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# An Enhanced Metabolization Protocol for In Vitro Genotoxicity Assessment of N-Nitrosamines in Mammalian Cells

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## ABSTRACT

N-Nitrosamines (NAs) are probable human carcinogens and were detected as impurities in pharmaceuticals, which led to a concern for human health. NAs require metabolic activation before they become mutagenic, and not all NAs are mutagenic since their reactivity is related to their structure. While some NAs are potent mutagens in vivo, in vitro metabolization with exogenous S9 liver extract is generally less efficient. While an enhanced bacterial mutagenicity protocol was recently developed, which uses increased concentrations of S9 liver extracts, there presently is not an improved metabolization protocol suitable for mammalian cell genotoxicity assays. Therefore, we optimized a hamster S9 liver extract-based protocol for in vitro NA metabolization and assessed the genotoxic potential of various NAs using ToxTracker. With this enhanced metabolization protocol (EMP), the genotoxic potency of N-nitrosodimethylamine (NDMA) increased approximately 200-fold compared with the standard S9 liver extract-based exposure protocol in ToxTracker. The EMP was further validated with seven additional mutagenic NAs to which humans are commonly exposed: N-nitrosodiethylamine (NDEA), N-nitrosodiethanolamine (NDELA), N-nitrosodibutylamine (NDBA), N-nitrosofluoxetine (NF), 1-nitrosopyrrolidine (NPYR), N-nitrosomorpholine (NMOR), and 1-cyclopentyl-4-nitrosopiperazine (CPNP), and two non-mutagenic NAs: N-nitrosobupropion (NBuPRO) and N-nitrosoproline (NPRO). Genotoxicity could be confirmed for six NAs using the EMP, demonstrating that mammalian cells and the new approach methodology (NAM) ToxTracker may have potential when investigating NA-related genotoxicity.

## 1 | Introduction

N-Nitrosamines (NAs) have been well known for their mutagenic potential for many years (IARC 1978). NA impurities can occur in a variety of sources, such as food, drinking water, cosmetics, tobacco products, and pharmaceuticals (EMA 2020b; Fahrer and Christmann 2023; Li and Hecht 2022). Since NAs are classified as probable carcinogens, they are part of the cohort of concern in the ICH M7(R2) guideline on impurities in pharmaceuticals (ICH 2023). Public concern was raised in 2018, when N-nitrosodimethylamine (NDMA) was detected as an impurity in Valsartan due to a change in the production process, leading to the withdrawal of sartans from the market (EMA 2018). More recently, nitrosamine drug substance-related impurities (NDSRIs) were detected in medicines, making it clear that NAs were not only product impurities but could also be formed by

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the nitrosation of the active pharmaceutical ingredients (API) (EMA 2020b). The genotoxic potency of these NDSRIs is often unknown, and about 40% of all drugs are at risk for NA formation (EMA 2020b; Schlingemann et al. 2023). The occurrence of NDSRIs has alerted regulatory agencies, which aim to identify the mutagenic risk that NAs pose and translate this into a daily acceptable intake (AI) (EMA 2024b; FDA 2024; Health Canada 2024).

Since 2023, EMA has adopted a novel structure–activity relationship (SAR)-based strategy based on known nitrosamines with carcinogenicity data using five categories (EMA 2023). For data-rich substances that are less potent, it can be argued that the daily AI could be raised or that these substances fall outside the cohort of concern. However, for many NDSRIs, no or limited data are available, and adherence to the lowest daily AI might lead to unnecessary withdrawal of valuable drugs from the market. Therefore, additional data are required to assess the potency of NAs (Burns et al. 2023).

NAs are metabolized by cytochrome P450 (CYP) enzymes. For example, NDMA is metabolized by CYP2E1 and CYP2A6 via alpha hydroxylation (Fahrer and Christmann 2023; Li and Hecht 2022). The CYP enzymes hydroxylate the alpha-carbon, leading to the formation of alkyldiazohydroxide species, which can form highly reactive diazonium ions after hydroxide loss (Ponting and Foster 2023). Diazonium ions are NA-specific and react with nucleophiles, including DNA, which results in alkylated bases that, when not repaired properly, can lead to gene mutations during DNA replication. The mutagenic and carcinogenic potential of an NA is related to its chemical structure. Especially, NAs with the nitroso groups attached to an alkyl group and, in particular, to an alpha-carbon are shown to have a high carcinogenic potency (Fahrer and Christmann 2023; Li and Hecht 2022; Ponting and Foster 2023).

Generally, to test compounds in vitro for their genotoxic potential, exogenous post mitochondrial supernatant from induced rat liver homogenate (liver S9 extract) is included to mimic metabolism in the liver as most used test systems lack endogenous CYP enzymes. However, metabolization of NAs using low percentages of rat S9 liver extract, as is the standard mammalian cell protocol, is inefficient, and high concentrations of the test substance are required to observe an effect (Tennant et al. 2023).

Recently, the metabolization protocol for the Ames test, a bacterial reverse mutation assay, has been optimized to address this limitation (EMA 2024a; Li et al. 2023). The enhanced Ames test (EAT) uses a 30-min pre-incubation of NA with a high percentage of rat or hamster S9 liver extract (30% v/v) (EMA 2024a). Mutagenicity detection was more sensitive with hamster S9 liver extract when compared to rat S9 liver extract, and using methanol or water as a solvent was preferred over DMSO (Li et al. 2023; Thomas et al. 2024). Since such a protocol does not yet exist for mammalian cells, we focused on optimizing a protocol for the metabolization of NAs using hamster S9 liver extract in mammalian cells and used the ToxTracker assay as a read-out for genotoxicity.

ToxTracker is a mammalian stem cell-based reporter assay for predicting primary and indirect genotoxicity. ToxTracker consists of six GFP reporter cell lines that are activated via different cellular stress pathways that survey the induction of DNA damage (Bscl2 and Rtkn), protein stress (Ddit3), cellular stress (Btg2), and oxidative stress (Srxn1 and Blvrb). Activation of the Bscl2 and/or Rtkn reporter predicts genotoxicity, while activation of the other reporters provides mechanistic insight, for example, if a substance is indirectly genotoxic due to high levels of oxidative DNA damage or protein stress (Hendriks et al. 2016, 2024).

