Metabolic activation of short-chain alkyl N-nitrosamines using Aroclor 1254 or phenobarbital/beta-naphthoflavone-induced rat or hamster S9 – A comparative analysis

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A B S T R A C T

N-nitrosamines, a very heterogeneous class of chemicals, may enter humans in small amounts through various sources and are produced endogenously, too. Some are known to be mutagenic carcinogens and have recently been detected as impurities in several marketed pharmaceuticals. Despite their known mutagenic properties, the suitability of the bacterial reverse mutation (Ames) assay and in particular the use of induced rat liver S9 to detect their mutagenic potential, is often discussed. Recently, it could be demonstrated that induced rat liver S9 is capable of metabolizing small alkyl nitrosamines to exert their mutagenic potential (Bringezu & Simon, 2022). In this project, the mutagenic potential of nitrosamines in vitro under different S9 conditions applying the preincubation protocol and OECD 471-compliant standard Ames test recommendations was investigated. These conditions included various amounts of S9 fraction from hamster and rat, uninduced or induced with Aroclor 1254 or Phenobarbital/beta-Naphthoflavone (PB/NF). The findings indicated that in addition to induced S9, uninduced hamster S9 also demonstrated effectiveness. Moreover, both rat and hamster S9 fractions exhibited suitable responses in terms of mutation frequencies. Increasing the S9 content did not increase the sensitivity of the Ames test. However, above 20% S9, reduced mutation frequency was observed in the higher concentration range suggesting cytotoxicity to the bacteria. Thus, limiting the S9 content to 10% provides reliable results and relates to a lower number of animals required for S9 production which is in concordance with the 3R principles (reduce, refine, replace) for animal testing. In addition, results obtained show that uninduced and induced hamster S9 are similarly effective, doubting the requirement of pretreating animals with enzyme inducers. Further investigations to compare mutagenicity data and rat and hamster S9 proteome analyses are ongoing.

1. Introduction

The bacterial reverse mutation test (Ames test) is a simple and rapid test used worldwide to detect the mutagenicity of chemical substances [13]. As the name implies, the principle of this test is that the bacteria used have mutations that can be reversed in the presence of mutagenic substances, thereby restoring their ability to synthesize an amino acid essential for bacterial growth. Typically, histidine-requiring *Salmonella typhimurium* and tryptophan-requiring *Escherichia coli* tester strains are used, as also recommended in the Organisation for Economic Cooperation and Development (OECD) Guideline 471 [18].

Some chemicals are mutagenic only after metabolic activation. Therefore, substances should be tested in the presence and absence of an appropriate metabolic activation system. Since bacteria are unable to perform an *in vivo*-like metabolic activation of compounds, an exogenous metabolizing system, called S9, is used. S9 can be prepared from liver tissue from different species. It is an enzymatic extract of microsomal and cytosolic fractions with a variety of drug-metabolizing enzymes. In the Ames test, S9 of rats pretreated with potent enzyme inducers, such as Aroclor 1254 or a combination of Phenobarbital and β-Naphthoflavone (PB/NF) is most frequently used applying a plate incorporation or preincubation protocol [6].

In recent years, there have been some discussions that induced rat S9 does not reliably detect certain mutagens, such as nitrosamines. Nitrosamines are characterized by a nitroso group bonded to an amine. Some are known rodent carcinogens and have been found as impurities in several marketed pharmaceuticals [3]. But they can also be present in food, alcohol products, drinking water, cosmetic products and cigarette

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smoke [8].

Based on work published in the 1970′s and 1980′s [11,12,17], there were concerns from regulatory bodies that the standard Ames test, using rat liver S9 and applying the plate incorporation protocol, is not sensitive enough to detect the mutagenic potential especially for nitrosamines with low molecular weight such as nitroso-dimethylamine (NDMA) [9]. Analysis of these publications showed that many of the reported Ames tests have limitations, such as e.g., uncommon strains, few strains or low concentrations. As no harmonized test guideline was available at these times, the comparability of the assays is questionable. Recently, the *European Medicines Agency* (EMA) updated the "Questions and answers for marketing authorisation holders/applicants on the Committee for Medicinal Products for Human Use (CHMP) Opinion for the Article 5(3) of Regulation (EC) No 726/2004 referral on nitrosamine impurities in human medicinal products" document introducing the *Enhanced Ames Test* (EAT) protocol for N-Nitrosamines with stricter and additional requirements [7]. These test conditions include (I) the five tester strains *S. typhimurium* TA98, TA100, TA1535, TA1537, and *E. coli* WP2*uvrA* (pKM101), (II) the 30-minute pre-incubation protocol, (III) testing of 30% induced rat liver S9 and 30% induced hamster liver S9, (IV) the use of two additional nitrosamine positive controls under the consideration of NDMA and 1-cyclopentyl-4-nitrosopiperazine, and nitrosamine drug substance-related impurities [7]. The selection of the test conditions is based on a paper published by the US Food and Drug Administration's (FDA) National Center for Toxicological Research [10]. Various other studies showed that the mutagenicity of short-chain nitrosamines can be detected in the Ames test when more sensitive assay conditions are used [1,12,16,19]. According to these studies, sufficient conditions for the detection of nitrosamines are (I) the pre-incubation protocol, (II) the use of rat or hamster S9 for metabolic activation, and (III) the correct solvent, all of which are consistent with the current OECD guideline. In addition, recent publications showed that applying the standard Ames conditions as described in the OECD 471 guideline is appropriate to detect the mutagenic activity of nitrosamines reliably [4, 19]. This led to an intensive discussion within the scientific community on whether the EAT conditions are necessary and scientifically justified. Considering published studies and the 3Rs animal welfare perspective, testing with 30% rat S9 *and* 30% hamster S9 is unlikely to increase sensitivity of the Ames test and might thus not be required for the reliable detection of mutagenic nitrosamines, leading to a reduced animal number needed for S9 preparation.

The present study examined 10%, 20%, and 30% rat and hamster S9 for the detection of four known mutagenic short-chain nitrosamines. The bacterial reverse mutation assays were performed using N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA), and N-nitrosodibutylamine (NDBA). These nitrosamines were selected as representative tool compounds for the current work because of their recognized and well described toxicological characteristics. The preincubation protocol was applied and various S9 fractions from rat and hamster, uninduced or induced with Aroclor 1254 or PB/NF, were used as exogenous metabolizing system. The tests were performed using the OECD 471 guideline recommendations [18]. As reported, *Salmonella typhimurium* TA100 and TA1535 and *Escherichia coli* WP2*uvrA* are very sensitive for the detection of nitrosamines [4,20]. Therefore, these strains were selected for the present study investigating the influence of the experimental conditions applied, such as S9 concentration, S9 induction method or plate incorporation versus incubation protocol, on the ability to detect the mutagenic potential of nitrosamines.

