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N-nitrosamine impurity risk assessment in pharmaceuticals: Utilizing In vivo mutation relative potency comparison to establish an acceptable intake for NTTP

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ABSTRACT

The finding of *N*-nitrosodiethylamine (NDEA) and *N*-nitrosodimethylamine (NDMA) in marketed drugs has led to implementation of risk assessment processes intended to limit exposures to the entire class of *N*-nitrosamines. A critical component of the risk assessment process is establishing exposure limits that are protective of human health. One approach to establishing exposure limits for novel *N*-nitrosamines is to conduct an in vivo transgenic rodent (TGR) mutation study. Existing regulatory guidance on *N*-nitrosamines provides decision making criteria based on interpreting in vivo TGR mutation studies as an overall positive or negative. However, point of departure metrics, such as benchmark dose (BMD), can be used to define potency and provide an opportunity to establish relevant exposure limits. This can be achieved through relative potency comparison of novel *N*-nitrosamines with model *N*-nitrosamines possessing robust in vivo mutagenicity and carcinogenicity data. The current work adds to the dataset of model *N*-nitrosamines by providing in vivo TGR mutation data for *N*-nitrosopiperidine (NPIP). In vivo TGR mutation data was also generated for a novel *N*-nitrosamine impurity identified in sitagliptin-containing products, 7-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo-[4,3-a]pyrazine (NTTP). Using the relative potency comparison approach, we have demonstrated the safety of NTTP exposures at or above levels of 1500 ng/day.

1. Introduction

N-nitrosamines as a structural class are considered potent mutagenic carcinogens that fall within the cohort of concern described by Kroes et al. (2004) and referenced in ICH M7 (ICH, 2023). Due to the potential for increased carcinogenic risk associated with *N*-nitrosamines, these compounds have historically received a higher level of scrutiny than other structural classes of mutagenic impurities. The unexpected presence of small molecular weight *N*-nitrosamines in tetrazole-containing sartans, metformin, and ranitidine (Tuesuwan and Vongsutilers, 2021) has resulted in increased focus on potential *N*-nitrosamine exposures in pharmaceuticals. Although this structural class includes potent compounds, there are numerous examples of less potent and/or non-carcinogenic *N*-nitrosamines (Thresher et al., 2020; Snodin, 2023). Regardless, current regulatory guidance calls for an evaluation of all potential *N*-nitrosamines across pharmaceuticals with the goal of ensuring patient safety.

A critical component of the *N*-nitrosamine evaluation process for pharmaceuticals is establishing exposure limits. ICH M7 recommendations can be followed to determine an acceptable intake (AI) for *N*-nitrosamines when robust rodent carcinogenicity data are available (e.g., extrapolate TD_{50} values to derive an AI associated with theoretical increased lifetime cancer risk of <1 in 100,00). Options to establish an AI for novel *N*-nitrosamines lacking rodent carcinogenicity data are described in current regulatory guidance issued by regional health authorities (e.g., EMA, 2024a; FDA, 2023, 2024; Health Canada, 2024a).

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These include structure-activity relationship (SAR)-based approaches [i.e., Carcinogenic Potency Categorization Approach (CPCA) and read-across] or mutagenicity testing [i.e., Enhanced Ames Testing (EAT) and in vivo transgenic rodent (TGR) mutation studies]. The development of the CPCA and EAT are significant advancements over previous options that were limited to read-across or application of a default AI equal to 18 ng/day or 26.5 ng/day (FDA). The ability to generate compound-specific data through in vivo TGR mutation testing represents a more refined approach to setting an AI for *N*-nitrosamines when robust rodent carcinogenicity data are not available. In vivo mutations serve as a surrogate endpoint to inform carcinogenic risk. The utility of these data for setting exposure limits has been described in both ICH M7 and the accompanying M7 Q&A document (ICH, 2022) as well as regional nitrosamine guidance (EMA, 2020, 2024a; Health Canada, 2024a). Recently updated guidance from EMA (2024a) and Health Canada (2024a) indicates that *N*-nitrosamines can be considered non-mutagenic and controlled per ICH Q3A/Q3B (ICH, 2006a, 2006b) if found to be negative in an in vivo TGR mutation study. In contrast, a positive in vivo result requires application of an AI derived from SAR-based approaches (i.e., CPCA assessment or read-across).

