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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

Frank Bringezu, Stephanie Simon *

Merck Healthcare KGaA, Frankfurter Straße 250, 64293, Darmstadt, Germany

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ABSTRACT

Humans are exposed to low levels of N-nitrosamines via different sources. N-Nitrosamines have recently been detected as impurities in various marketed drugs and they are known mutagenic carcinogens belonging to the cohort of concern as referred to in the ICH M7 guideline. Despite their well-known mutagenic properties, there is ongoing discussion on the suitability of the bacterial reverse mutation assay and using induced rat liver S9 as the external source of metabolism to detect their mutagenic potential. Therefore, we have investigated the mutagenic potential of N-nitrosodimethylamine, N-nitrosodiethylamine, N-nitrosodipropylamine, and N-nitrosodibutylamine *in vitro* under various conditions. Our work showed that the bacterial reverse mutation assay applying plate incorporation or preincubation protocols and using *Salmonella typhimurium* strains TA100 and TA1535 and *E. coli* WP2 uvrA is suitable to predict the mutagenicity of n-nitrosamines in the presence of phenobarbital/β-naphthoflavone induced rat liver S9.

1. Introduction

N-nitrosamines belong to a class of N-nitroso compounds that are potent human carcinogens that can induce tumors in organs of various animal species independent of the route of application [1,2]. They are formed by a nitrosating agent (e.g. N_2O_3, or N_2O_4) and amino groups in a pH dependent manner [3]. They are found in food, tobacco smoke, or industry [4]. Nitrosamines formed *in vivo* from active substances led to reformulation or withdrawals of marketed drugs [5].

Nitrosamines may also arise from chemical synthesis of active pharmaceutical ingredients (APIs) or drug formulation (e.g. due to use of nitrocellulose) and they have recently been reported as a result of process changes in API production [6–8].

Because some of them are mutagenic carcinogens of high potency that might also occur as impurities in drug substances, ICH M7 guidance referred these compounds as members of the cohort of concern carcinogens [9]. This means that acceptable levels of exposure are likely to be significantly lower than the TTC of 1.5 μg/day defined by the guideline. Therefore, their presence must be controlled on a case-by-case basis using appropriate carcinogenicity data including relevant data of closely related compounds. [10]. Bercu and coworkers have recently published a review on the application of the less-than-lifetime (LTL) approach outlined in ICH M7 that may be applied to control nitrosamine impurities as well as other classes of compounds which are potential mutagenic carcinogens [11].

The recent detection of nitrosamines in several commercial drug products including sartans, containing a tetrazole ring, pioglitazone, ranitidine, nizatidine, and metformin resulted in recalls of drug lots, review of the manufacturing processes by marketing authorization holders and in root-causes for the presence of nitrosamines in the chemical API synthesis and mitigation thereof [12–15].

N-Nitrosamines need activation by specific cytochrome P450 enzymes and it was shown that several enzymes, particularly CYP2E1, CYP2A6 catalyze metabolic activation of small to medium nitrosamine derivatives including N-Nitrosodialkylamines [16–18] and CYP3A4 catalyzes the activation of larger N-Nitrosamines [19]. Rodent CYP2E1 is known to metabolize many substrates, such as organic solvents, nitrosamines and drugs [20]. The variable levels of CYP2E1 availability and thus potency comparing rat, hamster and man resulted in different experimental results in both *in vivo* and *in vitro* studies suggesting induced hamster S9 being slightly more potent than induced rat S9 to activate nitrosamines including small N-nitrosodialkylamines [17,21,22].

Although N-Nitrosamines are mutagenic carcinogens, *in vitro*

* Corresponding author.
E-mail addresses: frank.bringezu@merckgroup.com (F. Bringezu), stephanie.simon@merckgroup.com (S. Simon).

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mutagenicity data have been seen problematic for the estimation on carcinogenic potential in man [23]. Additional concerns regarding the suitability of the bacterial reverse mutation assay to inform on the mutagenic activity of nitrosamines have been expressed. Main reasons were the CYP2E1 levels are not adequately provided by the rat liver S9, the variability and quality of the reported assay results, the different sensitivities and specificities of the strains used and the repair capacity of the alkyl transferase proficient bacteria strains that could effectively repair alkylated guanine caused by small alkyl-N-nitrosamines [24,25].