Like most cell-based genotoxicity assays, ToxTracker relies on the addition of rat S9 liver extract for the biotransformation of test articles and the ability to detect genotoxic metabolites. The standard metabolization protocol in ToxTracker utilizes a 24-h co-exposure to a low concentration (0.4% v/v) phenobarbital/beta-naphthoflavone-induced rat S9 liver homogenate. Although this protocol is sufficient for the metabolic activation of most genotoxic substances, it was deemed inefficient at bioactivating NAs as NDMA was not genotoxic even when tested up to 135.8 mM. Therefore, we first focused on improving the metabolization protocol by moving to an acute exposure (~4 h) with hamster S9 liver extract, additional cofactors, and a 30-min incubation with concentrated NA-S9 mix. Using this enhanced metabolization protocol (EMP), NDMA-induced genotoxicity was detected at 0.6 mM in the ToxTracker assay. To validate the EMP, another nine NAs were tested: five linear (NDEA, NDELA, NDBA, NBuPRO, and NF) and four cyclic NAs (NPYR, NMOR, NPRO, and CPNP, Figure 1). Two NAs were classified as non-genotoxic as expected, and seven out of eight genotoxic NAs were classified correctly. The genotoxic NAs were ranked by potency using Benchmark Dose (BMD) analysis, as previous work indicated that ToxTracker BMD values correlate well with those obtained from the in vivo micronucleus assay (Beal et al. 2023; Boisvert et al. 2023; Wills et al. 2021).

## 2 | Materials and Methods

### 2.1 | Compounds

The following N-nitrosamines were tested and purchased from EnamineStore: N-nitrosodimethylamine (NDMA, CAS no. 62-75-9), N-nitrosodiethylamine (NDEA, CAS no. 5-18-5), N-nitrosodiethanolamine (NDELA, CAS no. 1116-54-7), N-nitrosodibutylamine (NDBA, CAS no. 924-16-3), N-nitrosobupropion (NBuPRO, CAS no. 2763780-10-3), N-nitrosofluoxetine (NF, CAS no. 150494-06-7), 1-nitrosopyrrolidine (NPYR, CAS no. 930-55-2), N-nitrosomorpholine (NMOR, CAS no. 59-89-2), N-nitrosoproline (NPRO, CAS no. 7519-36-0), and 1-cyclopentyl-4-nitrosopiperazine (CPNP, CAS no. 61379-66-6). N-nitrosamines were diluted in MilliQ, except NF and NBuPRO, which were diluted in dimethyl sulfoxide (DMSO, 1.02952, Supelco). Cisplatin (PHR1624, Supelco) was diluted in phosphate buffered saline (PBS); diethyl maleate (DEM, D97703, Sigma-Aldrich), tunicamycin (T7765, Sigma-Aldrich), and cyclophosphamide (CP, PHR1404, Supelco) were diluted in DMSO.

### 2.2 | ToxTracker Assay

C57/Bl6 B4418 unmodified mES cells or GFP-reporter cells were cultured in complete mES cell culture medium (KnockOut

(A)

| Compound | | CAS number | MW (g/mol) | Conc. pos. in EAT (µg/plate)* | TD50 (mg/kg/day)** |
|---|---|---|---|---|---|
| N-nitrosodimethylamine | NDMA | 62-75-9 | 74.09 | 50 | 0.145 |
| N-nitrosodiethylamine | NDEA | 55-18-5 | 102.14 | 50 | 0.062 |
| N-nitrosodiethanolamine | NDELA | 1116-54-7 | 134.14 | N.D. | 38.9 |
| N-nitrosodi-n-butylamine | NDBA | 924-16-3 | 158.24 | 25 | 8.2 |
| N-nitrosobupropion | NBuPRO | 2763780-10-3 | 268.74 | N.D. | N.D. |
| N-nitrosofluoxetine | NF | 150494-06-7 | 338.32 | 25 | N.D. |
| N-nitrosopyrrolidine | NPYR | 930-55-2 | 100.12 | N.D. | 2.47 |
| N-nitrosomorpholine | NMOR | 59-89-2 | 116.12 | N.D. | N.D. |
| N-nitrosoproline | NPRO | 7519-36-0 | 144.13 | N.D. | Negative |
| 1-cyclopentyl-4-nitrosopiperazine | CPNP | 61379-66-6 | 183.24 | 10 | N.D. |