While existing regulatory guidance generally describes results of in vivo TGR mutation testing as a binary outcome, either positive or negative, the potential use of point of departure metrics from positive in vivo TGR mutation studies to support *N*-nitrosamine risk assessment is of interest. Interrogating point of departure for *N*-nitrosamines with a demonstrated threshold response in vivo would provide a means to establish exposure limits. One specific application being explored is using benchmark dose (BMD) modeling of in vivo TGR mutation data to compare the mutagenic potency of novel *N*-nitrosamines with well-studied model *N*-nitrosamines (Nudelman et al., 2023). This is consistent with the EMA assessment report which indicates comparison of BMDs as point of departure for in vivo TGR mutation data could provide meaningful contribution to *N*-nitrosamine risk assessment (EMA, 2020). To support this approach, potency ranking using in vivo TGR mutation data can be carried out by plotting and comparing BMD confidence intervals (BMD CI) (Wills et al. 2016, 2017; Hardy et al., 2017; Guo et al., 2018). The BMD CI are compared to each other, with overlapping BMD CI indicating equipotency, and non-overlapping BMD CI showing higher or lower potency of the chemicals.

The goals of the current publication are to 1) provide data to allow comparison of in vivo TGR mutation potencies for *N*-nitrosamines, and 2) apply relative potency comparison methodology to justify an AI for a novel *N*-nitrosamine impurity identified in sitagliptin-containing products, 7-nitroso-3-(trifluoromethyl)-5,6,7,8-tetrahydro-[1,2,4]triazolo-[4,3-a]pyrazine (NTTP).

In vivo TGR mutation data for model *N*-nitrosamines can provide the basis for relative potency comparison. *N*-nitrosodiethylamine (NDEA) and *N*-nitrosodimethylamine (NDMA) are considered model *N*-nitrosamines as each has robust carcinogenicity data with published exposure limits and in vivo TGR mutation data. Lifetime AIs of 26.5 ng/day and 96 ng/day have been established based on linear extrapolation of harmonic mean TD_{50} values from carcinogenicity studies for NDEA and NDMA, respectively, (EMA, 2024b; FDA, 2023; Health Canada, 2024b). Published in vivo TGR mutation data for liver is available from a study in Big Blue® transgenic rats administered NDEA (Bercu et al., 2023a) as well as mutant frequency in the *lacZ* gene from a Muta^{TM}Mouse study with NDMA (Lynch et al., 2024). BMD CI from these studies are summarized in Table 1. Mutant frequency at the *cII* gene from liver of Big Blue® rats treated with NDMA has also been determined by Gollapudi et al. (1998) with BMD CI subsequently reported by Johnson et al. (2021) ($BMDL_{50}$ = 0.06 mg/kg/day and $BMDU_{50}$ = 2.34 mg/kg/day). However, the Gollapudi et al. (1998) study was not conducted according to OECD TG 488 (OECD, 2022) (e.g., reduced treatment schedule with limited doses and a small number of animals) and was not considered in the current analysis. The OECD TG 488 compliant NDMA study in Muta^{TM}Mouse (Lynch et al., 2024) included a more robust study design and yielded a more precise BMD CI and was, therefore, more appropriate for relative potency comparison. Note that regardless of these differences in study design and data quality, BMD data in the Muta^{TM}Mouse (Lynch et al., 2024) fall within the BMD CI reported for the Big Blue® rats (Gollapudi et al., 1998; Johnson et al., 2021). Another well-studied *N*-nitrosamine, *N*-nitrosopiperidine (NPIP), has rodent carcinogenicity data supporting a lifetime AI of 1300 ng/day (EMA, 2024b; Health Canada, 2024b). Although NPIP is known to be mutagenic in *gpt delta* rats, dose-response data from that assessment is incomplete (Totsuka et al., 2019). Therefore, an in vivo mutation study was conducted in Big Blue® transgenic rats to support determination of BMD CI. The resulting database now includes in vivo TGR mutation data for 2 potent model *N*-nitrosamines (NDEA and NDMA) and a less potent model *N*-nitrosamine (NPIP), thereby, providing a robust basis for relative potency comparison with novel *N*-nitrosamines.