In literature, there exist several reports indicating that the activation of N-Nitrosamines by the liver microsomal fraction is species dependent [21,26,27]. In general, hamster S9 was shown to highly activate some N-Nitrosamines while rat S9 sometimes failed and, in particular, it was shown that N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA) were mutagenic in TA1535 with hamster S9 but not with rat S9 [28,29]. In contrast to these findings, Araki et al. reported a clear positive response in *E. coli* WP2 uvrA/pKM101 and TA100 with rat and hamster S9 [30].

In addition, exposure procedure related effects on the detection of the bacterial mutagenicity of Nitrosamines were reported. For NDMA, it was shown that the bacterial mutagenicity depends on the protocol performed. Here, the plate incorporation failed, while the preincubation protocol allowed to detect NDMA's mutagenicity [31]. In addition, NDEA was reported negative when tested with the plate incorporation protocol but positive with preincubation applied [32]. Protocol differences were confirmed by Araki et al., but detection of the mutagenic response was provided at all conditions [30].

The mixed results reported in literature together with the more recent discussions on the applicability of the bacterial mutagenicity assay to detect DNA reactivity of nitrosamines gained our interest in a robust evaluation of the applicability of the assay. Most data available for N-nitrosamines were published before the OECD 471 guideline was developed. Therefore, the requirements regarding strain selection, protocol, top dose, number of replicates, S9 concentration etc. were variable across the assays performed or not reported in the publications available.

A recent analysis revealed a clear correlation between carcinogenicity and bacterial mutagenicity calls for nitrosamines [33].

Therefore, we have performed mutagenicity tests using the plate incorporation and the preincubation protocol to test short chain N,N-dialkyl-nitrosamines with and without induced rat liver S9. The tests were performed using the OECD 471 guideline recommendations employing *Salmonella* and *E. coli* strains [34]. Our results indicate that mutagenicity of the nitrosamines investigated could be detected in presence of rat S9. In all cases, *E. coli* and TA1535 and TA100 were most sensitive and could thus be recommended in combination.

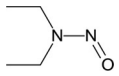
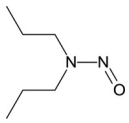
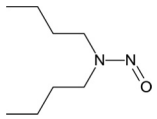
2. Materials and Methods

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 dimethylsulfoxide (DMSO) were used as solvents for the experiments. Table 1 lists the chemical structure and the purity of the batches used.

Male Wistar rats, Crl:WI (HAN) (Charles River, Germany), aged 6–8 weeks were pretreated with β-Naphthoflavone (NF) (100 mg/kg body weight) and Phenobarbital (PB) (80 mg/kg body weight) diluted in Miglyol 812 and administered orally once daily on three consecutive days. The animals received drinking water and a standard diet ad libitum. The body weight of the animals used was 238 ± 8.04 g. The study was conducted according to the license number DA4/1036 received from the respective authority (Regierungspräsidium Darmstadt, Germany).

Table 1
Test chemicals used for studies with identity, batch and purity as indicated.

Structure	Name/CAS	Batch/Purity
NDMA 	N-nitrosodimethylamine 62-75-9	LRAC4355, 92.7 %
NDEA 	N-nitrosodiethylamine 55-18-5	MKCM0585, 100 %
NDPA 	N-nitrosodipropylamine 621-64-7	LRAC6182, 99.95 %
NDBA 	N-nitrosodibutylamine 924-16-3	LRAC8566, 99.9 %

About 16 h before sacrifice, the rats remained without food. One day after the last administration of β-Naphthoflavone/Phenobarbital the animals were killed, their livers removed and homogenized in ice-cold 0.15 M KCl (3 mL KCl per g liver wet-weight). The homogenate was spun for 10 min at 9000 rpm and +4 °C. The supernatant fluid (S9) was decanted, transferred to sterile tubes and stored in liquid nitrogen.

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

Clear increases in the number of revertants for S. typhimurium TA98, TA100, and TA1537 with all positive controls and for TA1535 and *E. coli* WP2 uvrA with 2-aminoanthracene 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 % (v/v) S9 was thus applied in the S9 mix used.

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. 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.

