based on the author's conclusion. The data set included 88 and 64 compounds with both Ames test and rodent bioassay carcinogenicity calls for plate incorporation and preincubation methods, respectively. While the preincubation method showed a decrease in specificity compared to the plate incorporation method, there was little difference in the overall correlation with rodent carcinogenic activity in terms of BA (Fig. 1B). Overall, 82% and 78% of Ames test calls correctly matched the rodent carcinogenicity calls for the plate incorporation and preincubation subgroups, respectively. In addition, the sensitivity for plate incorporation versus preincubation was very similar, 84% versus 82%. When considering plate incorporation versus preincubation that used induced rat liver S9, the sensitivity was 68% and 67%, respectively.

A total of 46 compounds were tested for both plate incorporation and preincubation methods and included a rodent carcinogenicity call. The plate incorporation and preincubation methods produced the same Ames test call in 35 (76%) of these compounds. Of the 11 compounds (24%) where the Ames test calls differed, 7 were negative in plate incorporation but positive in preincubation protocols and 4 were positive in plate incorporation but negative in preincubation protocols. All but one of these compounds were considered positive for carcinogenicity in the rodent bioassay, and the non-carcinogen was predicted to be Ames test negative using the plate incorporation method but positive using the preincubation method. However, 5 of these mismatches are not strictly like-for-like comparisons, in that the study conditions producing the positive responses were not reproduced in both protocols. In one case, the positive plate incorporation response was observed in the bacterial strain TA1537 which was not tested in the preincubation protocol. Similarly, a further 3 compounds showed positive plate incorporation results only in the presence of hamster induced liver S9 fraction, which was not tested under the preincubation method.

To compare the effect of metabolic activation type on predictivity for carcinogenicity, Ames test calls were generated using only data from studies using either rat induced liver S9 or hamster induced liver S9 for metabolic activation. After removal of compounds which did not also have a rodent bioassay carcinogenicity call, there remained 89 Ames test calls with rat induced liver S9 and 34 with hamster induced liver S9. The rat induced liver S9 group appears to show comparable sensitivity with the hamster induced liver S9 group, which was 80% and 77% for rat and hamster, respectively (Fig. 1C). However, the specificity was much lower with hamster induced liver S9 (33% compared to 67% for rat).

A total of 22 compounds were tested with both rat and hamster induced liver S9 and have a rodent bioassay carcinogenicity call. Of these, 10 carcinogenic compounds (45%) showed different Ames test calls in the two subgroups, with 7 showing negative calls with rat induced liver S9 and positive calls with hamster induced liver S9, and 3 showing positive calls with rat induced liver S9 and negative calls with hamster induced liver S9. The 3 compounds showing positive rat S9 calls all had other protocol differences between the rat induced liver S9 and hamster induced liver S9 studies which may account for the divergent calls. In 2 cases the bacterial strain(s) showing the positive response (TA1535/TA1537) was not tested with hamster induced liver S9, while in the other case the positive rat induced liver S9 response was only observed when using a plate incorporation protocol, while the compound was only tested in the preincubation protocol with hamster induced liver S9. All 7 compounds showing negative rat induced liver S9 and positive hamster induced liver S9 responses were tested in directly comparable studies across the 2 subgroups, but with limited experimental conditions (e.g. either testing was limited to one or two bacterial strains, or treatments did not reach the limit concentration of 5000 μg/plate).

Finally, Ames test calls were generated for subgroups based on the solvent used. A total of 72 and 21 compounds contained both a rodent bioassay carcinogenicity call and an Ames test call using DMSO or water, respectively. Additional solvent types were not considered due to an insufficient amount of data available for analysis. The DMSO group shows a lower specificity (50%) compared to the water group (100%). The sensitivity was comparable between DMSO (87%) and water (80%) (Fig. 1D). However, the low number of compounds tested with water makes an accurate comparison difficult.

Thirteen compounds were present in both solvent subgroups and had a rodent bioassay carcinogenicity call, of which 10 showed concordant Ames test calls. Of the 3 compounds where the calls differed, 1 was negative in DMSO, positive in water and positive for rodent carcinogenicity, and 2 were positive in DMSO, negative in water (1 positive, 1 negative for carcinogenicity). However, positive responses for all 3 compounds were attained under conditions which were not replicated when using the other solvent, either differing in the use of hamster liver S9, the preincubation protocol or both.