In the absence of rodent carcinogenicity data for NTTP, regulatory agencies initially established a lifetime AI of 37 ng/day using read-across to *N*-nitroso-1,2,3,6-tetrahydropyridine (NTHP) (e.g., EMA, FDA, and Health Canada). The principal structural basis for correlating NTHP with NTTP appears to be the presence of unsaturation at the β-carbon, which is known to influence the rate of α-carbon hydroxylation leading to DNA-reactive species, and therefore carcinogenic potency (Ponting et al., 2022; Thomas et al., 2022). This characteristic is reflected in the CPCA framework where "benzylic or pseudo-benzylic substituents" are deemed to be activating features (Kruhlak et al., 2024). Note that several health authorities have recently updated the NTTP AI to 100 ng/day based on the CPCA assessment (e.g., EMA, 2024b; Health Canada, 2024b). The extent to which α-carbon hydroxylation is accelerated by the unsaturation is, however, expected to be strongly influenced by its electronic nature. This critical factor is not accounted for when using NTHP as the read-across analog for NTTP or in the CPCA assessment. For instance, the trifluoromethyltriazole substructure in NTTP is highly electron-deficient and significantly differentiated from the simple unsubstituted alkene in NTHP. The expectation is also consistent with results of quantum-mechanical SAR modeling described by Kostal and Voutchkova-Kostal (2023) which predicts a lower degree of carcinogenic potency for NTTP (i.e., Category 3 with TD50 $>$ 1.5 mg/kg/day) vs. NTHP (i.e., Category 2 with 0.15 mg/kg/day $<$ TD50 $\leq$ 1.5 mg/kg/day). Overall, an AI derived from read-across to NTHP or from the CPCA assessment would not be expected to accurately reflect the carcinogenic potential of NTTP. Therefore, compound-specific in vivo TGR mutation data are needed to more precisely characterize NTTP reactive potential and justify a scientifically valid exposure limit (Fig. 1).

2. Materials and methods

2.1. Test articles

NPIP (CAS #100-75-4) and NTTP (CAS # 2892260-32-9) were synthesized by Pharmaron Beijing Co., Ltd. (Beijing, P.R. China). Both test articles had purity of ≥99.9%.

2.2. Bacterial reverse mutation (Ames) Test

Testing was conducted using a 30-min preincubation procedure for in vitro bacterial reverse mutation assay as per OECD TG 471 (OECD, 2020). In this study, NTTP was tested as a solution in DMSO (14.3% final concentration in pre-incubation mix) at doses ≤5000 μg/plate in *Salmonella* strains TA97a, TA98, TA100, TA1535 and *E. coli* strain WP2 *uvrA* pKM101. The test was conducted with and without a liver microsomal enzyme activation system containing 10% S9 prepared from male rats or male hamsters treated with phenobarbital and beta-naphthoflavone (Molecular Toxicology, Inc., Boone, NC). The criteria for a positive result include a 2-fold or greater increase in revertants over concurrent vehicle control and evidence of a dose-related

increase in the number of revertant colonies.

2.3. In vivo testing

Procedures were performed in facilities using approved procedures by the respective laboratory institutional animal care and use committees.

2.3.1. Animals

Male Fischer Wild Type 344 rats from Envigo Global Services, Inc. (Frederick, MD, USA) were used in tolerability studies. Male Fischer 344 Big Blue® transgenic rats were obtained from Gentronix Limited (Macclesfield, Cheshire, UK) via Taconic Biosciences, Inc. (Germantown, NY, USA). Rats were between approximately 8 to 10 weeks of age at study start.

2.3.2. Tolerability studies

Two independent tolerability studies were conducted for NPIP and NTTP. NPIP tolerability was determined by evaluating mortality, clinical observations, body weights, food consumption, and toxicokinetics in male Fischer Wild Type 344 rats orally administered 50, 100, 150, or 300 mg/kg/day NPIP for 7 consecutive days or until dose group termination. The vehicle/control article was 10% Polysorbate 80 in deionized water (10% PS 80).

NTTP tolerability was determined by evaluating mortality, clinical observations, body weights, food consumption, serum biochemistry, limited gross pathology (liver, stomach, small intestine, and large intestine), and toxicokinetics in male Fischer Wild Type 344 rats orally administered 30, 300, 500, or 1000 mg/kg/day NTTP for 8 consecutive days. The vehicle/control article was 1% methylcellulose/0.1% sodium lauryl sulfate in deionized water (1% MC/0.1% SLS).

In both studies, animals sacrificed at the scheduled necropsy were anesthetized and subsequently euthanized by exsanguination on Day 8.

2.3.3. In vivo Big Blue® transgenic rat mutation studies

In vivo Big Blue® Transgenic rat mutation studies have the ability to detect minimal genetic damage that may be caused by mispairing or misincorporation of bases during replication (Lambert et al., 2005). Big Blue® transgenic rats have multiple copies of a λ shuttle vector with a *cII* reporter gene integrated into the genome of each cell in the body. Mutations are detected by recovering the *cII* gene and analyzing the phenotype of the reporter gene in a bacterial host deficient for the reporter gene (OECD, 2022). Studies for both NPIP and NTTP were conducted in GLP facilities in accordance with established standard operating procedures and current internationally recognized guidelines [e.g., OECD TG 488, ICH M7, and ICH S2(ICH, 2011)]; however, only the in vivo TGR mutation study with NTTP was GLP compliant.