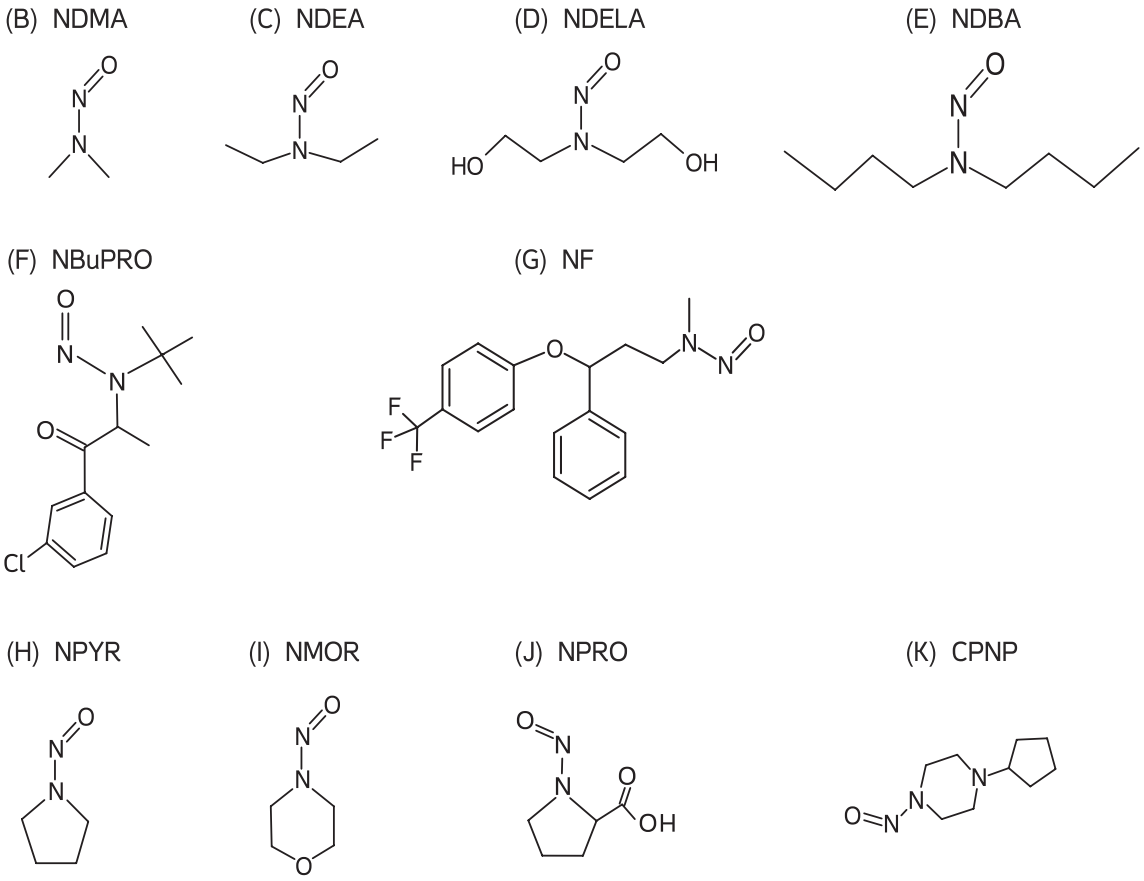



**FIGURE 1** | Overview of tested N-nitrosamines. (A) Table with the abbreviations, CAS numbers, molecular weight, concentration with a positive result in the EAT as determined by Heflich et al. (2024), and TD50 values. *Values shown are the lowest concentration (µg/plate) at which a positive effect was observed for any of the tested strains. N.D. not determined. **TD50 values in mg/kg/day based on the most sensitive species (Snodin et al. 2024). (B–I) Chemical structures of (B) NDMA: N-nitrosodimethylamine. (C) NDEA: N-nitrosodiethylamine. (D) NDELA: N-nitrosodiethanolamine. (E) NDBA: N-nitrosodi-n-butylamine. (F) NBuPRO: N-nitrosobupropion. (G) NF: N-nitrosofluoxetine. (H) NPYR: N-nitrosopyrrolidine. (I) NMOR: N-nitrosomorpholine. (J) NPRO: N-nitrosoproline. (K) CPNP: 1-cyclopentyl-4-nitrosopiperazine.

DMEM containing 10% fetal calf serum (FCS), 1% penicillin/streptomycin, 1× glutamax, 1 mM sodium pyruvate, 1× minimal essential medium non-essential amino acids, 100 µM β-mercaptoethanol, and 0.05% leukemia inhibitory factor (LIF)). Media components were acquired from Gibco, except for LIF, which was prepared in-house. Cells were maintained on feeders or on gelatin-coated plates according to established protocols (Hendriks et al. 2012, 2016). GFP-reporter cell lines were maintained in the presence of 0.2 mg/mL G418 (Sigma-Aldrich).

ToxTracker analysis was performed as previously described (Hendriks et al. 2016, 2024). Briefly, after exposure, the cells were washed twice with PBS, dissociated with Trypsin–EDTA (Gibco), resuspended in PBS supplemented with 2% FCS, and measured using a MACSQuant X (Miltenyi Biotech) or Guava EasyCyte (Millipore) flow cytometer with Argon excitation. GFP reporter activity was determined by measuring the mean fluorescent intensity (MFI) of 10,000 intact cells while simultaneously determining the absolute cell count to calculate

relative survival (RS). The induction levels were calculated as the MFI ratio of exposed cells over the corresponding vehicle control-treated cells, and a response was considered positive when GFP induction was at least 2-fold (Boisvert et al. 2023; Hendriks et al. 2024). RS was calculated by dividing the concentration of cells from exposed cultures by the number of cells in vehicle-treated samples. Measurements at concentrations that induced > 75% cytotoxicity after exposure were not considered when interpreting data. Three independent biological experiments were conducted, and positive control substances were measured in all experiments to assess assay validity.

### 2.3 | Hamster S9 Liver Extract-Based Metabolizing System

Cells were seeded on gelatin-coated plates in complete mES cell culture medium 24 h prior to exposure initiation. Cells were exposed to cisplatin, DEM, or tunicamycin for 4 h in the absence of hamster S9 liver extract, while cells were exposed to CP for 4 h in the presence of hamster S9 liver extract (described below). After the 4 h exposures, cells were washed once with PBS before adding fresh complete mES cell culture medium for 20 h.

To mimic metabolism in vitro, exogenous post-mitochondrial supernatant from phenobarbital/beta-naphthoflavone-induced hamster S9 liver homogenate was used (hamster S9 liver extract, protein concentration 33–38 mg/mL, Moltox). To optimize the hamster S9 liver extract concentration, a concentrated mix containing 10% hamster S9 liver extract and cofactor mix (15.3 mM $MgCl_2$, 29.7 mM KCl, 5.2 mM G-6-P, and 1.7 mM NADP, Moltox) was prepared. Cells were exposed for 4 or 24 h to increasing percentages of hamster S9 liver extract diluted in mES cell culture media with a maximum S9 percentage of 3% (0.99–1.14 mg/mL final protein concentration) for 4 h or 0.3% (0.10–0.11 mg/mL final protein concentration) for 24 h. After the 4 h exposure, cells were washed once with PBS before adding fresh complete mES cell culture medium for 20 h.

To optimize the exposure protocol, a mix containing the test compound and, where indicated, 3% hamster S9 liver extract (final protein concentration of 0.99–1.14 mg/mL) plus standard cofactor mix (final concentration 4.6 mM $MgCl_2$, 8.9 mM KCl, 1.6 mM G-6-P, 0.5 mM NADP, Moltox) was prepared and incubated for 30 min at room temperature. For the enhanced cofactor mix, $CaCl_2$ (423,520,250, ThermoScientific) was added to a final concentration of 2 mM based on previous literature (O'Neill et al. 1982). After the 30-min incubation, cells were exposed to the test conditions for 4 h, washed once with PBS, and fresh complete mES cell culture medium was added for 20 h.

### 2.4 | EMP

The EMP consists of a concentrated, 30-min incubation on the cells followed by a 4-h exposure. For that, a concentrated mix containing 3.3× concentrated compound, 10% hamster S9 liver extract, and 3.3× concentrated enhanced cofactor mix (15.3 mM $MgCl_2$, 29.7 mM KCl, 5.2 mM G-6-P, 1.7 mM NADP, and 6.6 mM $CaCl_2$) was prepared and incubated on the cells for 30 min at 37°C. The mix was then diluted 3.3 times with medium to final compound concentrations with 3% hamster S9 liver extract (final protein concentration of 0.99–1.14 mg/mL) in 4.6 mM $MgCl_2$, 8.9 mM KCl, 1.6 mM G-6-P, 0.5 mM NADP, and 2 mM $CaCl_2$. This final mix was incubated with cells for 4 h. In exposure without hamster S9 liver extract, an identical exposure scenario was followed, devoid of S9 and cofactor mix. After the 4 h exposure, cells were washed once with PBS before adding fresh complete mES cell culture medium for 20 h.