Bacteria were propagated from frozen stocks. For all frozen stocks, their genotype characteristics were confirmed as described by Mortelmans and Zeiger [35]. The Ames test was conducted following the recommendations of the OECD 471 test guideline and standard publications [34]. In brief, bacteria were grown in nutrient broth with shaking (200

Table 2
Composition of rat liver S9 mix.

Components	Quantity per mL S9 mix
S9	0.10 mL
Magnesium chloride/ potassium chloride solution (0.4 M/ 1.64 M)	0.02 mL
Glucose-6-phosphate x 1 H_2O, disodium salt	1.61 mg
Nicotinamide adenine dinucleotide phosphate, disodium salt	3.15 mg
Sodium phosphate buffer	0.50 mL
Ultra-pure water	0.38 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	Genotype	Type of mutations 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

rpm at 37 °C) until the exponential growth phase was reached. The cell density was determined by measurement of the optical density at 650 nm. For the plate incorporation experiments, 100 µL bacteria suspension, 10 µL test, 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 histidin- or tryptophane-containing molten top agar per plate were mixed and poured onto the selective minimal glucose agar plates. For the preincubation experiments, the same volumes of bacteria suspension, test or control materials and S9 mix or phosphate buffer were mixed and incubated for 60 min at 37 °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 °C. Counting of colonies was performed with the Ames Colony Counter 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 15.8–5000 µg/plate, separated by a $\sqrt{10}$ spacing.

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.

3. Results

3.1. Short chain N-Nitrosamines NDMA and NDEA require a preincubation step

Initial tests performed with NDEA using the preincubation and the plate incorporation protocols with and without metabolic activation confirmed that increases in mutation frequencies are obtained only with metabolic activation (data not shown). When metabolically activated, NDEA leads to an exponential increase in mutation frequencies for *E. coli* WP2 uvrA, TA1535, and TA100 while no response under any condition was observed for TA98 and TA1537.

Fig. 1 compares the fold increases obtained with NDEA after metabolic activation under different conditions as indicated.

Using the plate incorporation approach yields a dose related increase in mutation frequencies for *E. coli* WP2 uvrA, reaching 12-fold at the top concentration. The *Salmonella* strains TA1535 and TA100 show increases above the thresholds of 2- or 3-fold at 5000 µg/plate only (Fig. 1 A). The application of the preincubation protocol yields a concentration response relationship with fold increases of about 4, 5, and 30 for TA100, TA1535, and *E. coli* WP2 uvrA at the top concentration of 5000 µg/plate, respectively (Fig. 1 B). Both results have been obtained using DMSO as solvent. Exchanging DMSO with water confirms that TA100, TA1535 and *E. coli* WP2 uvrA could detect NDEA induced mutagenicity with slightly increased fold changes in TA1535 and *E. coli* WP2 uvrA, and no change with TA100. At 5000 µg/plate approximately 4 (TA100), 9 (TA1535) and 39-fold (WP2) have been obtained (Fig. 1 C).

3.2. Mutagenicity of NDPA and NDBA is reliably detected applying the plate incorporation protocol

After we could show that NDEA exposure results in a strain specific induction of mutation frequencies, the effect of the chain length on the pattern observed was of great interest. Therefore, we have investigated NDMA, NDPA, and NDBA using the preincubation and the plate incorporation protocols with metabolic activation. For NDMA we employed water as solvent, while DMSO was applied at longer chain length nitrosamines. Fig. 2 depicts the fold increases across all strains investigated comparing plate incorporation and preincubation protocol on the left and right column for the different nitrosamines investigated.

In general, the data show that the mutagenic response of all nitrosamines was detected, including dimethyl or diethyl substitutions. With increasing chain length, the compounds show bacterial toxicity when tested at high concentrations above 1580 µg/plate. Therefore, the highest fold increases are detected below the top concentration and the maximum fold increase thus shifts from preincubation to plate incorporation.

3.3. E. coli WP2 uvrA is highly sensitive to detect the mutagenic effects of N-Nitrosamines

The pattern of the strains detecting the mutagenicity includes TA100, TA1535, *E. coli* WP2 uvrA with a linear concentration response on the logarithmic scale. Using the preincubation protocol, *E. coli* WP2 uvrA could detect the mutagenicity for all nitrosamines investigated with relevant increases above the threshold as defined in the evaluation criteria.

