Risk (Re)Assessment of N-Methyl-N-nitrosophenethylamine for use in computing risk levels of N-Nitrosamine Drug Substance Related Impurities

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Abstract

Management of N-Nitrosamine impurity levels in pharmaceutical drug substances and products is guided by ICH M7 where N-nitrosamines are defined as Cohorts of Concern. Regulatory agencies have suggested using read-across of rodent carcinogenicity TD50 values for structurally similar compounds to assess the potency of various data-poor N-nitrosamines. The TD50 for N-Methyl-N-nitrosophenethylamine (NMPEA) as reported in the CPDB with a harmonic mean TD50 value 7.88 µg/kg/day (or an Acceptable Intake (AI) level of 8 ng/day) did not follow the recommendations of ICH M7. Mixed tissues (oesophagus, forestomach, tongue, and nasal cavity) were combined into a single group termed "upper gastro-intestinal tract". Upon examination of the original data, the oesophagus was considered the most sensitive organ of effect. The TD50 value for the oesophagus was recalculated to 40.1 µg/kg/day (or an AI of 40.1 ng/day). Subsequently, Benchmark Dose (BMD) analysis was performed on the same data set yielding a BMD10 of 3.06-17.6 µg/kg/day in rat (or Permitted Daily Exposure range of 306-1760 ng/day). Theses updated values are 5 times (or higher than) the current AI level of 8 ng/day and could result in significantly higher AI limits for marketed drug impurities that use NMPEA as a suitable analog (e.g., N-nitroso- nortriptyline) to derive an AI.

Keywords: N-Methyl-N-nitrosophenethylamine; *N*-nitrosamines; Acceptable Intake; Permitted Daily Exposure; Carcinogenicity Potency Database; ICH M7; Benchmark Dose (BMD); Cohort of Concern

Highlights:

- The derivation of the acceptable intake for NMPEA did not follow the methodology as recommended in the ICH M7 guidelines.
- An artificial point of departure does not accurately represent the carcinogenic potency and was based on a tissue grouping of convenience.
- A re-calculated acceptable intake of AI of 40.1 ng/day was derived.
- Subsequently, Benchmark Dose (BMD) analysis was performed on the same data set yielding a BMD_{10} of 3.06-17.6 µg/kg/day in rat (or Permitted Daily Exposure range of 306-1760 ng/day).

1. Introduction

Management of *N*-Nitrosamine impurity levels in pharmaceutical drug substances and products is guided by ICH M7 where they are defined as Cohorts of Concern (ICH, 2023). Exposure to *N*-Nitrosamine impurities and Nitrosamine Drug Substance Related Impurities in marketed pharmaceutical represents a potential carcinogenic risk. Their management is guided by ICH M7 (ICH, 2023) as well as publications generated by regulatory bodies [EMA, 2024; HC, 2024; FDA, 2024]. To assess risk for substances with robust toxicological data, a daily acceptable intake (AI) is derived from the dose giving a 50% tumour incidence (TD_{50}) adjusted for bodyweight. The TD_{50} value is calculated from linear extrapolation of dose-response data as documented in the Carcinogenicity Potency Database (CPDB) within the Leadscope Carcinogenicity Database (Leadscope Carcinogenicity Database, 2024) based upon the methodologies of Gold and Sawyer (Gold et al. 1984; Sawyer et al. 1984). The chosen TD_{50} value (in mg/kg bw/day) is adjusted for a 50 kg human bodyweight then divided by 50,000 to adjust from a 1:2 tumor incidence to a 1-in-100,000 excess cancer risk in humans (ICH, 2023). For substances lacking robust toxicological data, structure activity relationships (SARs) and read-across methods may be used to derive appropriate AIs (EMA, 2024). As a rapidly and constantly evolving area of risk assessment, it is important to identify regulatory cases where updates of TD_{50} values may impact the corresponding AI values of drug impurities including those derived for similar drugs using a read-across approach. N-Methyl-N-nitrosophenethylamine (NMPEA) has a derived AI of 8 ng/day based on a TD_{50} derived by Gold *et al* (Gold et al. 1984) based on experimental evidence in a study conducted by Lijinsky et al. 1982a. The point of departure for the AI derivation was the TD_{50} generated for the combined tissue presence of at least one tumor (mixed tumor types) in the oesophagus, tongue, forestomach, or nasal cavity (EMA, 2024).