### 2.5 | Benchmark Dose Analysis

BMD was conducted for the DNA damage reporters Rtkn and Bscl2 with PROAST (70.1, RIVM, Bilthoven, The Netherlands). ToxTracker MFI values obtained from three independent biological experiments were used for the analysis. NAs that did not induce the Rtkn or Bscl2 reporter and concentrations with > 75% cytotoxicity were excluded from the analysis. The dose–response analysis was performed with continuous data, and a benchmark response (BMR, critical effect size) of 100% was used for Rtkn, signifying a doubling of the response over background. For Bscl2, a BMR of 50% was used due to weaker responses, representing a 1.5-fold induction over background. From the analysis, the BMD 90% confidence intervals BMDL and BMDU were used to determine the relative potency of compounds.

## 3 | Results

### 3.1 | Establishing a Hamster S9 Liver Extract-Based Metabolizing System in ToxTracker

To improve mammalian cell-based toxicity testing of NAs, we first optimized a protocol for metabolizing NAs using hamster S9 liver extracts. Exposure to 3% hamster S9 liver extract (v/v) for 4 h or 0.3% hamster S9 for 24 h was well tolerated by the ToxTracker cell lines (Figure 2). No cytotoxicity was observed, and induction levels for the different GFP reporters in ToxTracker were below 2-fold. The validity of the test system was demonstrated with positive controls (representative responses in Figure S1). Following a 24-h exposure to

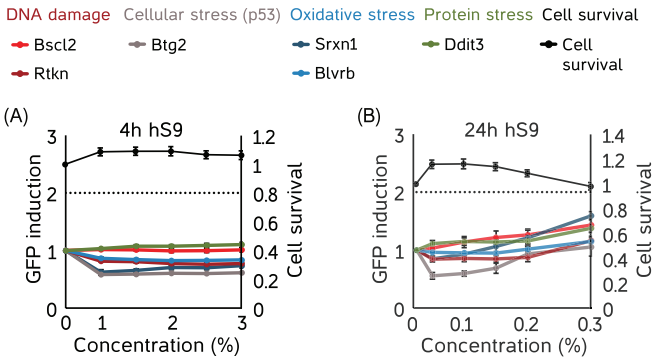



**FIGURE 2** | ToxTracker GFP reporter response and cell survival after treatment with increasing percentages of hamster S9 liver extract for 4 h (A, maximum of 3% hamster S9) or 24 h (B, maximum of 0.3% hamster S9). The dashed line indicates the 2-fold threshold for a positive response.

0.3% hamster S9 liver extract, a >1.5-fold increase in Srxn1 expression could be detected (Figure 2B), indicating that there is minor oxidative stress induced by these conditions. Considering that a 4-h exposure with higher concentrations of hamster S9 liver extract was tolerated, and that a 4-h exposure with 2% hamster S9 is used in the in vitro micronucleus assay for N-nitrosopropanolol (Li et al. 2023), we continued with this exposure duration and 3% hamster S9 liver extract for further optimization.

Next, NDMA was tested in ToxTracker using a 4-h exposure protocol with and without 3% hamster S9 liver extract and the standard cofactor mix. NDMA was incubated for 30 min with this S9/cofactor mix without cells prior to the 4-h exposure with cells to boost biotransformation. In the absence of hamster S9 liver extract, there was no reporter activation (Figure 3A). In the presence of hamster S9 liver extract, NDMA induced Rtkn-GFP (the DNA damage reporter) and Btg2-GFP (cellular stress, p53 analog) at least 2-fold at 135.8 or 67.9 mM, respectively (Figure 3B). These concentrations were very high (ICH 2011; Parry et al. 2010) and were not considered relevant. Since NDMA is a strong mutagen but genotoxicity was only observed in ToxTracker at 135.8 mM, the metabolization protocol required further optimization.

Previously, O'Neill et al. (1982) showed that addition of calcium chloride could significantly enhance the detection of mutagenicity induced by NDMA. This is why calcium chloride was previously added to different mammalian cell assays to improve mutagenicity and genotoxicity detection of nitrosamines (Li et al. 2023). Hence, to further enhance the in vitro metabolization of NAs by hamster S9 liver extract in ToxTracker, calcium chloride was added to the cofactor mix (referred to as the "enhanced cofactor mix"). Using the enhanced cofactor mix, NDMA induced a 2-fold increase in Rtkn-GFP at 8.5 mM instead of 135.8 mM, which was 16-fold lower in concentration (Figure 3C). Enhanced sensitivity was also confirmed by the activation of Btg2-GFP at a 2-fold lower concentration, and activation of both oxidative stress reporters, which was not previously observed.

With the enhanced cofactor mix, the Rtkn-GFP reporter was already activated at the lowest test concentration (8.5 mM). To be able to determine the lowest concentration that induced a 2-fold response, NDMA was re-tested with lower concentrations of 0.01 to 10 mM (Figure 3D). There was a more than 2-fold induction of Rtkn-GFP at 0.6 mM, which was more than a 200-fold lower concentration than initially observed. Additionally, there were ≥2-fold inductions of Btg2-, Srxn1-, and Bscl2-GFP at 1.3, 2.5, and 10 mM, respectively, indicating enhanced sensitivity across all ToxTracker cell lines.