Independent TGR mutation studies were conducted for NPIP and NTTP. The study with NPIP included evaluation of mortality, clinical observations, body weights, food consumption, serum biochemistry, limited gross pathology (liver and duodenum), limited histopathology (liver), and mutations at the *cII* locus (duodenum and liver) in male Fischer 344 Big Blue® transgenic rats orally administered 0.1, 1, 3, 10, 20, or 30 mg/kg/day NPIP for 28 consecutive days. The vehicle/control article was 10% PS 80. The NPIP high-dose was selected based on findings in the 7-day tolerability study with additional doses being chosen to allow investigation of dose-response over a wide range. Dosing holidays occurred on Days 6, 7, 13, and 14 for the 30 mg/kg/day group due to toxicity.

With NTTP, the evaluation included mortality, clinical observations, body weights, food consumption, serum biochemistry, limited gross pathology (liver and duodenum), and mutations at the *cII* locus (duodenum and liver) in male Fischer 344 Big Blue® transgenic rats orally administered 2, 16, 50, or 300 mg/kg/day NTTP for 28 days. The vehicle/control article was 1% MC/0.1% SLS. The NTTP high-dose was selected based on findings in the 8-day tolerability study with additional doses being chosen to allow investigation of dose-response over a wide range.

Both studies included positive controls orally administered 20 mg/kg/dose of ENU on Days 1, 2, 3, 12, 19, and 26. Animals sacrificed at the scheduled necropsy were anesthetized and subsequently euthanized by exsanguination on Day 31. At necropsy, median liver lobe and duodenum samples were collected for mutation frequency analysis. Duodenum was selected as it is a rapidly dividing tissue and also represents a site of contact following oral gavage. The liver represents a slow proliferating and metabolically competent tissue routinely selected to further investigate compounds which are Ames positive in the presence of S9. All tissue samples were flash frozen in liquid nitrogen and stored at −70 °C until being shipped on dry ice to Gentronix Limited.

2.3.4. Mutation assay

2.3.4.1. DNA Extraction, In vitro packaging, and plating of phage. Liver and duodenum tissue samples from the first 5 surviving animals per group were processed for DNA isolation and *cII* mutant analysis based on the Agilent Recover Ease DNA Isolation methods (Santa Clara, CA) (Agilent Technologies, 2018).

Frozen stocks of *E. coli* strain G1250 were used to prepare master bacterial plates. Packaged phage were adsorbed onto *E. coli* G1250 suspension cultures for at least 30 min, molten top agar was then added, and the cells subsequently plated onto bottom agar plates. Packaged phage were incubated overnight at 37 °C ± 2.0 °C, then scored for plaque formation and titre determination; *cII* mutant selection plates were incubated for approximately 2 day at 24 °C ± 0.5 °C, then scored for mutant plaque formation. At least 200,000 phage (plaque-forming units) were evaluated, from at least 2 packagings.

2.3.4.2. Data analysis. Mutant frequency, defined as the number of mutant phage/number of total phage screened, was calculated for each tissue specimen analyzed from each animal. An individual animal was considered the experimental unit. Statistical analyses were conducted for individual tissues.

NPIP- and NTTP-related increases in mutant frequency were determined by comparing $\log_{10}$ transformed data to the concurrent vehicle control using one-way ANOVA followed by one-sided Dunnett's test for multiple comparisons. In addition, dose-response was evaluated by additional trend test. P-values <0.05 were considered significant. The result of the assay was considered positive when a statistically significant increase in the frequency of *cII* mutants occurred, a dose-related increase was observed (trend), and the mutant frequency was outside the upper 95% control limits of the historical background mutant frequency range. The result of the assay was considered negative when no statistically significant increase in *cII* mutant frequency was observed.

Performance of the positive control was established by comparing with vehicle controls using a single one-sided ANOVA.

2.4. Benchmark dose modeling

PROAST v70.1 software was used for BMD model averaging (https://proastweb.rivm.nl/). BMD model averaging with 200 bootstraps was used to apply 4 default sets of statistical models to the data, which were exponential, Hill, inverse exponential, and lognormal dose response models. Weights were applied to each set models based on fit, and a single set of BMD CI calculated. A critical effect size (CES) of 50% was selected per published recommendations for transgenic gene mutation assays (Zeller et al., 2017; Johnson et al., 2021; White et al., 2024 manuscript in preparation).