2. Material and methods

The selective agar plates were purchased from Merck Life Science, article number 1466900120.

N-nitrosodimethylamine (NDMA), N-nitrosodiethylamine (NDEA), N-nitrosodipropylamine (NDPA) and N-nitrosodibutylamine (NDBA)

were purchased from Merck Life Science (Supelco/Sigma-Aldrich), Germany. Ultrapure water and dimethyl sulfoxide (DMSO) were used as solvents for the experiments.

2.1. Metabolic activation

Differently induced hamster and rat S9 batches were tested listed in Table 1.

Uninduced hamster S9, PB/NF-induced hamster S9 and Aroclor 1254-induced rat S9 were acquired from Trinova and Moltox®, respectively (Table 1).

Each S9 batch was tested for its metabolic activity using specific substrates, requiring different enzymes of the P450-isoenzyme family. The mutagenicity of 2-aminoanthracene (2-AA), benzo[*a*]pyrene (BAP), and 3-methylcholanthrene (3-MC) was thus determined.

Clear increases in the number of revertants above the two-fold or three-fold thresholds for *S. typhimurium* TA98, TA100, and TA1537 with all positive controls and for TA1535 and *E. coli* WP2*uvrA* with 2-AA were used as an acceptance criterion for each S9 batch.

Table 2 shows the composition of the S9 mix used for the experiments with metabolic activation. 10%, 20% and 30% (v/v) S9 were thus applied in the S9 mix used.

2.2. Bacterial strains

The origin of the bacterial strains applied during this study is summarized in Table 3. All frozen cultures used in the present investigation originated from these batches.

The bacterial strains' genotype causing different type of mutations is summarized in Table 4.

2.3. Bacterial reverse mutation assay

Bacteria were propagated from frozen stocks. For all frozen stocks, their genotype characteristics were confirmed as described by Mortelmans and Zeiger [13]. The Ames test was conducted following the recommendations of the OECD 471 test guideline and standard publications (*Test No. 471: Bacterial Reverse Mutation Test*, 2020). In brief, bacteria were grown in nutrient broth with shaking (200 rpm at 37 ± 2 °C) until the exponential growth phase was reached. The cell density was determined by measurement of the optical density at 650 nm. For preincubation experiments, 100 µL bacteria suspension, 10 µL test item, solvent, or positive control material, 500 µL S9 mix (for experiments with metabolic activation) or phosphate buffer (for experiments without metabolic activation) and 2 mL histidine- or tryptophane-containing molten top agar per plate were mixed and incubated for 60 min at 37 ± 2 °C. After this preincubation, 2 mL molten top agar were added, and the mixture poured onto the selective agar plates. The plates were then incubated for 2 days at 37 ± 2 °C. Counting of colonies was performed with the Ames Colony Counter (Instem, UK) and tables were generated using the Ames Study Manager software (Instem, UK).

The experiments were conducted using 3 replicates for each concentration level and the positive controls and the double number of negative control plates. Five concentration levels were applied. The test concentrations ranged from 5–5000 µg/plate, separated by a $\sqrt{10}$ spacing. The solvent (DMSO) concentration was 1.6% in the preincubation mix.

Table 1
Hamster and rat liver S9 fractions used for studies.

Species	Inducing agent	LOT	Supplier/Producer
Hamster	PB/NF	LOT4608	Trinova Biochem, Germany
	uninduced	LOT4448	Trinova Biochem, Germany
Rat	Aroclor 1254	LOT4287	Moltox®, USA
	Aroclor 1254	LOT4406	Moltox®, USA

Table 2
Composition of hamster and rat liver S9 mix.

Components	Quantity per mL S9 mix		
	10%	20%	30%
S9	0.10 mL	0.20 mL	0.30 mL
Magnesium chloride/ potassium chloride solution (0.4 M/1.64 M)	0.02 mL	0.02 mL	0.02 mL
Glucose-6-phosphate x 1 H2O, disodium salt	1.61 mg	1.61 mg	1.61 mg
Nicotinamide adenine dinucleotide phosphate, disodium salt	3.15 mg	3.15 mg	3.15 mg
Sodium phosphate buffer	0.50 mL	0.50 mL	0.50 mL
Ultra-pure water	0.38 mL	0.28 mL	0.18 mL

Table 3
Origin of strains.

Strain	Origin	Date of receipt
TA98	B. N. Ames, University of Berkeley, California, USA	August 15, 1985
TA100	Covance Laboratories Ltd., Harrogate UK	October 8, 2013
TA1535	Trinova Biochem GmbH, Giessen, Germany	October 18, 2018
TA1537	Sanofi-Aventis, Frankfurt am Main, Germany	November 24, 2016
WP2 uvrA	National Collections of Industrial & Marine Bacteria Ltd., Aberdeen, Scotland (NCIMB 11,188 from Aug. 18, 1977) and obtained from H. Träger, Knoll AG, Ludwigshafen, Germany	December 23, 1994

Table 4
Genotype of strains.

Strain	Gentyp	Type of mutation indicated
TA98	his D 3052, uvrB⁻, rfa⁻ + R-factor (pKM101)	Frameshift mutations
TA100	his G 46, uvrB⁻, rfa⁻, + R-factor (pKM101)	Base-pair substitutions
TA1535	his G 46, uvrB⁻, rfa⁻	Base-pair substitutions
TA1537	his C 3076, uvrB⁻, rfa⁻	Frameshift mutations
WP2 uvrA	Trp⁻, uvrA⁻	Base-pair substitutions and others

For evaluation of treatment-related effects, a fold increase was defined as mutagenic if a biologically relevant increase in the mean number of revertants above a threshold of 2-fold (TA98, TA100, WP2 *uvrA*) or 3-fold (TA1535, TA1537) as compared to the concurrent negative controls was observed. Historical negative and positive control (2-AA) values are shown in the appendix for rat and hamster S9 at 10%, 20% and 30%.

3. Results

3.1. Short-chain alkyl nitrosamines are reliably detected with 10% rat S9

As shown previously, certain short-chain alkyl nitrosamines need to follow the pre-incubation protocol for effective detection. Without implementing this protocol, such as in the case of NDMA, the plate incorporation design does not yield a distinct positive response [4].