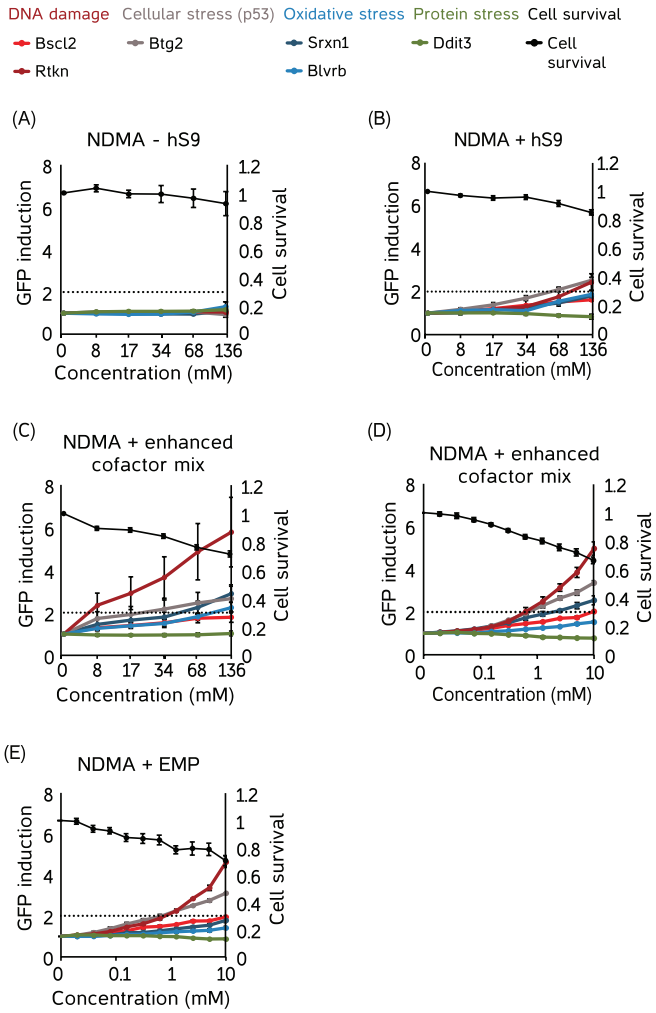



**FIGURE 3** | Optimization of the metabolization protocol for testing N-nitrosamines in the ToxTracker assay using hamster S9 liver extract and standard cofactor mix. The ToxTracker GFP reporter induction and relative cell survival is shown after treatment for 4 h with increasing concentrations of NDMA. The dashed line indicates the 2-fold threshold for a positive response. (A-C) Cells were treated with a maximum test concentration of 135.8 mM NDMA in the absence of hamster S9 liver extract (A), in the presence of hamster S9 liver extract and standard cofactor mix (B), or in the presence of hamster S9 liver extract and enhanced cofactor mix (C). (D) As C but with a maximum test concentration of 10 mM NDMA. (E) Reporter induction after treatment with NDMA using the EMP. Prior to the 4-h exposure, cells were incubated for 30 min with 10% hamster S9 liver extract, enhanced cofactor mix, and NDMA. Consequently, cells were treated for 4 h with a maximum test concentration of 10 mM NDMA in the presence of hamster S9 liver extract and enhanced cofactor mix.

## 3.2 | Optimizing the EMP for N-Nitrosamines

In the EAT (Li et al. 2023), a "pre-incubation" step is included where bacterial cells are incubated with concentrated N-nitrosamine, hamster S9 liver extract, and cofactor mix for 30–60 min before diluting and plating the cells. To determine if a pre-incubation with cells could further improve metabolization and reactivity of NAs in ToxTracker, we incubated the cells for 30 min with a concentrated mix of NA, 10% hamster S9 liver extract, and enhanced cofactor mix. This was diluted 3.3 times in medium (see Methods) and exposure was continued for 4 h. The lowest concentration that activated the genotoxicity reporter was the same when the 30-min incubation was performed with or without cells (Figure 3D,E). Since the half-life of the diazonium ion is short, the EAT includes a pre-incubation of NAs with S9 in the presence of cells prior to plating (Li et al. 2023; Li and Hecht 2022). Also, for mammalian cells, incubating NAs with S9 in the presence of cells was included in the ToxTracker

protocol from this point forward. Finally, we re-evaluated the final percentage of hamster S9 liver extract and confirmed that at least 3% hamster S9 liver extract (v/v) is required, as with 1% hamster S9 liver extract, there was no Rtkn-GFP induction (Figure S2A,B).

The final EMP consisted of a 30-min incubation (37°C) with 10% hamster S9 liver extract, 3.3 times concentrated enhanced cofactor mix, and concentrated NA on the cells, followed by a 4-h exposure at 37°C with 3% hamster S9 liver extract, enhanced cofactor mix, and 1x NA. After the 4-h exposure, the treatment medium was replaced by fresh cell culture medium, and reporter activation was determined 24 h after treatment initiation.

### 3.3 | Validation of the EMP in ToxTracker

The EMP was validated by testing seven additional mutagenic NAs (4 additional linear and 3 cyclic) and two non-mutagenic NAs (1 linear and 1 cyclic) in the ToxTracker assay. We first tested the linear N-nitrosamines NDEA, NDELA, NDBA, NF, and the non-mutagenic NBuPRO (Figures 1, 4, 5, and S3). NDEA did not induce responses or cytotoxicity in the absence of hamster S9 liver extract (Figure 4A). In the presence of S9, NDEA was not cytotoxic but induced at least 2-fold Rtkn-GFP at 0.6 mM and was therefore classified as genotoxic (Figure 4B). Like NDEA, NDBA was negative and non-cytotoxic in the absence of hamster S9 liver extract (Figure S3A). In the presence of hamster S9 liver extract, activation of the Rtkn-GFP reporter (at 0.3 mM) with limited cytotoxicity (at 10 mM) was observed for NDBA (Figure S3B). Hence, NDEA and NDBA were classified as genotoxic when using the EMP. Interestingly, NDELA did not induce any responses or cytotoxicity in the absence or presence of hamster S9 liver extract up to 10 mM and was classified as non-genotoxic (Figure 4C,D). Inherently, this nitrosamine was excluded from subsequent BMD analysis. For the non-mutagenic NDSRI NBuPRO, there was no reporter induction in the absence or presence of hamster S9 (Figure 5A,B).

Finally, we tested the NDSRI NF, the largest NA by molecular weight. In contrast to the other NAs, in the absence of hamster S9 liver extract, NF induced ≥ 50% cytotoxicity and activated the ToxTracker reporters for protein damage and oxidative stress, but none of the genotoxicity reporters (Figure 4E). In the presence of hamster S9 liver extract, NF was more cytotoxic and induced Rtkn-GFP, a genotoxicity reporter that indicates double-strand DNA break formation (Figure 4F). NF was therefore classified as genotoxic in the presence of hamster S9 liver extract.

For the cyclic N-nitrosamines NPYR, NMOR, and CPNP, there was no reporter induction or cytotoxicity in the absence of hamster S9 liver extract (Figure S3C,E,G). However, all three NAs induced Rtkn-GFP at least 2-fold without cytotoxicity in the presence of hamster S9 liver extract (Figure S3D,F,H). CPNP also induced cellular and oxidative stress and induced Bscl2-GFP at least 1.5-fold (Figure S3H). Hence, NPYR, NMOR, and CPNP were classified as genotoxic in the presence of hamster S9 liver extract. For the non-mutagenic cyclic NA NPRO, there was