Selection of a point of departure is a key part of risk assessment, and the choice must consider the best scientific rationale and available guidelines. Guidance is given by ICH M7 on selection of studies (ICH, 2023). This guideline indicated that "the lowest TD_{50} of a particular organ site for an animal species and sex was selected from the most robust studies". The guideline clarified using the single most sensitive tissue stating, "Data compiled as "all Tumor Bearing Animals" (TBA) were not considered in selecting an appropriate TD_{50} from the CPDB; mixed tumor types (e.g., adenomas and carcinomas) in one tissue (e.g., liver) were used where appropriate as this often gives a more sensitive potency estimate". Once an appropriate point of departure has been identified, the methodology used to calculate AI has a significant impact on the resulting value. Linear extrapolation from an appropriate TD_{50} is the most common methodology.

Alternatively, benchmark dose (BMD) modelling of *in vivo* dose-response data offers a scientifically valid and robust alternative methodology that takes direct account the shape of the response curve (Wills et al. 2016). As noted in the ICH M7 guideline, the Benchmark Dose Lower Confidence Limit ($BMDL_{10}$) provides an estimate of the lowest dose with which is 95% certain to cause no more than a 10% cancer

incidence in rodents. A linear extrapolation to a probability of 1 in 100,000 (i.e., the accepted lifetime risk level used) is achieved by simply dividing the $BMDL_{10}$ by 10,000 (ICH, 2023).

BMD modelling may be used to derive a permissible daily exposure (PDE) for carcinogens displaying threshold-based mutagenicity mechanisms (ICH, 2023). Specifically, the $BMDL_{10}$ value is also used as a point of departure (PoD), and can be substituted for the no observed effect level (NOEL) (EFSA, 2022). For genotoxic impurities, the permitted daily exposure (PDE) approach can be used if a 'threshold mechanism' can be defined (ICH, 2023). For mutagenic carcinogens acting via specific mutagenic DNA adducts, DNA repair is the threshold mechanism. As mentioned in the ICH M7 guidance, "Some published data give reliable experimental evidence for (practical) thresholds in the dose response for compounds that are positive for bacterial mutagenicity. This includes examples of thresholds in error-free repair capacity of the mutagenic DNA-ethylating agent ethyl methanesulfonate (EMS) (Müller et al. 2009) or similarly for methylating agents (Wirtz et al. 2010). NMPEA is positive in the Ames assay (Salmonella strains) demonstrating DNA mutation (Andrews and Lijinsky, 1984; Kier et al. 1986).

The present work examines relevant information on the carcinogenic potential of N-Methyl-N-nitrosophenethylamine (NMPEA; CAS No. 13256-11-6) shown in figure 1 and couples this with a study of current relevant guidelines and best scientific practice for the risk assessment of nitrosamines. Benchmark dose modelling is also employed to independently derive a biologically relevant PDE which is compared and contrasted with the AI value.

2. *In silico* methodologies and models

The original data used to calculate the Gold *et al* TD_{50} for NMPEA (Gold et al. 1984) was examined in the Leadscope Carcinogenicity Database (Leadscope Carcinogenicity Database 2024). The data reported in Table 1 was adapted from the Leadscope Carcinogenicity Database which maintains the original format of the CPDB (Gold et al. 1984; Gold et al. 1991; Gold et al. 2005]. The original Lijinsky study (Lijinsky et al. 1982a) on which the EMA AI limit was derived was conducted in male Fischer 344 rats (20 animals per group; 6 – 8 weeks old) with dosing (0.4, 1.1, 3.2, 9.5, 28 and 115 mg/l, with total doses of 1.3, 3.6, 19.5, 84 and 242 mg/rat over the dosing duration) occurring 5 days per week for up to 33 weeks via drinking water. Observations continued for up to 127 weeks. A concurrent study conducted with male Fischer 344 rats dosing daily at a single concentration for 104 weeks via drinking water with observations continuing for 127 weeks was also performed. A concurrent control group (untreated) was identified. Survival time was reduced significantly in the high dose groupings. Consequently, these groupings appear to have been excluded from the TD_{50} calculation in the study (Gold et al. 1984). The study grouped multiple separate tissues (oesophagus, forestomach, tongue, or nasal cavity) into a single group termed "upper gastro-intestinal tract". According to the original data as presented in the CPDB, the TD_{50} value was calculated using only 2 dose groupings (2.92 and 8.10 µg/kg-bw/day) and the control.