In this work, the fold increases in mutation frequencies after exposure to NDBA, NDPA, NDEA, and NDMA were examined using bacterial strains TA100, TA1535, and WP2*uvrA*. The preincubation protocol was applied, and the strains were metabolically activated with Aroclor 1254-induced rat liver S9. Fig. 1 summarizes the results obtained for the four

different short chain nitrosamines investigated at different S9 concentrations ranging from 10 to 30% respectively.

Under the conditions applied, NDBA exposure leads to a rapid increase in mutation frequencies for all tested strains (Fig. 1, top). Similar increases could be observed with NDPA using 10% Aroclor 1254-induced rat S9. Lower fold increase values were obtained with 10% S9 for NDEA and NDMA. With NDEA, the threshold of 2- (red line) or 3-fold (green line) could be reached with TA100 and WP2*uvrA*, whereas TA1535 was below the limit with a fold increase of 2.9. For NDMA, all tested bacterial strains were below the threshold at the lowest applied S9 concentration (TA100 (1.9), TA1535 (2.7), WP2uvrA (1.8)), but reached the limit using 20% S9 (TA100 (2.7), TA1535 (4.2), WP2uvrA (2.8)). Table 5.

In case of nitrosamines, increasing S9 concentration yields increasing absolute mutation frequencies. At 10% and 20% S9 concentration, all short chain nitrosamines investigated can be detected with the exception of NDMA, which did not induce significant mutation frequencies at 10%. A further increase to 30% does not improve sensitivity and may lead to decreasing mutation frequencies at higher substance concentrations as observed for NDPA and NDBA, respectively (Fig. 1). This trend holds true for the positive control 2-AA that yields a drop in mutation frequencies at higher S9 concentrations in all strains investigated (Table).

3.2. Increased hamster S9 concentration leads to increased fold induction, but not sensitivity

Further experiments focused on species differences investigating PB/NF-induced hamster S9 using 10% and 30% S9 in the S9 mix during the experiments. Fig. 2 summarizes the results obtained indicating the experimental conditions applied.

All nitrosamines showed an increase in fold induction reaching the threshold for a positive response using 10% and 30% hamster S9, respectively. Similarly, a concentration-dependent increase in fold induction was observed. In general, the lowest effective concentration leading to a positive response decreases with increasing chain lengths. NDMA requires a concentration of at least 1580 µg/plate for a positive response for each of the strains investigated. The mutagenic response caused by NDEA, NDPA and NDBA is detected at 500 µg/plate or depending on strain on condition even below.

In contrast to the observation for NDMA and NDEA, both, NDPA and NDBA showed a maximum or a plateau in the mutation frequencies. This could be explained by cytotoxicity leading to a drop in the fold increases observed. This effect is independent of the S9 concentration applied. A clear difference in fold increase values between 10% and 30% S9 was observed after exposure to NDBA (Fig. 2, top) in bacterial strain TA100 and for NDMA (Fig. 2, bottom) in both *S. typhimurium* strains. Otherwise, it should be noted that increasing the S9 concentration does not significantly increase the sensitivity. Table 6.

3.3. 10% uninduced hamster S9 is sufficient to activate 2-AA and BAP

2-AA and BAP are two of the most frequently used positive controls for the bacterial reverse mutation assay because of their high mutagenic activity in bacterial strains following metabolic activation. Thus, it also serves as evidence for the presence of metabolically active enzymes in the S9 fraction. To produce the hepatic S9 fraction, animals are usually treated with an enzyme inducer (PB/NF or Aroclor 1254) to increase enzyme and in particular CYP expression in the liver. In this study, it was investigated whether the use of induced hamster S9 compared to uninduced hamster S9 results in higher mutation frequencies. This was done by treating bacteria with varying concentrations of 2-AA and BAP and analyzing the outcomes. Fig. 3 shows a heatmap of the log10 mutation frequencies after metabolic activation of 2-AA (top) and BAP (bottom) using 10%, 20% and 30% of uninduced and PB/NF-induced hamster S9.

Both positive controls exerted the expected mutagenic responses under all conditions applied. However, with 10% PB/NF-induced
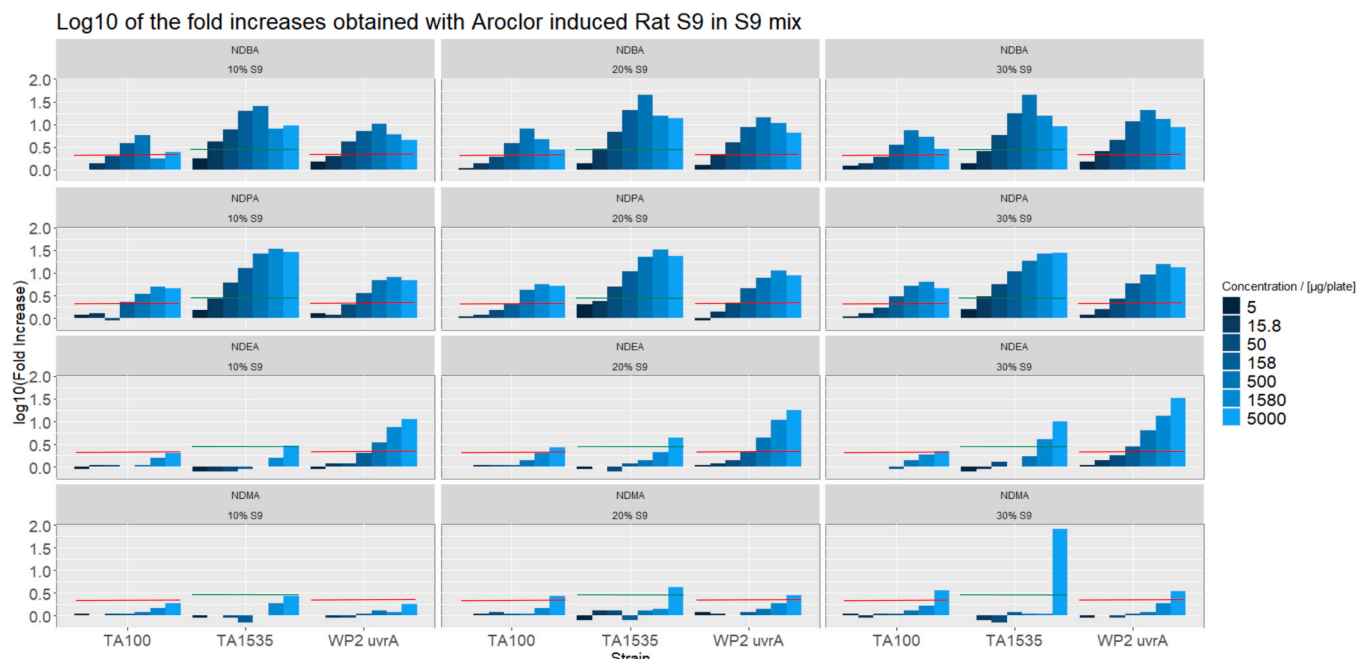
Fig. 1. Log fold increases obtained with metabolic activation using 10%, 20%, and 30% of Aroclor 1254-induced rat liver S9 mix in bacterial strains TA100, TA1535 and WP2*uvrA*. The preincubation design was applied. From top to bottom data is shown for NDBA, NDPA, NDEA and NDMA dissolved in DMSO. Red line marks a 2-fold induction and green line a 3-fold induction compared to the solvent control.