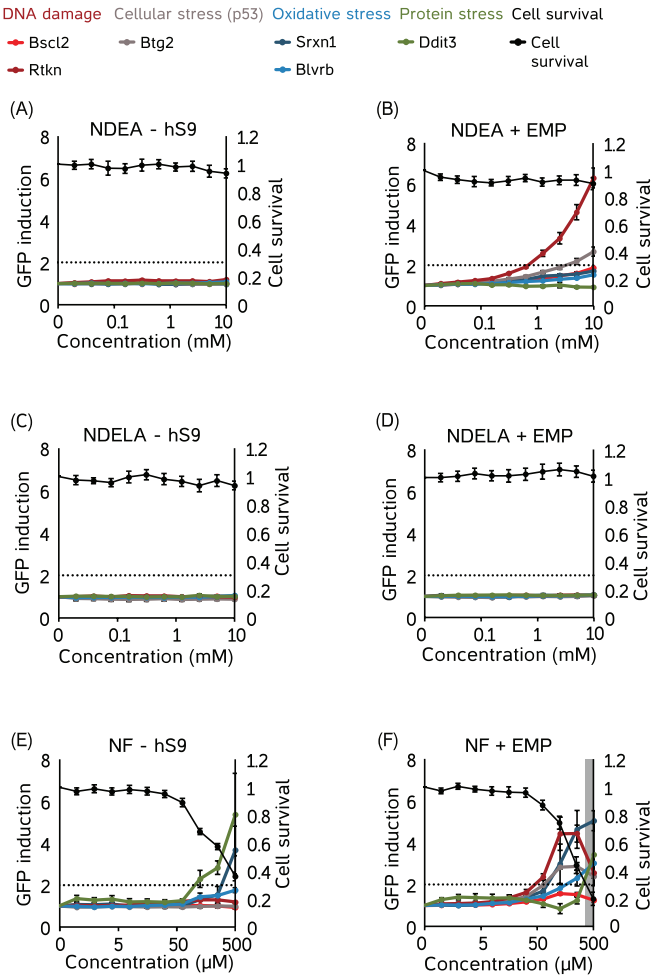



**FIGURE 4** | ToxTracker GFP reporter responses and relative survival of N-nitrosamines in the absence of hamster S9 liver extract or with the EMP for NDEA, NDELA, and NF. For the EMP, ToxTracker cells were treated for 30 min with 10% hamster S9, enhanced cofactor mix, and 3.3 times concentrated N-nitrosamine, followed by a 4-h exposure with 3% hamster S9, cofactor mix, and N-nitrosamine. The dashed line indicates the 2-fold threshold for a positive response. (A-B) ToxTracker result for N-nitrosodiethylamine (NDEA) in the absence of hamster S9 (A) or with the EMP (B). (C-D) ToxTracker result for N-nitrosodiethanolamine (NDELA) in the absence of hamster S9 (C) or with the EMP (D). (E-F) ToxTracker result for N-nitrosofluoxetine (NF) in the absence of hamster S9 (E) or with the EMP (F).

no reporter induction in the absence or presence of hamster S9 (Figure 5C,D).

Overall, seven of the eight tested in vivo mutagenic NAs were classified as genotoxic in ToxTracker in the presence of hamster S9 liver extract, and two non-mutagenic NAs were correctly classified as non-genotoxic, demonstrating that the EMP may be used to bioactivate NAs in mammalian test systems, which can support genotoxicity hazard assessment.

### 3.4 | Potency Ranking of NAs

BMD modeling was applied to the Rtkn-GFP and Bscl2-GFP reporters using a BMR of 100 or 50, respectively (Figure 6).

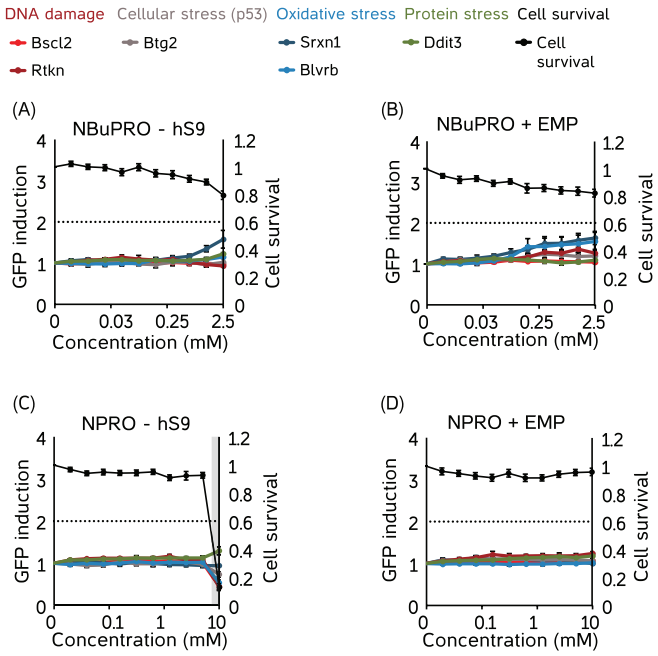



**FIGURE 5** | ToxTracker GFP reporter responses and relative survival of N-nitrosamines in the absence of hamster S9 liver extract or with the EMP for NBuPRO and NPRO. ToxTracker cells were treated without S9 or with the EMP. Therefore, cells were treated for 30 min with 10% hamster S9 liver extract, enhanced cofactor mix, and 3.3 times concentrated N-nitrosamine, followed by a 4-h exposure with 3% hamster S9 liver extract, cofactor mix, and N-nitrosamine. The dashed line indicates the 2-fold threshold for a positive response. (A-B) ToxTracker result for N-nitrosobupropion (NBuPRO) in the absence of hamster S9 liver extract (A) or with the EMP (B). (C-D) ToxTracker result for N-nitrosoproline (NPRO) in the absence of hamster S9 liver extract (C) or with the EMP (D).

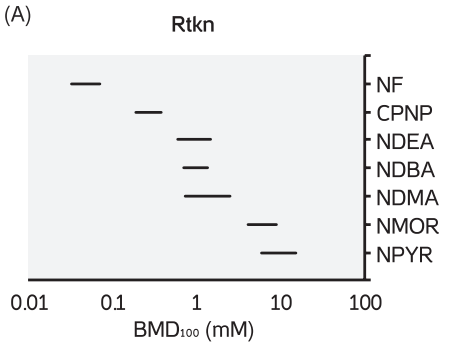
For Rtkn, NF was the most potent NA (BMD interval: 32.5 to 71.5 μM), followed by CPNP (BMD interval: 188 to 378 μM). The small linear NAs (NDEA, NDBA, and NDMA) were equipotent as their BMD intervals overlap. NMOR and NPYR were the least potent NAs (Figure 6A,B). For Bscl2, NF again was classified as the most potent NA (BMD interval: 91.5 to 154 μM), followed by CPNP, NDMA, NDEA, and NDBA (Figure 6C,D).

## 4 | Discussion

Here, we improved an in vitro metabolization protocol for predicting NA-induced genotoxicity in mammalian cells using the ToxTracker assay. The EMP uses hamster S9 liver extract, which has previously been shown to be more potent in metabolically activating NAs compared to rat S9 liver extract and thereby enhances the identification of mutagenic NAs in vitro (Tennant et al. 2023). The addition of calcium chloride to the hamster S9 cofactor mix further improved the metabolic activation of NAs by 200-fold for NDMA when comparing the lowest observed positive concentrations in the ToxTracker assay. This was not novel as calcium chloride has previously been shown to improve the sensitivity of detecting NDMA-induced mutagenicity in vitro but is lacking from present-day standard cofactor mixes (Li et al. 2023; O'Neill et al. 1982). Possibly, calcium chloride enhances the influx of NA metabolites into the cells thereby improving assay sensitivity (O'Neill et al. 1982).