In this analysis, the data considered for calculating the TD_{50} value were re-assessed according to ICH M7 criteria relating to the choice of the point of departure for derivation of the AI. The guideline states that "The lowest TD50 of a particular organ site for an animal species and sex was selected from the most robust studies. When more than one study exists, the CPDB provides a calculated harmonic mean TD_{50}, but in this Addendum the lowest TD_{50} was considered a more conservative estimate. Data compiled as "all Tumor Bearing Animals" (TBA) were not considered in selecting an appropriate TD_{50} from the CPDB; mixed tumor types (e.g., adenomas and carcinomas) in one tissue (e.g., liver) were used where appropriate as this often gives a more sensitive potency estimate." (ICH, 2023). In this study, the oesophagus was considered the most sensitive single organ (ICH, 2023). The TD_{50} value was recalculated using only oesophagus tissue study data.

Subsequently, BMD analysis was performed using PROAST online version 70.1 software [PROAST BMD, 2024] following the methodology described in EFSA's, "Guidance on the use of the benchmark dose approach in risk assessment" (EFSA, 2022). Its use in assessing carcinogenic risk as an alternative method to employing TD_{50} values is supported in ICH M7.

The results from BMD graph fitting of the NMPEA oesophagus study data using a critical effect size (CES) of 10% is presented in figures 3 and 4. Seven different models were fit to the data and are individually displayed: two stage, log logistic, Weibull, log probit, gamma, exponential, and Hill models (figure 3). 200 bootstrap curves were then generated using model averaging resulting in weights calculated for each model and listed in the figure 4 caption (figure 4). The BMD_{10} confidence interval (CI) between the BMD_{10} lower and upper bounds for the study data was then calculated.

The BMD-based PDE approach for risk assessment of nitrosamines is more completely described in Johnson et al. 2021. Calculation of the PDE upper and lower limits from the BMD limits is:

Permitted Daily Exposure (PDE):

- F1: species extrapolation values employed to determine the human equivalent dose. The analyses here employed a default value of 5 for rats based on standard allometric scaling factors.
- F2: interindividual variability. A maximum value of 10 was used to reflect the assumed variability in DNA repair proficiency as the major factor.
- F3: exposure duration. A factor of 1 was used for the long-term study duration (over 1 year of continuous exposure in rodents cancer bioassay data) [ICH, 2024; ICH, 2024].
- F4: severity of effect. Since cancer is considered an irreversible severe effect, a maximum value of 10 was used [ICH, 2024].
- F5: factor to compensate for database insufficiency (e.g., cases where a NOEL cannot be determined). This was set to 1 because the analyses are based on a BMDL values, which is considered a superior metric.

PDE = $\frac{\text{BMD x human weight}}{\text{F1xF2xF3xF4xF5}}$

PDE = $\frac{\text{BMD x 50kg}}{\text{5 x 10 x 1 x 10 x 1}}$

3. Animal study toxicity analysis

The Lijinsky study undertaken for assessment of a TD_{50} value of NMPEA (Lijinsky et al. 1982a) was less robust in comparison to the ideal carcinogenicity study design parameters described in ICH M7 guidance in terms of dosing duration, sex, and animal numbers according to the default criteria specified in ICH M7. The EMA considered the study to be sufficiently robust to derive a TD_{50} value (EMA, 2024). The study examined a range of doses as well as clinical signs, histopathological examination of carcinogenicity in a range of organs and although the dosing was not conducted over the duration of the study, animals were examined on death and the authors conducted appropriate analyses on survival and tumour incidence. The concurrent 104-week study, conducted at single dose level, also adds weight to the robustness of the study. Data in the TD_{50} calculations, taken from the 33-week study only, is standardized to account for differences in dose duration, frequency of dosing and survival time. ICH M7 notes "Use of less robust data can sometimes be considered acceptable when no more complete data exist, given the highly conservative nature of the risk assessment in which TD_{50} was linearly extrapolated to a 1 in 100,000 excess cancer risk." These deficiencies are further compensated by the presence of the additional, concurrent 104-week study.

3.1. Regarding the relevance and non-relevance of organs and multiple tissues

Lijinsky et al 1982a concluded that NMPEA should be considered carcinogenic even though the most sensitive organ was not identified according to conditions specified in the ICH M7 guidelines since the study grouped multiple separate tissues (oesophagus, forestomach, tongue, or nasal cavity) into a single group termed "upper gastro-intestinal tract". However, in a separate paper the "upper gastro-intestinal tract" tissue group is defined as "includes animal with 1 tumor or more of the esophagus, forestomach, tongue, oropharynx, or oral cavity" (Lijinsky et al. 1982b). While, in a third paper reviewed the "upper gastro-intestinal tract" is defined as "esophagus, forestomach, tongue and oropharynx" then separately within the same paper as "esophagus, forestomach, tongue, oropharynx and epiglottis" (Lijinsky et al. 1982c). This indicates that the grouping is not a defined tissue set but potentially a convenient statistical artefact.