Table 5

Mutation frequencies of bacterial colonies obtained with metabolic activation using 10%, 20% and 30% of Aroclor 1254-induced rat liver S9 mix in bacterial strains TA100, TA1535 and WP2*uvrA* using the preincubation protocol. For DMSO and 2-AA, the table shows the mean values (n = 4). For NDBA, NDPA, NDEA and NDMA, the highest mutation frequencies observed, and the respective concentration is shown.

Pre-incubation	Induced rat liver S9								
	10%			20%			30%		
Strain	TA100	TA1535	WP2	TA100	TA1535	WP2	TA100	TA1535	WP2
Solvent	158 ± 14	17 ± 5	48 ± 8	185 ± 15	18 ± 5	52 ± 8	194 ± 14	19 ± 6	49 ± 10
NDBA	936 ± 81 (500 µg/plate)	593 ± 11 (500 µg/plate)	453 ± 1 (500 µg/plate)	1426 ± 35 (500 µg/plate)	998 ± 34 (500 µg/plate)	741 ± 12 (500 µg/plate)	1467 ± 97 (500 µg/plate)	1165 ± 172 (500 µg/plate)	916 ± 44 (500 µg/plate)
NDPA	817 ± 35 (1580 µg/plate)	447 ± 46 (1580 µg/plate)	481 ± 94 (1580 µg/plate)	1087 ± 195 (1580 µg/plate)	478 ± 27 (1580 µg/plate)	756 ± 68 (1580 µg/plate)	1247 ± 89 (1580 µg/plate)	442 ± 114 (5000 µg/plate)	919 ± 9 (1580 µg/plate)
NDEA	290 ± 9 (5000 µg/plate)	54 ± 8 (5000 µg/plate)	475 ± 57 (5000 µg/plate)	440 ± 38 (5000 µg/plate)	77 ± 17 (5000 µg/plate)	742 ± 43 (5000 µg/plate)	392 ± 68 (5000 µg/plate)	159 ± 16 (5000 µg/plate)	1360 ± 173 (5000 µg/plate)
NDMA	312 ± 20 (5000 µg/plate)	37 ± 5 (5000 µg/plate)	86 ± 11 (5000 µg/plate)	532 ± 45 (5000 µg/plate)	71 ± 8 (5000 µg/plate)	131 ± 12 (5000 µg/plate)	720 ± 63 (5000 µg/plate)	1527 ± 1640 (5000 µg/plate)	184 ± 12 (5000 µg/plate)
2-AA	2176 ± 236 (2 µg/plate)	250 ± 24 (5 µg/plate)	260 ± 39 (10 µg/plate)	1040 ± 74 (2 µg/plate)	187 ± 24 (5 µg/plate)	110 ± 12 (10 µg/plate)	708 ± 49 (2 µg/plate)	132 ± 19 (5 µg/plate)	78 ± 11 (10 µg/plate)

hamster S9, after treatment with 10 µg/plate 2-AA, the threshold of 3-fold was not reached in bacterial strains TA1535 and TA1537. In general, highest mutation frequencies are observed already at the lowest 2-AA concentration of 2.5 µg/plate applied. Increasing S9 concentrations from 10% to 30% reduces the mutation frequencies for all *S. typhimurium* strains. In *E. coli* WP2*uvrA,* the frequencies were independent from the S9 concentrations. With the exception of the two highest concentrations (10 and 7.5 µg/plate) with 30% S9 in TA1535 (FI = 0.0–1.4) and TA1537 (FI = 0.8–1.4), 2-AA was detectable in all strains when using uninduced hamster S9. Here, the lowest 2-AA concentration led to almost comparable mutation frequencies across S9 concentrations applied. However, at higher 2-AA concentrations, the mutation frequencies decreased with increasing uninduced S9 concentration in *S. typhimurium* strains TA98, TA1535 and TA1537.

Treatment with BAP yields similar patterns of mutation frequencies comparing both conditions applied, i.e., uninduced and PB/NF-induced hamster S9 (Fig. 3, bottom). The bacterial strains TA1535 and WP2*uvrA* do not reach the threshold of 2-fold and 3-fold at any concentration independent of the S9 condition. However, the detection of BAP-related positive response can be similarly observed for all other strains with S9 induced by PB/NF or uninduced S9.