Fig. 3 compares the maximum fold increases obtained for the different strains under preincubation or plate incorporation conditions applied in dependence on the alkyl chain length of the different molecules investigated. The figure shows that with increasing chain lengths the maximum fold inductions shift to higher values. For NDMA, the preincubation protocol allows the detection of mutagenicity while the plate incorporation protocol fails. For NDEA, *E. coli* WP2 uvrA is the most sensitive strain showing fold inductions above 10-fold (about 30 and 12). Using the preincubation protocol, NDEA's mutagenicity can be detected with TA1535 and TA100 with fold increases of 4 and 5 above the defined thresholds for positive responses, respectively. Going to higher chain lengths at NDPA and NDBA relevant increases are obtained independent on the protocol conditions applied with TA1535, TA100 and *E. coli* WP2 uvrA. With increasing chain lengths of the residues, the maximum fold changes obtained with plate incorporation increase and exceed the values obtained for preincubation conditions. Comparing preincubation with plate incorporation for NDBA, *E. coli* WP2 uvrA and TA1535 show higher fold inductions using the plate incorporation protocol indicating that the protocol related effects become less important for those nitrosamines.

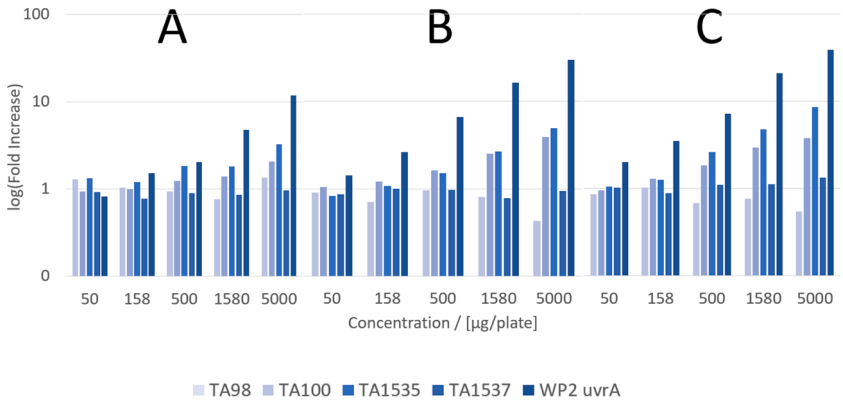


Fig. 1. Log fold increases obtained after NDEA exposure for the different strains as indicated using metabolic activation by rat liver S9 and different assay conditions (A: plate incorporation, solvent: DMSO; B: preincubation, solvent: DMSO; C: preincubation, solvent: ultrapure water).

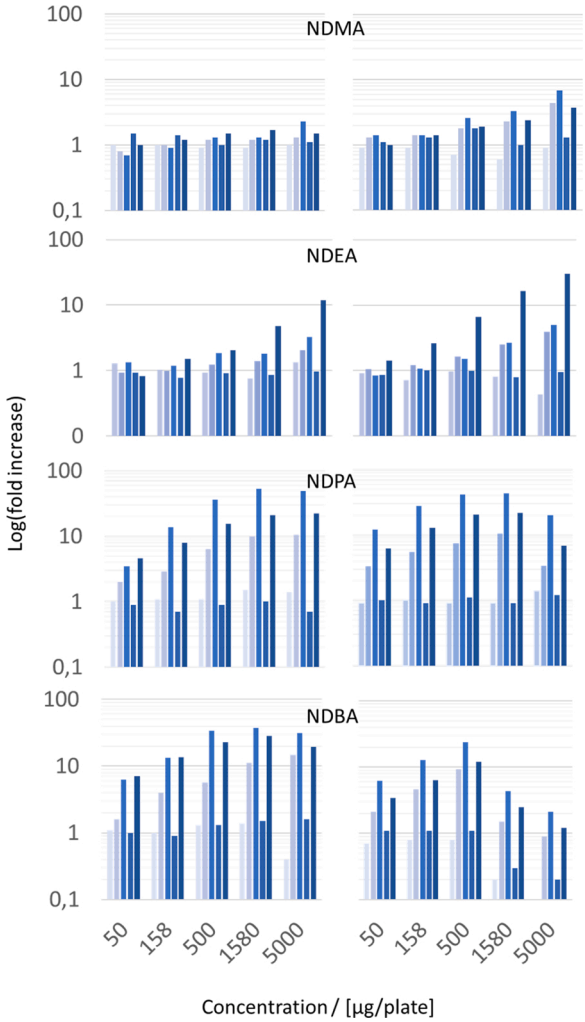


Fig. 2. Log fold increases obtained with metabolic activation using rat liver S9 mix for the different nitrosamines investigated. The plate incorporation protocol (left) is compared to the preincubation protocol (right). From bottom to top the data are shown for NDBA, NDPA, NDEA, and NDMA, respectively.