3.2. Leadscope genotoxicity and carcinogenicity database

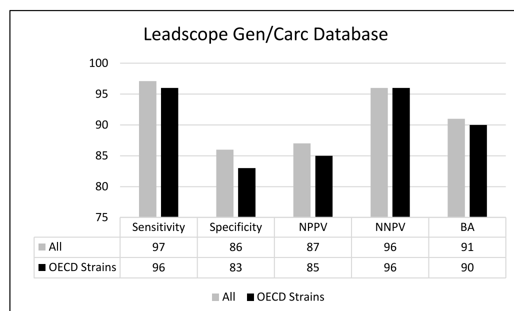
The Leadscope Genotoxicity database contained Ames test data for 225 selected NA compounds having one or more study calls. However, only 70 of these included an activity call for both the Ames test and rodent bioassay for carcinogenicity after removing those containing either equivocal or conflicted calls. There was a high level of correlation between the Ames test calls and rodent carcinogenic activity, with 91% BA (Fig. 2A). When the data set was restricted to include only those bacterial strains listed in the current OECD guideline 471, there remained a total of 63 compounds with both Ames test and rodent carcinogenicity calls. While this classification reduced the number of compounds analyzed in the data set, none of the Ames test activity calls of any compounds were altered by the removal of the non-OECD recommended bacterial strain data. Therefore, the predictivity of the Ames test data for carcinogenic activity remains unchanged. The correlation between Ames test calls and rodent bioassay carcinogenic activity were similar (91% versus 90%). Sensitivity was extremely high when considering all or only OECD 471 recommended bacterial strains for NAs (97% versus 96%).

To compare the predictivity of different Ames test protocols for rodent carcinogenicity, Ames test study calls of compounds were considered only for studies performed according to either plate incorporation or preincubation methods. No study calls were rescored or changed. Existing study calls were merely compared between the two methods. The data set included 19 and 30 compounds with both Ames test and rodent carcinogenicity calls for plate incorporation and preincubation methods, respectively. The specificity for both plate incorporation and preincubation was not meaningful due to the presence of few negative studies in the data set which hindered this analysis (Fig. 2B). Sensitivity remained high amongst both sets at 89% with BA at 94% and 70%, respectively. In a separate analysis of the Ames test data that compared plate incorporation versus preincubation methods that used rat induced liver S9, the sensitivity was 87% for plate incorporation and 80% for preincubation, respectively.

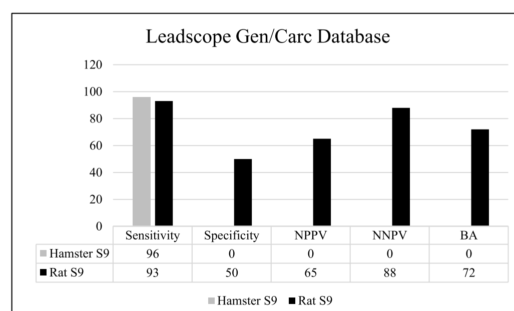
A total of 18 compounds with rodent bioassay carcinogenicity calls were tested using both Ames test plate incorporation and preincubation methods. The plate incorporation and preincubation methods produced the same Ames test call for all compounds.

To compare the effect of metabolic activation on predictivity for rodent carcinogenicity, Ames test study calls were considered from OECD 471 compliant studies using liver S9 derived from induced rats or hamster. Ames test study calls using rat induced liver S9 were considered separately from those using hamster induced liver S9. In cases where both species were used in a single study, test calls particular to each species were used to construct a species-specific study call. After

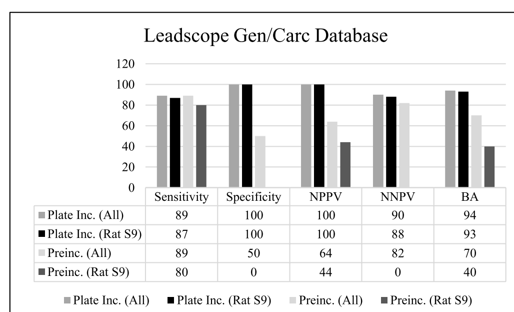
A. Strains (All Data (n = 70) vs OECD Strains (n = 63))



C. Metabolic activation (Hamster S9 (n = 23) vs Rat S9 (n = 45))



B. Methods (Plate Incorporation All (n = 19) /Rat S9 (n = 16)) vs Ames (Preincubation All (n = 30) /Rat S9 (n = 26))



D. Solvent (DMSO (n = 18) vs Water (n = 21))

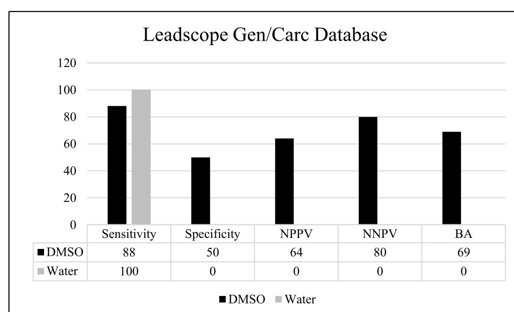


Fig. 2. Leadscope Performance Metrics of Ames calls vs Carcinogenicity
A. Strains (All Data (n = 70) vs OECD Strains (n = 63))
B. Methods (Plate Incorporation All (n = 19)/Rat S9 (n = 16)) vs Ames (Preincubation All (n = 30)/Rat S9 (n = 26))
C. Metabolic activation (Hamster S9 (n = 23) vs Rat S9 (n = 45))
D. Solvent (DMSO (n = 18) vs Water (n = 21)).