4. Discussion

This study investigated the additional benefit of increasing the S9 concentration for the detection of four known mutagenic short-chain nitrosamines in the bacterial reverse mutation test. The present investigation was conducted according to current OECD 471 "state-of-the-art" conditions to overcome limitations of early investigations of nitrosamine mutagenicity [18]. The preincubation protocol was applied and various
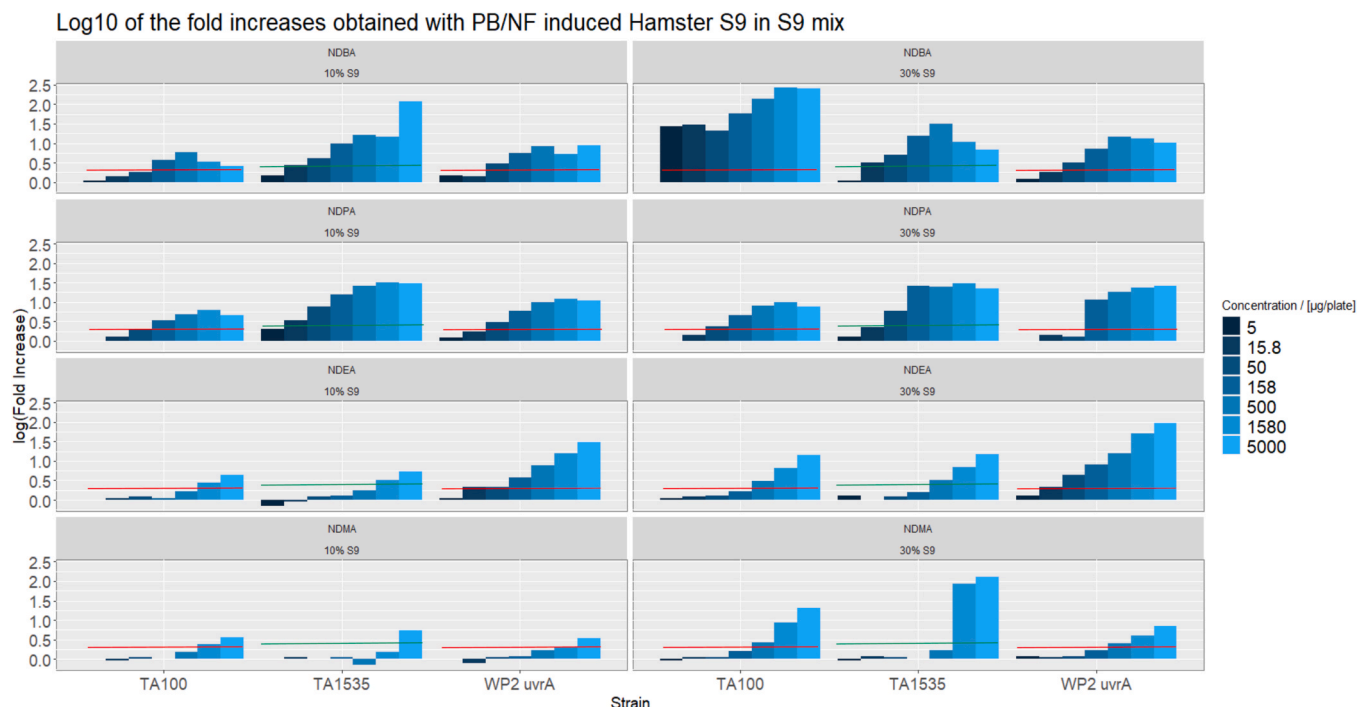
Log10 of the fold increases obtained with PB/NF induced Hamster S9 in S9 mix

Fig. 2. Log fold increases obtained with metabolic activation using 10% and 30% of PB/NF-induced hamster liver S9 mix in bacterial strains TA100, TA1535 and WP2*uvrA*. The preincubation design was applied. From top to bottom data is shown for NDBA, NDPA, NDEA and NDMA dissolved in DMSO. Red line marks a 2-fold induction and green line a 3-fold induction compared to the solvent control.

Table 6

Absolute numbers of bacterial colonies obtained with metabolic activation using 10% and 30% of PB/NF-induced hamster liver S9 mix in bacterial strains TA100, TA1535 and WP2*uvrA*. The preincubation design was applied. For DMSO and 2-AA, table shows the mean value of n = 4. For the nitrosamines NDBA, NDPA, NDEA and NDMA, the highest absolute number reached, and the respective concentration is given.

Pre-incubation	PB/NF-induced hamster S9					
	10%			30%		
Strains	TA100	TA1535	WP2	TA100	TA1535	WP2
Solvent	155 ± 15	26 ± 7	36 ± 8	184 ± 23	36 ± 10	37 ± 9
NDBA	876 ± 83	2901 ± 759	300 ± 65	1049 ± 264	967 ± 97	579 ± 57
	(500 µg/plate)	(5000 µg/plate)	(5000 µg/plate)	(1580 µg/plate)	(500 µg/plate)	(500 µg/plate)
NDPA	917 ± 51	706 ± 78	427 ± 52	1732 ± 136	1173 ± 58	934 ± 178
	(1580 µg/plate)	(1580 µg/plate)	(1580 µg/plate)	(1580 µg/plate)	(1580 µg/plate)	(5000 µg/plate)
NDEA	678 ± 19	158 ± 13	1102 ± 77	2581 ± 536	533 ± 50	3176 ± 135
	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)
NDMA	594 ± 2	139 ± 46	136 ± 5	3724 ± 204	4762 ± 250	263 ± 16
	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)	(5000 µg/plate)
2-AA	5034 ± 298	211 ± 33	886 ± 81	2024 ± 233	428 ± 37	340 ± 37
	(2 µg/plate)	(5 µg/plate)	(10 µg/plate)	(2 µg/plate)	(5 µg/plate)	(10 µg/plate)

S9 fractions from rat and hamster, uninduced or induced with Aroclor 1254 or PB/NF, were used as exogenous metabolizing system.

In the first part of the study, it could be shown that 10% of induced rat liver S9 mix combined with the preincubation design activates most shorter chain substituted N-nitrosamines sufficiently to exert their mutagenic potential. *Salmonella* strains TA100 and TA1535 and *E. coli* WP2*uvrA* are specifically sensitive to detect the mutagenicity of shorter chain N-Nitrosamines as already previously described [4]. The current EMA questions-and-answers document recommends testing of 30% induced rat liver S9 and 30% induced hamster liver S9 [7]. In comparison to the recommendation, the data presented here show that the sensitivity is not necessarily increasing with increasing the S9 concentration for both hamster and rat liver S9. In contrast, the results obtained indicate that with 30% S9 the mutation frequency decreased in some strains due to cytotoxic effects. For some nitrosamines, i.e., NDMA, increasing the rat liver S9 concentration to 20% may be beneficial, but a further increase to 30% S9 does not improve sensitivity. Using lower S9