4. Discussion

In comparison to the literature data, we could show that all nitrosamines investigated are detected in the bacterial reverse mutation assay using OECD 471 compliant conditions starting from shortest dimethyl to dibutyl substitutions. For NDMA, the preincubation protocol is required as no clear positive response was detected with plate incorporation. It is well established that small nitrosamines are preferentially activated by CYP2E1 [18] that shows a small active site. For medium and larger alkyl residues at the N-nitrosamine alternative forms of CYPs are also relevant for their activation, e.g. CYP2A6, CYP2C19, or CYP3A4 [36]. These findings are supported by our results showing improved activation and thus mutagenic response with increasing chain length.

Nitrosamines require metabolic activation and there were different publications showing that species differences exist suggesting hamster S9 for this class of compounds [26,28,37]. Moreover, there exist data indicating that rat S9 failed to activate nitrosamines to exert mutagenic activity [38]. In these investigations, the induction of the rat liver enzymes was performed using phenobarbital 1% (v/v) in drinking water. We have applied a combination of phenobarbital/β-naphthoflavone (PB/NF) as inducing agents administered via oral gavage that might lead to higher exposure of the rats as compared to application via drinking water. Our data could clearly demonstrate that PB/NF induced rat liver S9 is capable to metabolize these compounds appropriately and thus allows detection of mutagenic activity *in vitro*. The differences in mutagenic response using induced rat liver S9 might be explained by differences in the induction procedures performed.

The solvent comparison between DMSO and water performed with NDEA showed slightly higher mutation frequencies when using water for all three positive strains. This result may easily be explained as DMSO is known to inhibit CYPs and in particular CYP2E1 [39]. However, vehicle independent significant increases above the thresholds from the evaluation criteria were observed in our studies for TA1535, TA100 and *E. coli* WP2 uvrA, respectively. Previous investigations in mammalian cells showed that DMSO did also not modify NDEA-induced gene mutations or micronuclei in human CYP2E1 competent V79 cells [40]. This is in line with our data suggesting that DMSO does neither lead to a significant inhibition of the induced rat liver S9 overall activity profile nor to a deactivation of the nitrosamine related mutagenicity in bacteria and may thus be applied for this class of compounds up to a concentration of 1% (v/v).

The strains discussed in literature include TA1535 and expand into specifically modified strains like the alkyl-transferase deficient *Salmonella* strain YG7108 being specifically sensitive for N-nitrosamine mutagenicity. Our investigation demonstrated that the strains outlined in the OECD 471 guideline are sufficient to record the mutagenic response. Indeed, TA100 and TA1535 are capable detecting the activity. In addition, we could show that *E. coli* WP2 uvrA is highly sensitive to detect the mutagenic activity and should be combined with TA1535 and TA100 for appropriate testing.

The clear correlation between carcinogenicity and bacterial mutagenicity calls for nitrosamines reported in literature showed to be stronger for nitrosamines than for non-nitrosamines [33]. Our data