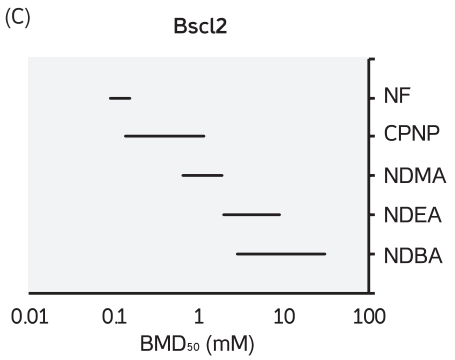
The EMP was validated using ToxTracker by testing a variety of linear and cyclic NAs. For the linear NAs, we tested NDMA, NDEA, NDBA, NDELA, NF, and non-mutagenic NBuPRO. NDEA is a data-rich, small linear N-nitrosamine (MW 102 g/mol) that has previously been found in cosmetics, food, and as an impurity in drugs such as valsartan (EMA 2020b; Li and Hecht 2022). NDBA (MW 158 g/mol) has been found in food products and was identified during the production of ranitidine, a drug for reducing stomach acid (EMA 2020a, 2020b; Li and Hecht 2022). In the ToxTracker assay, both NDEA and NDBA were classified as genotoxic (induced Rtkn-GFP) which is consistent with data from the Ames test and rodent carcinogenicity assay (Bringezu and Simon 2022; Dieckhoff et al. 2024; EMA 2020b; Heflich et al. 2024; Thomas et al. 2024). NDEA and NDBA were equipotent to NDMA in ToxTracker based on their overlapping Rtkn $BMD_{100}$ confidence intervals. Conversely, in the Ames test, NDBA induced revertants at lower concentrations than NDMA and NDEA, suggesting that NDBA may be more potent (Figure 1A) (Dieckhoff et al. 2024; Heflich et al. 2024). Interestingly, the TD50 of NDBA (8.2 mg/kg/day based on the most sensitive species) is higher compared to the TD50 of NDMA (0.145 mg/kg/day) and NDEA (0.062 mg/kg/day) suggesting that NDBA is a less potent mammalian carcinogen (Snodin et al. 2024). Interestingly, the published AI levels of NDEA and NDBA are 3-fold lower (26.5 ng/day) than NDMA (96 ng/day) despite NDBA having a notably higher TD50 value (Bercu et al. 2023; EMA 2020b; Health Canada 2024). Of these 3 linear NAs, NDMA had the highest BMDL for Rtkn-GFP, which correlates with the ranking observed in AI.

NDELA is larger than NDEA (MW 134 g/mol and 102 g/mol, respectively), can be found in cosmetics, and has previously been classified as possibly carcinogenic to humans (EMA 2020b; Li and Hecht 2022). NDELA has previously shown conflicting results in the Ames test, was carcinogenic in rats, and tested negative in vivo in both micronucleus and chromosomal aberration endpoints in mice (CPDB 2008; EMA 2020b; Gilbert et al. 1981; Li and Hecht 2022; Lijinsky and Reuber 1984). NDELA did not activate any ToxTracker reporters when tested up to 10 mM. In vitro detection of NDELA's mutagenicity may be difficult as it not only requires CYP-mediated metabolism but also alcohol dehydrogenases (ADH) activity (Eisenbrand et al. 1984; Liu and Glatt 2008). NAD is the cofactor for ADH and is not included in the EMP, which may explain why NDELA remained undetected. Another explanation could be that NDELA is a less potent mutagen and therefore was not classified as genotoxic in ToxTracker. The TD50 for NDELA is reportedly 38.9 mg/kg/day (based on the most sensitive species), indicating that it is a less potent carcinogen than, for example, NDMA (0.145 mg/kg/day) (Snodin et al. 2024). This is also reflected in the published AI levels. For NDELA, the AI is much higher (1900 ng/day) than for the other linear NAs evaluated herein that have AI levels below 100 ng/day (Bercu et al. 2023; EMA 2020b; Health Canada 2024). As NDMA, NDEA, and NDBA are only activating the DNA damage reporters at higher concentrations




(B)

| | Rtkn | |
|---|---|---|
| Compound | BMDL (mM) | BMDU (mM) |
| NF | 0.0325 | 0.0715 |
| CPNP | 0.188 | 0.378 |
| NDEA | 0.598 | 1.48 |
| NDBA | 0.709 | 1.37 |
| NDMA | 0.738 | 2.5 |
| NMOR | 4.08 | 8.95 |
| NPYR | 5.86 | 15 |




(D)

| | Bscl2 | |
|---|---|---|
| Compound | BMDL (mM) | BMDU (mM) |
| NF | 0.0915 | 0.154 |
| CPNP | 0.136 | 1.16 |
| NDMA | 0.642 | 1.84 |
| NDEA | 1.98 | 8.9 |
| NDBA | 2.87 | 30.2 |

**FIGURE 6** | Benchmark dose response modeling of Rtkn-GFP and Bscl2-GFP (DNA damage reporters) data using PROAST software. (A) Potency ranking of the quantitative BMD confidence limits for Rtkn with the most potent N-nitrosamine on top. A benchmark response of 100% was used for Rtkn, indicating a 2-fold increase in GFP reporter expression. (B) Lower and upper 90% BMD confidence limits. (C) Potency ranking of the quantitative BMD confidence limits for Bscl2 with the most potent N-nitrosamine on top. A benchmark response of 50% was used for Bscl2, indicating a 1.5-fold increase in GFP reporter expression. (D) Lower and upper 90% BMD confidence limits.

(> 0.3 mM), reporter activation by NDELA could simply be missed due to a lower potency.

NF is an NDSRI and the largest NA that was tested in this study (MW 338 g/mol). Its parent is the drug Fluoxetine (i.e., Prozac) with an AI of 100 ng/day based on similarity to its surrogate 4-(methylnitrosoamino)-1-(3-pyridinyl)-1-butanone (NNK) (FDA 2024). NF, in the presence of hamster S9 liver extract, was the most potent of all NAs tested based on $BMD_{100}$ analysis. The large size of NF could play a role in inducing cytotoxicity as larger NAs are predicted to establish larger (and more lethal) DNA adducts compared to smaller NAs whose adducts may not interfere as severely with replication (Fine et al. 2023). However, this was not found in the Ames test where NF was less potent than CPNP and equipotent to NDBA (Heflich et al. 2024). This suggests that although NF is a potent genotoxic agent in mammalian cells, it may be a less potent mutagen in bacterial cells. Alternatively, the inherent methodological differences between the exposure paradigms may account for part of (or all of) this discrepancy. Regardless, NF was the only NA that showed a clear induction of cytotoxicity and reporter activation (oxidative stress and protein stress) in the absence and presence of hamster S9 liver extract.