concentrations would reduce the amount of S9 required and thus the number of animals needed while maintaining the reliable detection of the mutagenic potential and thus the carcinogenic hazard of short-chain nitrosamines, considering the 3 R aspects for animal welfare. Recently published data show that there is no significant difference in sensitivity for the detection of N-nitrosamines between induced rat and hamster liver S9 [19]. However, the present study reveals that at the same S9 concentration of 10%, the mutagenic properties of NDMA were clearly detected in all strains tested with hamster but did not reach the threshold with Aroclor-induced rat S9. This is in contrast to the results reported earlier, where 10% PB/NF-induced rat S9 sufficiently metabolized NDMA to produce significantly increased mutation frequencies [4]. This difference might be due to a slightly different induction pattern of metabolizing enzymes in the rat liver when using either Aroclor 1254 or PB/NF. To investigate this effect further, in-house proteomic analyses have been started to assess the protein expression levels of relevant CYPs, such as 2A6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 in various rat and
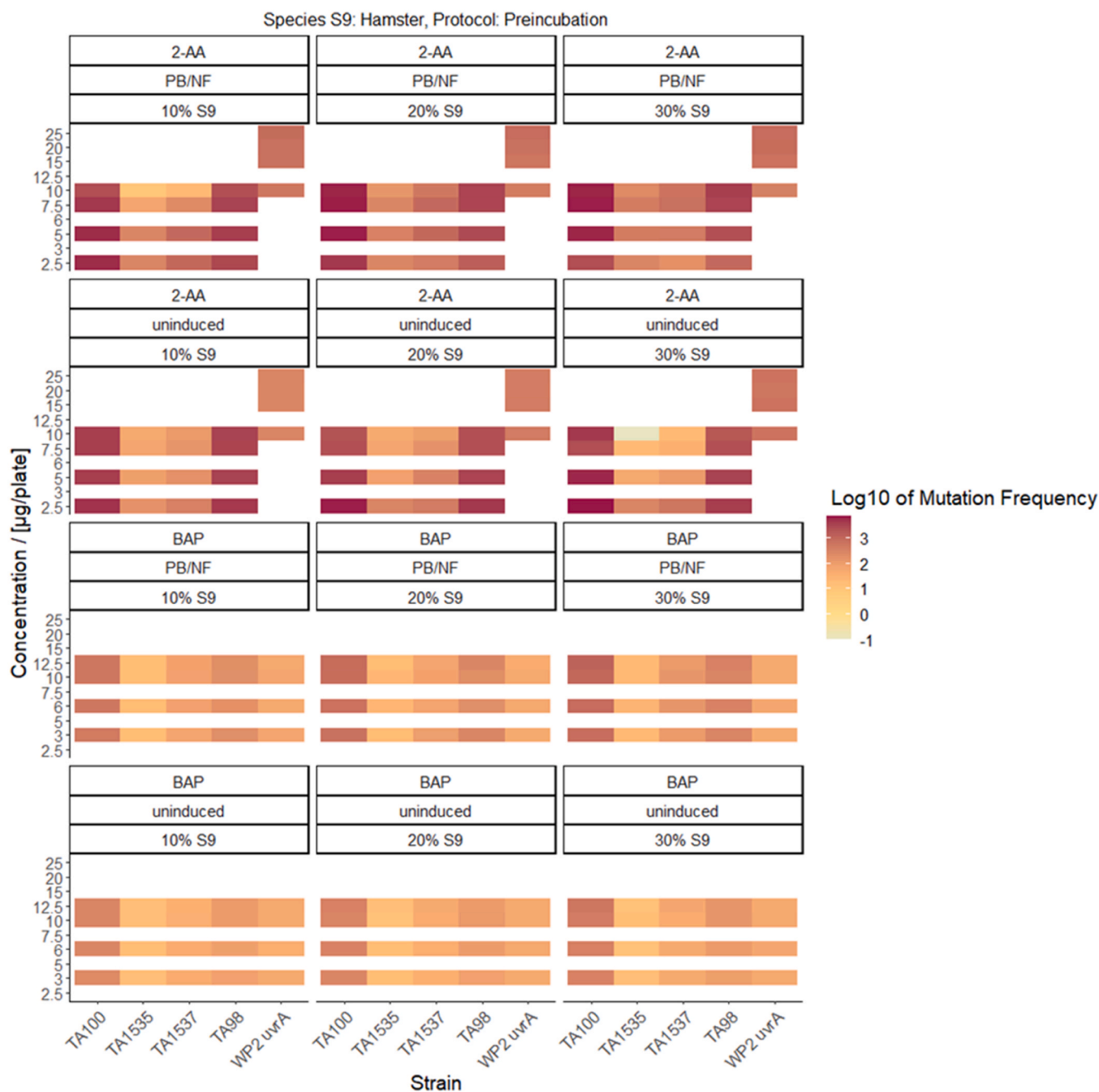
Fig. 3. Log10-fold increases of mutation frequencies after 2-AA and BAP exposure compared to the solvent control DMSO. Several concentrations of 2-AA and BAP were metabolically activated using 10%, 20% and 30% of PB/NF-induced or uninduced hamster liver S9 mix in bacterial strains TA100, TA1535, TA1537, TA98 and WP2*uvrA*. The preincubation design was applied.

hamster S9 fractions. The observation that hamster liver S9 as a metabolic activator of nitrosamines is more active than rat liver S9 has already been described in several publications [12,15]. Depending on their length, small to medium nitrosamines are mainly metabolized via CYP2E1 and CYP2A6 and large molecules via CYP3A4 enzymes [5,14, 21,22]. It has already been described in the literature that hamster-induced S9 can metabolize some short-chain nitrosamines more efficiently than rat S9 [2,12]. Possibly, the amount of the responsible metabolizing enzymes is higher in the hamster S9 fraction than in the rat fraction. Further investigations on the proteomic analysis of different S9 fractions will be performed to get further insights on this assumption.

In general, rat and hamster liver S9 fractions were suitable to detect all short-chain alkyl nitrosamines. Increasing the S9 concentration leads

to increased maximum fold inductions. The data showed that for very small nitrosamines, testing with induced hamster S9 might be preferable as it resulted in a higher number of revertant colonies.

In the second part of the study, it was tested whether higher induction rates are obtained using increasing induced S9 concentrations rather than uninduced S9 after treatment of bacteria with positive control substances, namely 2-AA and BAP. The presented data showed that an induction with PB/NF does not lead to a significant increase in maximum fold inductions after treatment with 2-AA and BAP. In addition, 10% of S9 was sufficient to reliably activate 2-AA and BAP to their mutagenic metabolite.

In contrast to BAP, 2-AA was mutagenic in all bacterial strains commonly used for the OECD 471-compliant Ames test (TA98, TA100, TA1535, TA1537 and WP2*uvrA*). BAP is not mutagenic in *E. coli*

WP2*uvrA* because this bacterial strain does not have the *rfa* mutation, which increases the permeability of the bacteria's cell membrane, allowing large molecules, such as BAP, to enter [13].