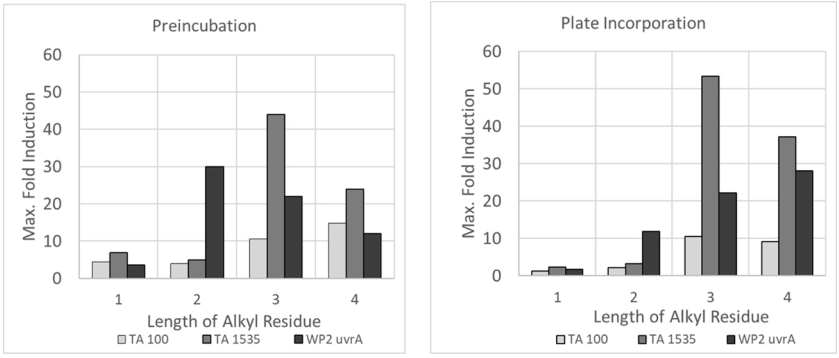


Fig. 3. Maximum Fold induction values obtained with metabolic activation using rat liver S9 mix for the different nitrosamines investigated is shown as function of the chain length of the alkyl residues at the nitrogen. The figure compares the results obtained using the preincubation protocol (left) with the plate incorporation protocol (right) for three strains that show increased mutation frequencies, i.e. TA100, TA1535, and E. coli WP2 uvrA as indicated.

suggest that appropriate testing for bacterial mutagenicity using the OECD 471 protocol and considering preincubation for short chain substitution gives the responses expected. Therefore, the Ames assay is highly relevant for nitrosamines and may thus be used for evaluation of their mutagenic activity.

5. Conclusion

In conclusion, our results demonstrate that the mutagenicity of N-nitrosamines can be reliably detected when applying OECD 471 guideline recommendations regarding strain selection, protocol conditions, top concentrations, and metabolizing system.

Data Availability

Data will be made available on request.

Conceptualization: Frank Bringezu and Stephanie Simon.

Project Administration, Methodology, Investigation and Validation: Stephanie Simon.

Data Curation, Formal Analysis, Software and Visualization: Frank Bringezu.

Supervision: Stephanie Simon.

Writing – original draft: Frank Bringezu.

Writing - review and editing: Frank Bringezu and Stephanie Simon.

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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.

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References

[1] H. Druckrey, R. Preussmann, S. Ivankovic, D. Schmähl, [Organotropic carcinogenic effects of 65 various N-nitroso- compounds on BD rats], Z. Krebsforsch. 69 (1967) 103–201.

[2] J.M. Barnes, P.N. Magee, Some toxic properties of dimethylnitrosamine, Br. J. Ind. Med. 11 (1954) 167–174, https://doi.org/10.1136/oem.11.3.167.

[3] L.K. Keefer, P.P. Roller, N-nitrosation by nitrite ion in neutral and basic medium, Science. 181 (1973) 1245–1247, https://doi.org/10.1126/science.181.4106.1245.

[4] J.H. Hotchkiss, Sources of N-nitrosamine contamination in foods, Adv. Exp. Med. Biol. 177 (1984) 287–298, https://doi.org/10.1007/978-1-4684-4790-3_14.

[5] G. Eisenbrand, B. Spiegelhalder, J. Kann, R. Klein, R. Preussmann, Carcinogenic N-nitrosodimethylamine as a contamination in drugs containing 4-dimethylamino-2,3-dimethyl-1-phenyl-3-pyrazolin-5-one (amidopyrine, aminophenazone), Arzneimittelforschung. 29 (1979) 867–869, https://doi.org/10.1002/chin.197938327.

[6] D.J. Snodin, D.P. Elder, Short commentary on NDMA (N-nitrosodimethylamine) contamination of valsartan products, Regul. Toxicol. Pharmacol. 103 (2019) 325–329, https://doi.org/10.1016/j.yrtph.2019.01.007.

[7] F. Sörgel, M. Kinzig, M. Abdel-Tawab, C. Bidmon, A. Schreiber, S. Ermel, J. Wohlfart, A. Besa, O. Scherf-Clavel, U. Holzgrabe, The contamination of valsartan and other sartans, part 1: new findings, J. Pharm. Biomed. Anal. 172 (2019) 395–405, https://doi.org/10.1016/j.jpba.2019.05.022.

[8] O. Scherf-Clavel, M. Kinzig, A. Besa, A. Schreiber, C. Bidmon, M. Abdel-Tawab, J. Wohlfart, F. Sörgel, U. Holzgrabe, The contamination of valsartan and other sartans, Part 2: untargeted screening reveals contamination with amides additionally to known nitrosamine impurities, J. Pharm. Biomed. Anal. 172 (2019) 278–284, https://doi.org/10.1016/j.jpba.2019.04.035.