3. Results

3.1. Bacterial reverse mutation (Ames) Test

NTTP was negative in TA97a, TA98, TA100, and *E coli* WP2 *uvrA* pKM101 with and without metabolic activation as well as TA1535 without metabolic activation. In contrast, a positive response was noted in TA1535 with metabolic activation at doses ≥3000 μg/plate with rat S9 and ≥1000 μg/plate with hamster S9. Positive results in TA1535 with metabolic activation following pre-incubation is consistent with literature reports for other *N*-nitrosamines (e.g., Tennant et al., 2023).

3.2. In vivo testing

3.2.1. NPIP

NPIP-related effects in male Fischer Wild Type 344 rats demonstrated a lack of tolerability at all dose levels tested (≥50 mg/kg/day). The severity of these effects resulted in mortality and dose group termination at ≥ 100 mg/kg/day, and the only surviving dose group (50 mg/kg/day) also had severe clinical signs. Therefore, it was determined that NPIP was not tolerated at dose levels ≥50 mg/kg/day. Meaningful toxicokinetic data could not be obtained due to the lack of tolerability. Based on results from this study, a high-dose of 30 mg/kg/day was selected for the definitive mutation study.

The NPIP Big Blue® transgenic rat mutation study used dose levels of 0.1, 1, 3, 10, 20, or 30 mg/kg/day orally administered for 28 days followed by a 3-day treatment-free period. NPIP-related toxicity included dose-dependent body weight changes and decreased food consumption at doses ≥20 mg/kg/day. Dose-dependent effects on serum biochemistry included increases in AST and ALT activities at ≥ 20 mg/kg/day and increased AP and TBIL at 30 mg/kg/day. Gross observations of pale liver and microscopic lesions in liver were noted at ≥ 20 mg/kg/day with effects being more pronounced at 30 mg/kg/day. The gross and microscopic hepatic effects were not observed at doses ≤10 mg/kg/day. Due to the significant effects on body weight, animals in the 30 mg/kg/day dose group were given dosing holidays on Days 6, 7, 13, and 14.

NPIP did not induce a statistically significant increase in mutant frequency in the duodenum at the highest dose tested (i.e., 30 mg/kg/day). However, a statistically significant increase in mutant frequency over concurrent control was observed in the liver at doses ≥1 mg/kg/day. Note that the increases in mutant frequency at 1 and 3 mg/kg/day were within the 95% control limits of the historical control data. The increases at these dose levels were, therefore, deemed to have questionable biological relevance. Note that the increase in mean mutant frequency at 1 mg/kg/day was less than 1.5-fold over the concurrent controls (i.e., increase was less than the CES). In addition, the mean mutant frequency at this dose level was below the mean for historical control data and all individual animals had mutant frequency values within the 95% control limits of the historical control data. A statistically significant increase in mutant frequency was not observed in the liver at 0.1 mg/kg/day. The vehicle control and positive control responses confirmed appropriate performance of the test system. Overall, the no observable effect level for genotoxicity, commonly referred to as

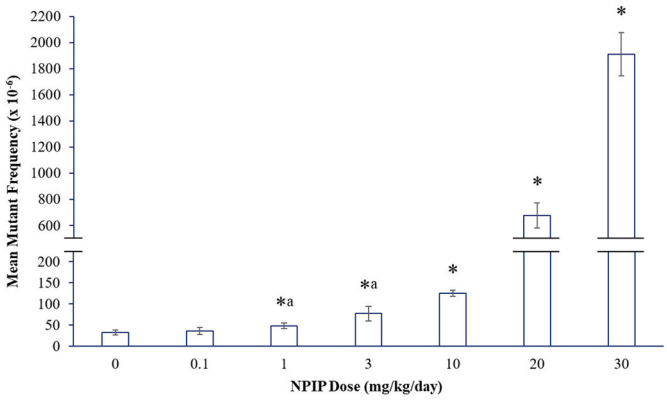

Fig. 2. Summary of Big Blue® transgenic rat mutation data for NPIP in liver. Vehicle control and NPIP treated animals were dosed daily for 28 days (note - dosing holidays occurred on Days 6, 7, 13, and 14 for the 30 mg/kg/day group) and tissues collected following necropsy on Day 31. Data are presented as mean mutant frequency (x 10^{-6}) ± SD with * indicating a statistically significant difference vs. concurrent vehicle control ($P < 0.001$; 1-Way ANOVA) and [a] indicating mean values were within the 95% control limits of the historical control data.