removal of compounds not having a rodent bioassay carcinogenicity call, there remained 45 compounds with rat induced liver S9 and 23 with hamster induced liver S9 Ames test calls. The rat induced liver S9 study results compared favorably with the hamster induced liver S9 studies with a sensitivity of 93% and 96% for rat and hamster liver preparations, respectively (Fig. 2C). The specificities and negative predictivities between the two groups could not be compared as the hamster liver S9 group contained no negative rodent bioassay carcinogenicity studies.

A total of 22 compounds with rodent carcinogenicity calls were evaluated in the Ames test (using both rat and hamster induced liver S9). All 22 rodent bioassay carcinogenicity calls were positive: 21 tested positive with hamster induced liver S9 while 19 tested positive with rat induced liver S9. All 19 positive tests with rat induced liver S9 were also positive when tested with hamster induced liver S9. Only two compounds showed different Ames test calls between the two subgroups, with both compounds testing negative with rat induced liver S9. These 2 compounds were both tested in directly comparable studies and conditions across the 2 subgroups. The first compound was tested with rat and hamster induced liver S9 in TA100 in water using preincubation. The second compound was tested with rat and hamster induced liver S9 in TA1535 in water using preincubation. In this head-to-head comparison the sensitivity of both species' liver S9 was very high and comparable. However, with no negative rodent bioassay carcinogenicity studies for comparison, the specificity of the two subgroups could not be determined.

Finally, existing Ames test study calls were compared for subgroups based on the vehicle solvent used. A total of 18 and 21 compounds contained both a rodent bioassay carcinogenicity call and an Ames test call using a DMSO or water vehicle, respectively. The sensitivity was comparable between DMSO (88%) and water (100%) (Fig. 2D). However, the low number of compounds tested, including few or no negative studies, makes an accurate comparison difficult.

A total of 9 compounds with rodent bioassay carcinogenicity calls were tested in both solvent subgroups. All 9 compounds were positive for rodent carcinogenicity and were Ames test positive with both solvents. The performance between the two subgroups was identical with equal sensitivity for both subgroups, but with no negative carcinogenicity results, the specificity of the two subgroups could not be compared.

4. Discussion

Despite previous concerns about the sensitivity of the Ames test to predict the carcinogenic potential of NAs, this retrospective analysis has found that the Ames test is highly sensitive when conducted under current OECD 471 guidelines. Previous reports such as Rao et al. (1979) reported a low sensitivity of the Ames test for NA. However, the Ames test procedures reported in this study were not conducted in accordance with current OECD 471 guidelines; specifically, the top dose was not tested up to 5000 μg/plate and the Ames tests conducted did not use bacterial strains that can detect mutations at the AT base pair primary reversion site (i.e., *E. coli* WP2 or *S. typhimurium* TA102). Since this publication, several of the NAs that were considered negative in the original Rao study were subsequently reported to be positive in the Ames test in later studies (Andrews and Lijinsky 1980; Rao, 1981; Mori et al., 1985). The original conclusion regarding the sensitivity of the Ames test for NAs as a result of the Rao et al. (1979) publication was based on a limited number of NAs (25 compounds). Further analysis in the performance of the Ames test is presented herein using data extracted from the Vitic and Leadscope databases which contained 131 and 70 NAs, respectively, all with Ames test and corresponding rodent bioassay

carcinogenicity data. There was significant overlap between the Vitic and Leadscope databases. 61 NAs overlapped in both databases. The number was reduced to 114 for Vitic and 63 for Leadscope databases when considering only those NA compounds which have Ames test studies compliant with OECD 471 test guideline and have used the currently recommended bacterial tester strains. The sensitivity of the Ames test results was comparable for the Leadscope and Vitic databases. These results are consistent with, and further expand upon, earlier work that shows the Ames test is more sensitive for NAs than other chemical classes (Thresher et al., 2020).