[9] ICH, Assessment and control of DNA reactive (mutagenic) impurities in pharmaceuticals to limit potential carcinogenic risk. ICH harmonised tripartite guideline, step 4, 31 March 2017, International Conference on Harmonisation of Technical Requirements for Registration of Pharmaceuticals for Human Use (2017). https://database.ich.org/sites/default/files/M7_R1_Guideline.pdf.

[10] J.P. Bercu, S.M. Galloway, P. Parris, A. Teasdale, M. Masuda-Herrera, K. Dobo, P. Heard, M. Kenyon, J. Nicolette, E. Vock, W. Ku, J. Harvey, A. White, S. Glowienke, E.A. Martin, L. Custer, R.A. Jolly, V. Thybaud, Potential impurities in drug substances: compound-specific toxicology limits for 20 synthetic reagents and by-products, and a class-specific toxicology limit for alkyl bromides, Regul. Toxicol. Pharmacol. 94 (2018) 172–182, https://doi.org/10.1016/j.yrtph.2018.02.001.

[11] J.P. Bercu, M. Masuda-Herrera, G. Johnson, A. Czich, S. Glowienke, M. Kenyon, R. Thomas, D.J. Ponting, A. White, K. Cross, F. Waechter, M.A.C. Rodrigues, Use of less-than-lifetime (LTL) durational limits for nitrosamines: case study of N-Nitrosodiethylamine (NDEA), Regul. Toxicol. Pharmacol. RTP. 123 (2021), https://doi.org/10.1016/j.yrtph.2021.104926, 104926.

[12] EMA, Temporary Interim Limits for NMBA, DIPNA and EIPNA Impurities in Sartan Blood Pressure Medicines, 2019. https://www.ema.europa.eu/en/documents/other/temporary-interim-limits-nmba-dipna-eipna-impurities-sartan-blood-pressure-medicines_en.pdf.

[13] Health Canada, Impurities Found in Certain Angiotensin II Receptor Blocker (ARB) Products, Also Known As Sartans, Impurities Found Certain Angiotensin II Recept. Blocker ARB Prod. Also Known Sartans, 2019 (accessed April 29, 2019), https://www.canada.ca/en/health-canada/services/drugs-health-products/compliance-enforcement/information-health-product/drugs/angiotensin-receptor-blocker.html.

[14] N.A. Charoo, A.A. Ali, S.K. Buha, Z. Rahman, Lesson learnt from recall of Valsartan and other angiotensin II receptor blocker drugs containing NDMA and NDEA impurities, AAPS PharmSciTech 20 (2019), https://doi.org/10.1208/s12249-019-1376-1, 166.

[15] FDA, Control of Nitrosamine Impurities in Human Drugs, 2021 (accessed February 24, 2021), https://www.fda.gov/regulatory-information/search-fda-guidance-documents/control-nitrosamine-impurities-human-drugs.

[16] 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 (1992) 979–985, https://doi.org/10.1093/carcin/13.6.979.