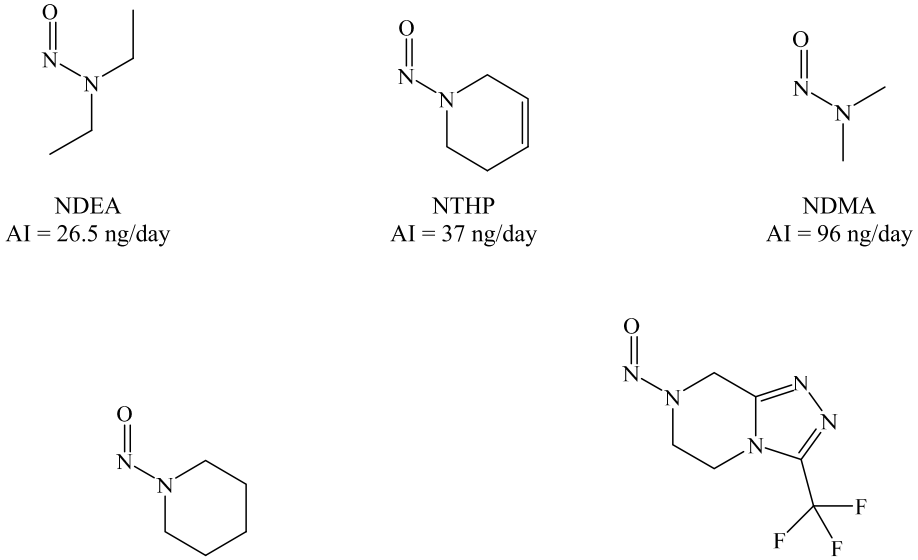

Fig. 1. *N*-nitrosamine structures and associated regulatory lifetime AI. AI limits are published by regulatory agencies using rodent carcinogenicity data from the Lhasa Carcinogenicity Database (https://carcdb.lhasalimited.org/). a The AI for NTTP is based on read-across to NTHP or CPCA.

the no observed genotoxic effect level (NOGEL) for *cII* mutations in the liver was 1 mg/kg/day. Data are presented in Fig. 2 with detailed information provided in Table S1.

3.2.2. NTTP

NTTP-related findings in male Fischer Wild Type 344 rats included unscheduled deaths at ≥ 500 mg/kg/day, dose-dependent increases in serum AST and ALT activities at ≥ 300 mg/kg/day, as well as dose-dependent antemortem changes at ≥ 300 mg/kg/day that consisted of correlated mean body weight losses, decreased food consumption, and adverse clinical signs. Due to mortality at ≥ 500 mg/kg/day and antemortem findings that were slight and/or transient at 300 mg/kg/day, 300 mg/kg/day was considered the highest tolerated dose of NTTP. These data were used to support dose selection for the definitive in vivo mutation study.

Toxicokinetic analysis demonstrated dose-proportional exposure in plasma and liver. In addition, $C_{1\ hr}$ was similar in plasma (Day 7) and liver (Day 8).

The NTTP Big Blue® transgenic rat mutation study evaluated dose levels of 2, 16, 50, or 300 mg/kg/day orally administered for 28 days followed by a 3-day treatment-free period. NTTP-related toxicity observed in male Fischer 344 Big Blue® transgenic rats was limited to a dose-dependent, transient effect on body weight and/or food consumption at ≥ 50 mg/kg/day. NTTP was not associated with a statistically significant increase in mutant frequency in duodenum at the highest dose tested (i.e., 300 mg/kg/day). In contrast, a statistically significant increase in mutant frequency was noted for liver at doses ≥50 mg/kg/day. A statistically significant increase in mutant frequency was not observed in the liver at doses ≤16 mg/kg/day. The vehicle control and positive control responses confirmed appropriate performance of the test system. Overall, the NOGEL for induction of *cII* mutations in the liver was 16 mg/kg/day. Data are shown in Fig. 3 with detailed information provided in Table S1.

3.3. BMD modeling

Using *cII* mean mutant frequency data from liver, a BMD lower bound ($BMDL_{50}$) of 1.89 mg/kg/day and BMD upper bound ($BMDU_{50}$) of 3.61 mg/kg/day was established for NPIP. The same approach was used to derive a $BMDL_{50}$ of 9.17 mg/kg/day and $BMDU_{50}$ of 27.3 mg/kg/day for NTTP. The precision of the BMD analysis is provided by the width of the CI. BMD CI width below 100x is considered suitable for use while anything below 10 is considered a very precise and useable BMD analysis (White et al., 2019). In the case of both NPIP and NTTP, the BMD CI width was ≤3 which demonstrated excellent precision.