The OECD 471 bacterial reverse mutation assay (Ames test) represents a cornerstone of genetic toxicology used to de-risk impurities that are predicted mutagens from computational models and expert review (Amberg et al., 2016). For NAs, performing an *in vitro* assay to understand carcinogenic potential is critically important. Many NAs have already been evaluated in the Ames test. There are other nitrosamine drug substance-related impurities (NDSRIs) where Ames testing will be critical to understanding their corresponding hazards. NDSRIs may be present in final drug products due to the prevalence of secondary or tertiary amines in drug substances and the introduction of nitrite sources by excipients. Given the low level assumed AIs for NAs where experimental data is not available, there may be situations where the mutagenic hazards are necessary to mitigate risks to patients from exposure to NA impurities. Without *in vitro* methodologies available, such as the Ames test, the default would be animal-based mutagenicity or carcinogenicity testing. The Ames test has high sensitivity but lower specificity, meaning that it predicts well the carcinogenic compounds but less so the non-carcinogens. From a patient safety perspective prediction of carcinogenic compounds is more relevant. Testing NAs in the Ames test allows for a more rapid assessment of carcinogenicity potential and would save the use of animals required for *in vivo* transgenic mutation assay or 2 year rodent carcinogenicity studies, consistent with the 3Rs principles of animal testing (replace, reduce, and refine) (Wange et al., 2021).

In order to understand if Ames test methodology should be modified for NAs, we focused our attention to the effects of method, metabolic activation, solvents and bacterial strains. An analysis of the Ames test to the rodent bioassay carcinogenicity showed performance of plate incorporation versus preincubation methods was comparable, both methods resulting in sensitivity above 80%. However, certain smaller molecular weight aliphatic NAs have been reported in the literature to be more efficiently detected for mutagenicity with the pre-incubation method, possibly due to the high concentrations of liver S9 and NA maintained during the initial step of the preincubation assay compared to the concentrations in the plate incorporation method (Prival et al., 1979) or to the reaction of the active metabolite with the agar in the plate incorporation method (Bartsch et al., 1976).

The comparison between hamster and rat induced liver S9 fractions use in the Ames test showed comparable sensitivities for carcinogenicity outcome. However, lower specificity was observed with hamster induced liver S9. These results were surprising since several early reports concluded that hamster induced liver S9 is more sensitive than rat as a metabolic activator of NAs in the Ames test in the *Salmonella* strains (Lijinsky and Andrews, 1983; Phillipson and Ioannides, 1984; Yahagi et al., 1977). Consequently, we expected a significant difference in the sensitivity for Ames test results with NAs between hamster and rat induced liver S9 in the Vitic and Leadscope datasets. In these legacy publications, the hamster induced liver S9 fraction produced a higher number of revertants compared with rat liver S9 (Lijinsky and Andrews, 1983; Phillipson and Ioannides, 1984; Yahagi et al., 1977). These publications compared the activation of several known rodent carcinogenic NAs using hamster and rat liver S9 but their experimental protocols did not comply with OECD 471 guidelines, for example NAs were tested in just one or two *Salmonella* strains (TA1535 or TA100) and only at concentrations up to 2000 μg/plate, and in some cases no cytochrome P450 inducer was used in the production of the S9 liver fraction. When taking into consideration data sets from Vitic and Leadscope which included Ames experimental results with rat induced liver S9 metabolic activation and testing concentrations up to 5000 μg/plate, similar sensitivities between rat and hamster induced liver S9 were observed. These results imply that although the hamster induced liver S9 may induce a stronger revertant colony response, treatment with rat induced liver S9 will also provide a weaker, but still positive, response.

The use of DMSO as a solvent has previously been shown to give rise to negative Ames test results for NDMA and NDEA which are carcinogenic in rodents (Mori et al., 1985; Yahagi et al., 1977). The inhibition of mutagenicity is believed to be the result of solvent interference with metabolic activation (Mori et al., 1985) and is consistent with DMSO inhibition of P450 CYP2E1 which is the dominant liver metabolizing enzyme for very small NAs (Cross and Ponting, 2021; Chauret et al., 1998). However, for slightly larger molecular weight NAs e.g., *N*-Nitroso-di-n-propylamine, *N*-Nitroso-di-n-butylamine, *N*-Nitroso-di-n-isobutyl amine, the use of DMSO as a solvent resulted in a positive Ames test (Andrews and Lijinsky, 1980). Despite the results for NDMA and NDEA, the sensitivity of the Ames test was high when using DMSO, thus supporting its use with the exception of certain small aliphatic NAs.

5. Conclusions

Our results showed that an Ames test following current OECD 471 recommendations (bacterial strains and both plate incorporation and preincubation methodologies) can be used to identify most NAs. For short-chain aliphatic NAs, preincubation protocols are recommended. Most NAs require metabolic activation for mutagenicity. For metabolic activation, rat induced liver S9 was as effective as hamster induced liver S9 to determine the mutagenic potential of NAs. While hamster induced liver S9 may result in a greater number of revertant colonies, our data indicated there was little improvement on assay sensitivity and there was reduced assay specificity. DMSO has been shown to be an effective solvent for most NAs, apart from very short-chain aliphatic NAs like NDMA and NDEA, where a reduction in mutagenicity was observed.

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CRediT authorship contribution statement

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Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Alejandra Trejo-Martin reports a relationship with Gilead Sciences Inc that includes: employment.

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Appendix A. Supplementary data

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