In this study, higher S9 concentrations often reduced the number of relative mutation frequencies to such an extent that the threshold for mutagenic response was no longer reached. This was observed for 2-AA in TA1535 and TA1537 using uninduced hamster S9 at 30%. Testing with the EAT recommendations using 30% rat liver and 30% hamster liver S9 could result in the situation, that the standard positive control 2-AA does not exhibit in a significant increase in colony counts. In conjunction with the fact that the mutagenic potential of the four short-chain nitrosamines tested here could also be reliably demonstrated with 10% and 20% rat and hamster S9, the necessity of using 30% liver S9 is questionable. Furthermore, in the interests of 3R animal welfare aspects, fewer animals would have to be used with lower S9 concentrations without compromising the sensitivity and detection quality of the test. The EAT protocol of the EMA recommends including positive controls such as NDMA, 1-cyclopentyl-4-nitrosopiperazine, and the nitrosamine drug substance related impurity, in addition to the experimental conditions. In the light of NDMA's properties as a volatile, known mutagen, its use as a positive control in routine testing is a hurdle for many laboratories due to occupational safety considerations and is thus less preferable. The data presented here suggest that NDPA or NDBA are very well suited to be used as additional positive controls in the EAT.

5. Conclusion

In conclusion, the results of the present investigation demonstrate that the mutagenic potential of short chain alkyl nitrosamines can reliably be detected using 10% rat or hamster S9. For NDMA, higher Aroclor-induced rat S9 concentrations might be more appropriate. In addition, higher S9 concentrations do not necessarily increase sensitivity. This work also showed that uninduced hamster S9 is as efficient as induced hamster S9 to activate the standard positive controls 2-AA and BAP.

In this present study, all four tested short chain alkyl nitrosamines could be detected using hamster and rat liver S9 and applying the pre-incubation protocol. Therefore, they would all be generally suitable to be used as additional positive controls in the EAT described by EMA, as they are already well characterized at this stage. However, for occupational safety and health reasons the data would rather suggest using NDEA, NDPA or NDBA as a positive control.

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CRediT authorship contribution statement

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

Data will be made available on request.

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Appendix

Historical control data Ames test with 10% rat and hamster S9.

Table 7
Negative (solvent) controls.

Strain	TA 98		TA 100		TA1535		TA1537		WP2 uvrA	
S9 Mix Total Plates	rat 42	hamster 22	rat 54	hamster 46	rat 54	hamster 46	rat 42	hamster 18	rat 60	hamster 18
Number of Experiments	7	4	9	8	9	8	7	3	10	3
Minimum	37	33	104	100	8	10	13	18	30	18
Maximum	61	49	186	163	28	30	27	25	62	25
Mean	52	44	146	134	16	19	21	22	47	22
Standard Deviation	9.4	7.3	24.4	24.3	6.7	7.3	4.8	3.6	10.8	3.6

Table 8
Positive control 2-AA.

Strain	TA98		TA100		TA1535		TA1537		WP2 uvrA	
S9 Mix	rat	hamster	rat	hamster	rat	hamster	rat	hamster	rat	hamster
Total Plates	21	11	33	11	30	11	21	9	30	9
Number of Experiments	7	4	11	4	10	4	7	3	10	3
Minimum	161	2275	499	2275	138	2275	135	296	111	296
Maximum	1085	3340	2077	3340	460	3340	571	905	296	905
Mean	738	2855	1191	2855	313	2855	372	695	196	695
Standard Deviation	358.7	442.6	495.8	442.6	81.0	442.6	158.1	346.0	59.9	346.0

Historical control data Ames test with 20% rat and hamster S9.

Table 9
Negative (solvent) controls.

Strain	TA 100		TA1535		WP2 uvrA	
S9 Mix	rat	hamster	rat	hamster	rat	hamster
Total Plates	24	8	24	8	24	4
Number of Experiments	4	2	4	2	4	1
Minimum	161	44	13	114	34	12
Maximum	199	46	17	114	63	12
Mean	177.3	45	15.8	114	46.5	12
Standard Deviation	17.3	1.0	1.9	0.0	12.1	NA

Table 10
Positive control 2-AA.

Strain	TA100		TA1535		WP2 uvrA	
S9 Mix	rat	hamster	rat	hamster	rat	hamster
Total Plates	12	4	12	4	12	2
Number of Experiments	4	2	4	2	4	1
Minimum	455	2161	145	2954	91	302
Maximum	651	4223	204	8139	116	302
Mean	537.3	3192	169.8	5547	104.3	302
Standard Deviation	83.9	1458.1	26.9	3666.4	13.1	NA

Historical control data Ames test with 30% rat and hamster S9.

Table 11
Negative (solvent) controls.

Strain	TA 98		TA 100		TA1535		TA1537		WP2 uvrA	
S9 Mix	rat	hamster	rat	hamster	rat	hamster	rat	hamster	rat	hamster
Total Plates	18	22	48	46	48	46	24	18	42	42
Number of Experiments	3	4	8	8	8	8	4	3	7	7
Minimum	23	36	91	115	10	11	15	23	32	34
Maximum	70	48	200	192	19	39	28	30	52	49
Mean	42.3	41	158	161	14.4	25	21	27	44.3	40
Standard Deviation	24.6	5.5	34.2	27.0	3.1	12.4	6.1	3.8	7.5	4.7

Table 12
Positive control 2-AA.

Strain	TA98		TA100		TA1535		TA1537		WP2 uvrA	
S9 Mix	rat	hamster	rat	hamster	rat	hamster	rat	hamster	rat	hamster
Total Plates	9	11	21	23	27	23	9	9	18	21
Number of Experiments	4	4	7	8	9	8	3	3	6	7
Minimum	194	754	161	1352	93	310	67	470	41	205
Maximum	488	2058	491	2327	654	476	100	797	442	483
Mean	296.3	1298	328.7	1878	180.9	393	87.7	610	150.8	323
Standard Deviation	166.1	547.8	104.5	391.9	178.9	62.1	18.0	168.4	153.4	97.5

References

[1] Araki, A., Kamigaito, N., Sasaki, T., & Matsushima, T. (2004). Mutagenicity of Carbon Tetrachloride and Chloroform in Salmonella typhimurium TA98, TA100, TA1535, and TA1537, and Escherichia coli WP2uvrA/pKM101 and WP2/pKM101, Using a Gas Exposure Method. https://doi.org/10.1002/em.20005.

[2] A. Araki, M. Muramatsu, T. Matsushima, Comparison of mutagenicities of N-nitrosamines on Salmonella typhimurium TA100 and Escherichia coli WP2 uvrA/pKM101 using rat and hamster liver s9, GANN Jpn. J. Cancer Res. 75 (1) (1984) 8–16, https://doi.org/10.20772/CANCERSCI1959.75.1_8.

[3] J.C. Beard, T.M. Swager, An organic chemist's guide to n-nitrosamines: their structure, reactivity, and role as contaminants, J. Org. Chem. 86 (3) (2021) 2037, https://doi.org/10.1021/ACS.JOC.0C02774.