According to the original data as presented in the CPDB, the TD_{50} value was calculated using only 2 dose groupings (2.92 and 8.10 µg/kg-bw/day) and the control. Such a combination artificially inflated the tumour occurrence (the composition of the tumours differs at each treatment group), removed individual organ significance, and erroneously removed considerations of dose dependence and survivability (Gold et al. 1984).

Basal carcinomas were identified in the tongue but only occurred in the mid-dose group (44.6 µg/kg-bw/day) and showed no dose-dependence. Additionally, the dose where carcinomas occurred was apparently excluded from the overall calculation of TD_{50} in the combined organ assessment. Similarly, only a single animal (in group 4) was reported to have shown an adenocarcinoma in the nasal cavity. Animals in the high dose group also developed lung damage and subsequently pneumonia which reduced survival time. On this basis, this review focuses on the TD_{50} values derived for the Oesophagus (multiple tumor types) and forestomach (Forestomach: Carcinoma – basal cell; Forestomach: Papilloma – basal cell).

The p-value for the results identified in the forestomach (Forestomach: Carcinoma – basal cell; Forestomach: Papilloma – basal cell) indicates no significance of effect in the original CPDB. Lijinsky *et al* reported significance in the 44.6 µg/kg-bw/day group as well as when the results of forestomach carcinoma and papilloma are combined - however the latter lacks dose dependence and was apparently excluded from TD_{50} calculations. The human relevance of the carcinogenicity of the forestomach should also be considered. In rodents the oesophagus leads to the forestomach, which subsequently empties into the glandular stomach. The forestomach is a glandular organ and does not contain protective mucus secretions. The contents of the forestomach may have significant retention time and thus contact with the epithelium. By contrast, in humans the oesophagus leads directly to the stomach which is a glandular organ that produces mucus to provide lubrication and protection. Although the human distal oesophagus and rodent forestomach have some histological similarities, the functional anatomical differences render any such comparisons non-relevant. Differences in pH may also play a role in the relevance of nitrosamine formation – the rat forestomach has a median pH ranging from 4.5 – 6.0, while the human esophagus has a median pH of 7.0 (Proctor et al. 2007; Wester & Kroes, 1988).

The ICH M7 guideline details multiple considerations when assessing tumours of the rodent forestomach:

> "Chemicals that induce tumors associated with local irritation/inflammation (such as rodent forestomach tumors) and are site-of-contact carcinogens may be considered not relevant to human exposure at low, non-irritating concentrations as potential impurities in pharmaceuticals (e.g., benzyl chloride)."

The guideline further notes that:

> "Forestomach tumors in rodents have been the subject of much discussion in assessment of risk to humans. With non-mutagenic chemicals, it is recognized that after oral gavage administration, inflammation and irritation related to high concentrations of test materials in contact with the forestomach can lead to hyperplasia and ultimately tumors. Material introduced by gavage can remain for some time in the rodent forestomach before discharge to the glandular stomach, in contrast to the rapid passage through the human esophagus. Such tumor induction is not relevant to humans at non-irritating doses. The same inflammatory and hyperplastic effects are also seen with mutagenic chemicals, where it is

more complex to determine relative contribution to mode of action of these non-mutagenic, high-dose effects compared with direct mutation induction. However, often a strong case can be made for site-of-contact tumorigenesis that is only relevant at concentrations that cause irritation/inflammation, potentially with secondary mechanisms of damage. Cell proliferation is expected to play an important role in tumor development such that there is a non-linear dose response and the forestomach (or other site-of-contact) tumors are not relevant to low-dose human exposure."

"Proctor *et al* (2007) proposed a systematic approach to evaluating the relevance of forestomach tumors in cancer risk assessment, taking into account whether any known genotoxicity is potentially relevant to human tissues (this would include whether a compound is genotoxic *in vivo*), whether tumors after oral administration of any type are specific to forestomach, and whether tumors are observed only at doses that irritate the forestomach or exceed the MTD."