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

[18] 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 (1992) 1789–1794, https://doi.org/10.1093/carcin/13.10.1789.
[19] K.P. Cross, D.J. Ponting, Developing structure-activity relationships for N-nitrosamine activity, Comput. Toxicol. 20 (2021), https://doi.org/10.1016/j.comtox.2021.100186, 100186.
[20] M. Martignoni, G.M.M. Groothuis, R. de Kanter, Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction, Expert Opin. Drug Metab. Toxicol. 2 (2006) 875–894, https://doi.org/10.1517/17425255.2.6.875.
[21] C.E. Phillipson, C. Ioannides, A comparative study of the bioactivation of nitrosamines to mutagens by various animal species including man, Carcinogenesis. 5 (1984) 1091–1094, https://doi.org/10.1093/carcin/5.8.1091.
[22] S. Synder, I.C. Hsu, B.F. Trump, Comparison of metabolic activation of carcinogens in human, rat, and hamster hepatocytes, Mutat. Res. 182 (1987) 31–39, https://doi.org/10.1016/0165-1161(87)90005-7.
[23] J. McCann, L.S. Gold, L. Horn, R. McGill, T.E. Graedel, J. Kaldor, Statistical analysis of Salmonella test data and comparison to results of animal cancer tests, Mutat. Res. 205 (1988) 183–195, https://doi.org/10.1016/0165-1218(88)90017-1.
[24] E.D. Wagner, J. Osiol, W.A. Mitch, M.J. Plewa, Comparative in vitro toxicity of nitrosamines and nitramines associated with amine-based carbon capture and storage, Environ. Sci. Technol. 48 (2014) 8203–8211, https://doi.org/10.1021/es5018009.
[25] EMA, Nitrosamine Impurities in Human Medicinal Products, 2020. https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-assessment-report_en.pdf.
[26] R. Raineri, J.A. Poiley, A.W. Andrews, R.J. Pienta, W. Lijinsky, Greater effectiveness of hepatocyte and liver S9 preparations from hamsters than rat preparations in activating N-nitroso compounds to metabolites mutagenic to Salmonella, J. Natl. Cancer Inst. 67 (1981) 1117–1122.
[27] W. Lijinsky, Carcinogenicity and mutagenicity of N-nitroso compounds, Mol. Toxicol. 1 (1987) 107–119.
[28] W. Lijinsky, A.W. Andrews, The superiority of hamster liver microsomal fraction for activating nitrosamines to mutagens in Salmonella typhimurium, Mutat. Res. 111 (1983) 135–144, https://doi.org/10.1016/0027-5107(83)90058-1.
[29] B.N. Ames, J. Mccann, E. Yamasaki, Methods for detecting carcinogens and mutagens with the Salmonella/mammalian-microsome mutagenicity test, Mutat. Res. 31 (1975) 347–364, https://doi.org/10.1016/0165-1161(75)90046-1.
[30] 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, Gan. 75 (1984) 8–16.
[31] H. Bartsch, C. Malaveille, R. Montesano, In vitro metabolism and microsome-mediated mutagenicity of dialkylnitrosamines in rat, hamster, and mouse tissues, Cancer Res. 35 (1975) 644–651.
[32] T. Yahagi, M. Nagao, Y. Seino, T. Matsushima, T. Sugimura, Mutagenicities of N-nitrosamines on Salmonella, Mutat. Res. 48 (1977) 121–129, https://doi.org/10.1016/0027-5107(77)90151-8.
[33] A. Thresher, R. Foster, D.J. Ponting, S.A. Stalford, R.E. Tennant, R. Thomas, Are all nitrosamines concerning? A review of mutagenicity and carcinogenicity data, Regul. Toxicol. Pharmacol. RTP. 116 (2020), https://doi.org/10.1016/j.yrtph.2020.104749, 104749.
[34] OECD, Test No, 471: Bacterial Reverse Mutation Test, 2020. https://www.oecd-ilibrary.org/content/publication/9789264071247-en.
[35] K. Mortelmans, E. Zeiger, The Ames Salmonella/microsome mutagenicity assay, Mutat. Res. 455 (2000) 29–60, https://doi.org/10.1016/s0027-5107(00)00064-6.
[36] D.F. Lewis, Y. Ito, Human CYPs involved in drug metabolism: structures, substrates and binding affinities, Expert Opin. Drug Metab. Toxicol. 6 (2010) 661–674, https://doi.org/10.1517/17425251003674380.
[37] M.J. Prival, V.D. King, A.T. Sheldon, The mutagenicity of dialkyl nitrosamines in the Salmonella plate assay, Environ. Mutagen. 1 (1979) 95–104, https://doi.org/10.1002/em.2860010202.
[38] T.K. Rao, B.E. Allen, W. Winton, W. Lijinsky, J.L. Epler, Nitrosamine-induced mutagenesis in Escherichia coli K12 (343/113), Mutat. Res. Toxicol. 89 (1981) 209–215, https://doi.org/10.1016/0165-1218(81)90239-1.
[39] L. Jia, X. Liu, The conduct of drug metabolism studies considered good practice (II): in vitro experiments, Curr. Drug Metab. 8 (2007) 822–829, https://doi.org/10.2174/138920007782798207.
[40] G. Jin, L. Cai, K. Hu, Y. Luo, Y. Chen, H. Glatt, Y. Liu, Mutagenic activity of *N*-Nitrosodiethylamine in cell lines expressing human CYP2E1-Adequacy of dimethylsulfoxide as solvent: DMSO compatible with CYP2E1-activated toxicity, Environ. Mol. Mutagen. 60 (2019) 214–226, https://doi.org/10.1002/em.22264.