[4] F. Bringezu, S. Simon, 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. 9 (2022) 250, https://doi.org/10.1016/J.TOXREP.2022.02.005.

[5] K.P. Cross, D.J. Ponting, Developing structure-activity relationships for N-nitrosamine activity, Comput. Toxicol. 20 (2021) 100186, https://doi.org/10.1016/J.COMTOX.2021.100186.

[6] J. Easterbrook, D. Fackett, A.P. Li, A comparison of aroclor 1254-induced and uninduced rat liver microsomes to human liver microsomes in phenytoin O-deethylation, coumarin 7-hydroxylation, tolbutamide 4-hydroxylation, S-mephenytoin 4′-hydroxylation, chloroxazone 6-hydroxylation and testoste, Chem. -Biol. Interact. 134 (3) (2001) 243–249, https://doi.org/10.1016/S0009-2797(01)00159-4.

[7] EMA. (2023). *Questions and answers for marketing authorisation 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.* 5(726), 28. https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53–1490-questions-answers-marketing-authorisation-holders/applicants-chmp-opinion-article-53-regulation-ec-no-726/2004-referral-nitrosamine-impurities-human-medicinal-products_en.pdf.

[8] A.J. Gushgari, R.U. Halden, Critical review of major sources of human exposure to N-nitrosamines, Chemosphere 210 (2018) 1124–1136, https://doi.org/10.1016/J.CHEMOSPHERE.2018.07.098.

[9] T. Kameswar Rao, J.A. Young, W. Lijinsky, J.L. Epler, Mutagenicity of aliphatic nitrosamines in Salmonella typhimurium, Mutat. Res. /Genet. Toxicol. 66 (1) (1979) 1–7, https://doi.org/10.1016/0165-1218(79)90002-8.

[10] X. Li, Y. Le, J.E. Seo, X. Guo, Y. Li, S. Chen, R.A. Mittelstaedt, N. Moore, S. Guerrero, A. Sims, S.T. King, A.H. Atrakchi, T.J. McGovern, K.L. Davis-Bruno, D.A. Keire, R.K. Elespuru, R.H. Heflich, N. Mei, Revisiting the mutagenicity and genotoxicity of N-nitroso propranolol in bacterial and human in vitro assays, Regul. Toxicol. Pharmacol. 141 (2023) 105410, https://doi.org/10.1016/J.YRTPH.2023.105410.

[11] Lijinsky, W., Reuber, M.D., & Riggs, C.W. (1981). *Dose Response Studies of Carcinogenesis in Rats by Nitrosodiethylamine1.* http://aacrjournals.org/cancerres/article-pdf/41/12_Part_1/4997/2409139/cr04112p14997.pdf.

[12] William Lijinsky, A.W. Andrews, The superiority of hamster liver microsomal fraction for activating nitrosamines to mutagens in Salmonella typhimurium, Mutat. Res. /Fundam. Mol. Mech. Mutagen. 111 (2) (1983) 135–144, https://doi.org/10.1016/0027-5107(83)90058-1.

[13] K. Mortelmans, E. Zeiger, The Ames Salmonella/microsome mutagenicity assay, Mutat. Res. /Fundam. Mol. Mech. Mutagen. 455 (1–2) (2000) 29–60, https://doi.org/10.1016/S0027-5107(00)00064-6.

[14] V. Nedelcheva, I. Gut, P450 in the rat and man: methods of investigation, substrate specificities and relevance to cancer, Xenobiotica 24 (12) (1994) 1151–1175, https://doi.org/10.3109/00498259409038673.

[15] C.E. Phillipson, C. Ioannides, A comparative study of the bioactivation of nitrosamines to mutagens by various animal species including man, Carcinogenesis 5 (8) (1984) 1091–1094, https://doi.org/10.1093/CARCIN/5.8.1091.

[16] M.J. Prival, V.D. King, A.T. Sheldon, The mutagenicity of dialkyl nitrosamines in the salmonella plate assay, Environ. Mutagen. 1 (2) (1979) 95–104, https://doi.org/10.1002/EM.2860010202.

[17] T.K. Rao, B.E. Allen, W. Winton, W. Lijinsky, J.L. Epler, Nitrosamine-induced mutagenesis in Escherichia coli K12 (343/113): 1. mutagenic properties of certain aliphatic nitrosamines, Mutat. Res. /Genet. Toxicol. 89 (3) (1981) 209–215, https://doi.org/10.1016/0165-1218(81)90239-1.

[18] Test No. 471: Bacterial Reverse Mutation Test. (2020). https://doi.org/10.1787/9789264071247-EN.

[19] A. Trejo-Martin, J.P. Bercu, A. Thresher, R.E. Tennant, R.F. Thomas, K. Cross, A. Czich, K. Waese, J.J. Nicolette, J. Murray, P. Sonders, A. Kondratiuk, J.R. Cheung, D. Thomas, A. Lynch, J. Harvey, S. Glowienke, L. Custer, P.A. Escobar, Use of the bacterial reverse mutation assay to predict carcinogenicity of N-nitrosamines, Regul. Toxicol. Pharmacol. 135 (2022) 105247, https://doi.org/10.1016/J.YRTPH.2022.105247.

[20] T. Yahagi, M. Nagao, Y. Seino, T. Matsushima, T. Sugimura, M. Okada, Mutagenicities of N-nitrosamines on salmonella, Mutat. Res. /Fundam. Mol. Mech. Mutagen. 48 (2) (1977) 121–129, https://doi.org/10.1016/0027-5107(77)90151-8.

[21] H. Yamazaki, Y. Inui, C.H. Yun, F.P. Guengerich, T. Shimada, Cytochrome P450 2E1 and 2A6 enzymes as major catalysts for metabolic activation of N-nitrosodialkylamines and tobacco-related nitrosamines in human liver microsomes, Carcinogenesis 13 (10) (1992) 1789–1794, https://doi.org/10.1093/CARCIN/13.10.1789.

[22] H. Yamazaki, Y. Oda, Y. Funae, S. Imaoka, Y. Inui, F.P. Guengerich, T. Shimada, Participation of rat liver cytochrome P450 2E1 in the activation of N-nitrosodimethylamine and N-nitrosodiethylainine to products genotoxic in an acetyltransferase–overexpressing Salmonella typhimurium strain (NM2009), Carcinogenesis 13 (6) (1992) 979–985, https://doi.org/10.1093/CARCIN/13.6.979.