Table 1 summarizes relevant TD_{50} values derived by Gold *et al* (Gold et al. 1984). It illustrates that the potencies of the forestomach and oesophagus are distinct. Furthermore, as stated in ICH M7, combinations of mixed tumor types are appropriate as they give a more sensitive potency estimate. As NMPEA is a genotoxic carcinogen [Andrews et al. 1984], exclusion of the results from the rodent forestomach based on potential irritation is not appropriate. However, the forestomach is not the most appropriate organ to choose as a point of departure as its TD_{50} value is greater than the Oesophagus: multiple tumor types derived value (2.57 mg/kg-bw/day versus 0.0401 mg/kg-bw/day respectively). The lower TD_{50} value for the Forestomach: Papilloma – basal cell is not considered valid due to a lack of dose response in the dataset. As discussed, the ICH M7 guideline notes that "the lowest TD_{50} of a particular organ site for an animal species and sex was selected from the most robust studies". For comparison, Thresher *et al* calculated a TD_{50} value of 0.0405 mg/kg-bw/day for NMPEA when using an updated methodology to reproduce CPDB TD_{50} results (Thresher et al. 2019).

The weight of evidence indicates that the most appropriate point of departure for derivation of an AI was the oesophagus. The data for the oesophagus, unlike the other organs shows good dose response in the treatment groups used in the calculation, and also shows a clear treatment related effect in the excluded high dose groups that was not observed in the forestomach. This indicates it is the most sensitive and biologically relevant organ. The oesophagus should thus be considered the most sensitive organ of effect and the derived TD_{50} of 0.0401 mg/kg-bw/day represents the most scientifically valid point of departure for derivation of an acceptable intake. Using linear extrapolation and accounting for a 50kg bodyweight, an acceptable lifetime intake of 40.1 ng/day may be calculated:

$$\text{Lifetime AI} = \frac{0.0401 \text{ mg/kg-bw/day} \times 50 \text{ kg}}{50000} = 40.1 \text{ ng/day}$$

4. Benchmark dose analysis

The results from BMD graph fitting of the NMPEA oesophagus study data using a critical effect size (CES) of 10% are presented in figures 2 and 3. A BMD_{10} confidence interval (CI) of 3.06-17.6 µg/kg/day was calculated from the oesophagus study data for NMPEA. The BMD model averaging approach was employed as it represents the most advanced and precise method for calculating BMD metrics (EMA, 2024). The individual exponential and Hill models both fit the data nearly equally well with regression lines falling within the uncertainty bars of all data points. These two models contributed nearly all the weight (93%) to the average model fit, therefore determining the BMD CI. The BMD CI ratio (BMDU/BMDL) is below 10, indicating good precision, that is, a small confidence interval.

Based upon the BMD CI determined from the average model, the Permitted Daily Exposure range for NMPEA was calculated at 306-1760 ng/day as discussed in the material and methods section.

5. Structure-Activity Relationship considerations

The carcinogenic potencies of *N*-nitrosamines spans a range of at least 4 orders of magnitude (Thresher et al. 2020) which can be characterized by the chemical features of the compounds (Cross & Ponting, 2021). Structure-Activity Relationships (SARs) have been developed to help understand how structural features, i.e. substructures, contribute towards increasing or decreasing mutagenicity, individually or in combination with other features, including their effects on physiochemical properties such as solubility, polarity, cell permeability, etc. (Cross & Ponting, 2021). Understanding the statistical significance of a specific feature present in its local environment and its impact upon carcinogenic potency allows for the development of heuristics explaining the potency of compounds with measured carcinogenicity as well as predicting the potency of compounds lacking data (Cross & Ponting, 2021; Dobo et al. 2022; Thomas et al. 2022).

The mutagenicity mechanism for potent *N*-nitrosamine carcinogens, like NMPEA, is dominated by metabolic activation facilitated by Cytochrome (CYP) P450; specifically α-carbon hydroxylation of one or more of the carbon atom substituents immediately adjacent to the *N*-nitroso subgroup. This mechanism ultimately results in the formation of a diazonium ion after one of the *N*-nitrosamine substituents is cleaved through heterolysis. The diazonium ion can then directly form DNA adducts (SN_2 reaction) or first lose the diazonium group resulting in a carbocation which then forms DNA adducts (SN_1 reaction). More details of this mechanism and characteristics that are key in determining the type and extent of DNA adduct formation are described in Cross & Ponting 2021.