In addition to the in vivo genotoxic linear NAs tested, the expected non-mutagenic linear NA NBuPRO was tested. NBuPRO is an NDSRI and marked by the EMA and Health Canada as a non-mutagenic impurity (EMA 2024b; Health Canada 2024). It did not induce reporter activation in the ToxTracker, further showing the specificity of the EMP in ToxTracker.

Regarding the cyclic NAs evaluated herein (NPYR, NMOR, CPNP, and non-mutagenic NPRO), NPYR was the smallest (MW 100 g/mol) and it has previously been shown to occur in food, water, and tobacco products. It is classified as a mutagen in the Ames test and as carcinogenic in rats, mice, and Syrian golden hamsters (CPDB 2008; EMA 2020b; Li and Hecht 2022). NPYR induced a 2-fold increase in Rtkn-GFP only at the highest test concentration (10 mM) and was the least potent of all evaluated NAs as determined by $BMD_{100}$ analysis. This aligns with carcinogenicity potency, as the TD50 for NPYR was determined to be 1.59 mg/kg/day, which is approximately 10-fold higher than the TD50 for NDMA (Snodin et al. 2024). Similarly, NMOR occurs in water and food but also is found in cosmetics and occupational airspaces (Li and Hecht 2022). It is one of the fastest formed NAs (Li and Hecht 2022) and was mutagenic in the Ames test (CPDB 2008; EMA 2020b). NMOR also only induced Rtkn-GFP at least 2-fold at 10 mM and was the second least potent NA tested. CPNP is the largest cyclic NA evaluated herein (MW 183 g/mol), is mutagenic in the Ames test, and has been detected in rifapentine, an anti-tuberculotic drug (CPDB 2008; EMA 2020b; FDA 2021; Li et al. 2023). Specifically in this cited work, a mutagenic response in TA100, TA1537, and WP2 uvrA was observed at ≥ 100 μg/plate, whereas NDMA showed a mutagenic response in these strains starting from ≥ 2500 μg/plate. This potency is likely why CPNP is recommended as a positive control for the EAT (Kajavadara et al. 2024). Furthermore, in strain TA100, Heflich et al. (2024) showed the lowest positive concentration of CPNP to be 10 μg/plate, whereas NDMA induced a mutagenic response at 50 μg/plate. In ToxTracker, CPNP induced ≥ 2-fold Rtkn-GFP at 250 μM and was the second

most potent NA determined by $BMD_{100}$ analysis. Together, this indicates that CPNP is more potent than NDMA when considering genotoxicity in vitro.

In addition to the cyclic genotoxic NAs tested, the non-mutagenic and cyclic NPRO was tested. NPRO is a member of the family of pyrrolidines and was previously tested negative for mutagenicity in the Ames test and negative for carcinogenicity in rats (CPDB 2008; EMA 2024b; Rao et al. 1981). Here, it did not induce reporter activation in ToxTracker, further showing the specificity of the EMP in ToxTracker.

All NAs require metabolization by CYP450 enzymes to form the highly reactive diazonium ion that can react with DNA and consequently lead to gene mutations. Reactivity in the absence of hamster S9 liver extract can often be explained by spontaneous decomposition (Li and Hecht 2022; Tennant et al. 2023). In the case of NF, there is protein stress and oxidative stress in the absence of S9, possibly due to a mechanism that is unrelated to the diazonium ion as there is no metabolization. A similar mechanism was previously shown for N-nitroso-hydrochlorothiazide (NO-HCTZ) (Gandhi et al. 2024). Gandhi et al. (2024) detected mutagenicity in the absence of CYP enzymes, and this was attributed to the formation of formaldehyde after spontaneous decomposition, thereby establishing that NO-HCTZ was not directly DNA reactive. Identification of such an indirect DNA reactive mechanism of action will allow better safety predictions and may help to set more realistic AI levels.

When ranking NAs by their potency to induce the Rtkn-GFP and Bscl2-GFP genotoxicity reporters in ToxTracker, the larger NAs (NF and CPNP) were the most potent. The potency to activate these DNA damage reporters might not directly correlate with mutagenicity, as DNA repair also plays a role in the detection and removal of adducted bases. Generally, small NAs are assumed to be more mutagenic (Fine et al. 2023). ToxTracker predicts genotoxicity (and genotoxic MoA) but does not directly detect mutagenicity. Classically, an HPRT assay or MLA assay could be performed to detect mutagenicity, but these identify mutants based on phenotypical changes and suffer from low specificity (Kirkland et al. 2005). The development of error-corrected next generation sequencing (ecNGS) allows sensitive detection of single nucleotide variants (SNVs) and can concomitantly determine the mutation frequency and mutation spectrum (Marchetti et al. 2023; Maslov et al. 2022; Schmitt et al. 2012). This approach would provide further insight into the types of mutations formed and can assist in determining DNA reactivity.

## 5 | Conclusion

We have shown that the genotoxicity of NAs can be assessed in mammalian cell culture when using an EMP that mimics exposure paradigms used in the EAT. The EMP is a valuable protocol for hazard identification of NAs in mammalian cells and could be applied to other mammalian toxicity assays or NAMs. ToxTracker may lend itself as a useful in vitro tool to follow up on EAT negative test results in mammalian cells and further prioritize compounds for additional in vivo testing. While additional NAs will need to be tested, this pilot study is the first step towards developing a robust mammalian cell genetic toxicity endpoint for NA hazard identification and may be useful for subsequent risk assessment of this class of compounds.

### Author Contributions

M.E.G. and G.H. designed the study. A.M.G. and N.M. performed the experimental work. A.M.G. and M.E.G. analyzed the data, prepared draft figures and tables. M.E.G. prepared the manuscript with input from I.B. and G.H. All authors approved the final manuscript.

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### Conflicts of Interest

M.E.G., A.M.G., N.M., and I.B. are employed by Toxys B.V., a Dutch company that offers ToxTracker as a commercial service. G.H. is a shareholder of Toxys B.V.

### Data Availability Statement

The data that support the findings of this study are available from the corresponding author upon reasonable request.

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**Supporting Information**

Additional supporting information can be found online in the Supporting Information section.