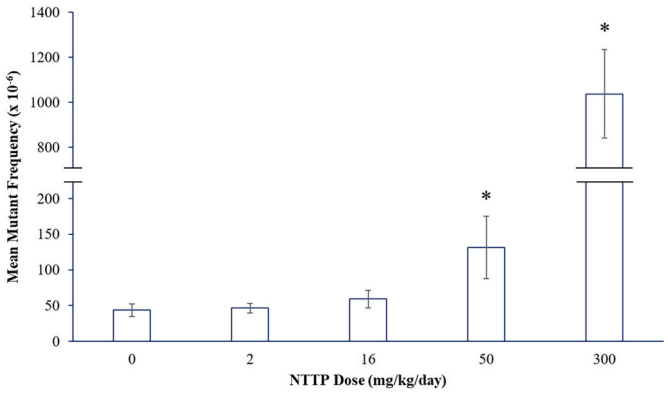

Fig. 3. Summary of Big Blue® transgenic rat mutation data for NTTP in liver. Vehicle control and NTTP treated animals were dosed daily for 28 days and tissues collected following necropsy on Day 31. Data are presented as mean mutant frequency (x 10^{-6}) ± SD with * indicating a statistically significant difference vs. concurrent vehicle control ($P < 0.001$; 1-Way ANOVA).

4. Discussion

Establishing AIs for novel *N*-nitrosamine impurities has been among the key challenges faced by regulatory agencies and the pharmaceutical industry. The development of the CPCA and EAT represent significant advancements; however, there is still a need to develop additional strategies for deriving relevant AI that are protective of patient health. One potential option is to leverage BMD CI from in vivo TGR mutation studies conducted with novel *N*-nitrosamines and compare to similar datasets from well-studied model *N*-nitrosamines. To this end, the 1st goal of the current work was to provide data to allow comparison of in vivo TGR mutation potencies. The existing dataset was previously limited to NDEA and NDMA, both potent *N*-nitrosamines with low AIs. To expand this dataset, we conducted an in vivo Big Blue® transgenic rat mutation study with NPIP to characterize the mutagenic response for an *N*-nitrosamine with a relatively high AI. Evaluations of NDEA (Bercu et al., 2023a), NDMA (Lynch et al., 2024), and NPIP were conducted in compliance with the current OECD TG 488 and utilized similar study design, making them appropriate for comparison. The resulting database of well-studied model *N*-nitrosamines represents a diverse set covering an AI range from 26.5 ng/day to 1300 ng/day.

The 2nd step in the current work was to apply relative potency comparison to justify a scientifically valid AI for NTTP that is protective of patient health. To achieve this, we conducted an in vivo Big Blue® transgenic rat mutation study with NTTP. A comparison of the $BMDL_{50}$ for NTTP with NDEA, NDMA, and NPIP indicated substantial differences in mutagenic potency. BMD CI from OECD TG 488-compliant studies with NDEA, NDMA, NPIP, and NTTP are provided in Table 1 and Fig. 4. NOGEL for these compounds as well as AIs published by regulators as well as Bercu et al. (2023b) are also included in Table 1.

The lack of overlapping BMD CI shows a clear difference in mutagenic potency between NTTP and the model *N*-nitrosamines. More specifically, a comparison of $BMDL_{50}$ values for in vivo mutant frequency in the liver demonstrates that NTTP is ~40–80 times less potent than NDEA and NDMA and ~5 times less potent than NPIP. It is noteworthy that the rank order of potency is consistent with quantum-mechanical SAR modeling developed by Kostal and Voutchkova-Kostal (2023). Application of this model predicted NDEA to be potent (i.e., Category 1 with TD50 ≤ 0.15 mg/kg/day), NDMA and NPIP to be less potent (i.e., Category 2 with 0.15 mg/kg/day < TD50 ≤ 1.5 mg/kg/day), and NTTP to fall outside of the cohort of concern (i.e., Category 3 with TD50 > 1.5 mg/kg/day).

The substantial differences in mutagenic potency determined through in vivo testing as well as predicted carcinogenic potency are not reflected in the current AI for NTTP. Instead of relying on a read-across to NTHP or a CPCA assessment, the recently generated compound-specific in vivo mutation data for NTTP serve as a more appropriate basis to derive an exposure limit. These data provide scientific justification for an exposure limit exceeding the current lifetime limit of 37

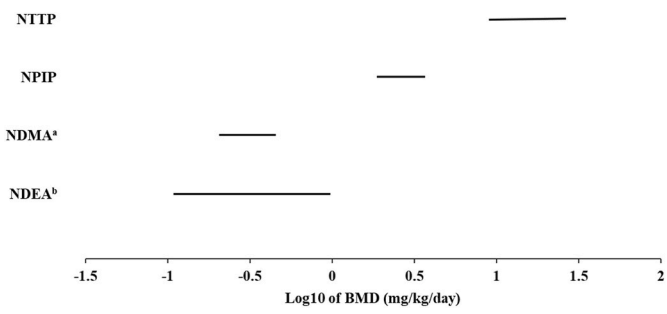

Fig. 4. Comparison of BMD confidence intervals for model *N*-nitrosamines and NTTP from in vivo transgenic mutation studies. Data represent log_{10} transformed BMD confidence interval data from Table 1 a Data from Lynch et al. (2024). b Data from Bercu et al. (2023a).

Table 1
BMD confidence intervals, NOGEL, and AI for model *N*-nitrosamines and NTTP.

N-Nitrosamine	In Vivo TGR Mutation Data (mg/kg/day)[a]			AI (ng/day)	
	$BMDL_{50}$	$BMDU_{50}$	NOGEL	Regulatory	Bercu et al. (2023b)
NDEA	0.11	0.96	0.1	26.5[b]	62[c]
NDMA	0.21	0.46	0.36	96[b]	145[c]
NPIP	1.89	3.61	1	1300[c]	1300[c]
NTTP	9.17	27.3	16	37/100[d]	N/A

[a] In vivo TGR mutation data for NDEA and NDMA from Bercu et al. (2023a) and Lynch et al. (2024), respectively.
[b] AI based on compound-specific rodent carcinogenicity data using harmonic mean TD_{50}.
[c] AI based on compound-specific rodent carcinogenicity data using most sensitive TD_{50} from most robust study.
[d] AI based on read-across to NTHP or CPCA.

ng/day. Overall, the weight of evidence supports the safety of NTTP exposures at, or even above, the default ICH M7 TTC-based AI of 1.5 μg/day or 1500 ng/day (calculations in Table S2). It is noteworthy that the variability of calculated relative potency comparison values correlates with the source of model *N*-nitrosamine data. For instance, there is a higher degree of variability when using regulatory AIs as several of these were derived from harmonic mean TD_{50} values. In contrast, the variability in the calculations was lower when using model *N*-nitrosamine AI derived from the most robust carcinogenicity data (Bercu et al., 2023b). It is also notable that the calculated relative potency values for NTTP did not substantially differ across TGR models considered in our evaluation (e.g., Big Blue® for NDEA and NPIP vs. Muta™Mouse for NDMA). Although based only on a limited number of compounds, this observation suggests selection of TGR model has limited impact on relative potency comparison. A similar conclusion was previously reported for a comparison of TGR mouse models (Wills et al., 2017).

5. Conclusions

The current work demonstrates how in vivo TGR mutation data can be utilized to establish an AI for novel *N*-nitrosamines based on relative potency comparison with model compounds. This approach provides an opportunity to use compound-specific data instead of relying on conservative SAR-based methodologies, such as read-across or the current CPCA framework, that may not accurately reflect the carcinogenic potential of a specific *N*-nitrosamine. In addition, the current work adds to the knowledge of well-studied, model *N*-nitrosamines by providing in vivo TGR mutation data and associated potency metrics for NPIP. The resulting database of in vivo mutation data and carcinogenicity data for NDEA, NDMA, and NPIP can serve as basis for comparison to novel *N*-nitrosamines. Using the relative potency comparison approach, we have demonstrated the safety of NTTP exposures at or above levels of 1500 ng/day.

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CRediT authorship contribution statement

Mark W. Powley: Writing – original draft, Methodology, Formal analysis, Conceptualization. **Zhanna Sobol:** Writing – review & editing, Methodology, Formal analysis, Conceptualization. **George E. Johnson:** Writing – review & editing, Methodology, Formal analysis. **Robert W. Clark:** Writing – review & editing. **Stephen M. Dalby:** Writing – review & editing. **Bridget A. Ykoruk:** Writing – review & editing, Methodology. **Alema Galijatovic-Idrizbegovic:** Writing – review & editing, Conceptualization. **Mark D. Mowery:** Writing – review & editing. **Patricia A. Escobar:** Writing – review & editing, Methodology, Formal analysis, Conceptualization.

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

The data described in this publication are proprietary.

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Appendix A. Supplementary data

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