Three characteristics affecting metabolism of *N*-nitrosamines (and hence its potency) are the presence of α-carbon hydrogens, accessibility of these hydrogens for hydroxylation, and the electrostatic nature of the reaction site (Cross & Ponting, 2021). NMPEA, structurally, is a relatively featureless compound consisting of a phenyl ring with an ethyl chain linking it to the *N*-nitroso group on one side, and a methyl group on the other (see figure 1). The methyl group on one side has three hydrogens and the ethyl

group on the other has two hydrogens at the α-carbon site. Neither site is sterically hindered, nor is their reactivity diminished by adjacent chemical functionality (such as strong electron withdrawing groups). Hence NMPEA contains no features that would slow or prevent its metabolism to DNA adducts.

Additionally, there are two characteristics of NMPEA known to be prevalent in higher potency *N*-nitrosamine. The first is the presence of a methyl-only substituent on one side. This characteristic has been shown to be statistically significant in higher potency *N*-nitrosamines structures (Cross & Ponting 2021; Thomas et al. 2022). The second is the presence of a benzylic group (phenyl-CH_2) where the benzylic carbon is particularly reactive due to conjugation with the aromatic system and therefore increases potency (Thomas et al. 2022; Hanzlik & Ling, 1990]. While for NMPEA, the ethyl group does not directly conjugate with the *N*-nitroso group α-carbon atom, the β-carbon atom is conjugated and can lead to increased β-carbon hydroxylation resulting in DNA methylation (Li & Hect, 2022a). Secondary α-carbon hydroxylation frequently follows β-carbon hydroxylation albeit both result in the same adduct formation (Li & Hect, 2022a).

α-carbon metabolic hydroxylation of NMPEA could result in two different types of DNA adducts formed, one created from each substituent: methyl adducts, and ethyl-phenyl adducts. The presence of a benzylic group suggests that DNA methylation predominates (Hodgson et al. 2022). The DNA repair mechanism available to repair this type of damage are the alkylguanine-DNA alkyltransferease (AGT) and would be the threshold mechanism affecting its dose-response. While the DNA adducts and repair mechanisms for NMPEA have yet to be published, examination of the evidence suggests that they would likely follow the same as for NMPEA's mechanistically similar, well-studied tobacco-related *N*-nitrosamine analogs 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL; CAS No 76014-81-8) having a TD_{50} of 103 µg/kg/day and 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; CAS No 64091-91-4) having a TD_{50} of 99.9 µg/kg/day. NNAL and NNK displayed oesophageal tumours similar to those reported for NMPEA.

The structural similarity of NNAL and NNK relative to NMPEA is illustrated in Figure 2. NNAL and NNK have butyl chains connecting their aromatic rings with the *N*-nitroso group instead of the ethyl chain for NMPEA. They also have an additional alcohol or carboxylic group attached to the "benzyl" carbon adjacent to the aromatic ring. However, α-carbon hydroxylation of the α-carbons on both substituents for both NNAL and NNK is thought to be the primary bioactivation mechanism with pyridylhydroxybutyl and methyl DNA adducts formed for NNAL and pyridyloxobutyl and methyl DNA adducts formed for NNK (Li & Hect, 2022b). NMPEA would have similar activity based on its structural commonalities that define the nature and relative rate of metabolic mechanism.

Discussion

The TD_{50} for (NMPEA) as reported in the CPDB with a harmonic mean TD_{50} value 7.88 µg/kg/day (or an Acceptable Intake (AI) level of 8 ng/day) did not follow the recommendations of ICH M7. Mixed tissues (oesophagus, forestomach, tongue, and nasal cavity) were combined into a single group termed "upper gastro-intestinal tract". Upon examination of the original data, the oesophagus was considered the most sensitive organ of effect. The TD_{50} value for the oesophagus was recalculated to 40.1 µg/kg/day (or an AI of 40.1 ng/day). Subsequently, Benchmark Dose (BMD) analysis was performed on the same data set yielding a BMD_{10} of 3.06-17.6 µg/kg/day in rat resulting in a Permitted Daily Exposure range of 306-1760 ng/day).

The AI calculated following the ICH M7 methodology is 5 times higher than the current AI published by regulatory authorities while the PDE range is substantially higher. The TD_{50} of 40.1 µg/kg/day is in better agreement with that of its mechanistically similar analogs NNAL (TD_{50} 103 µg/kg/day) and NNK (TD_{50} 99.9 µg/kg/day). ICH M7 recommends using AI limits calculations for mutagenic carcinogens that do not demonstrate evidence for a threshold mechanism whereas it defines using PDE calculations for mutagenic compounds with evidence for threshold mechanisms, such as a non-linear dose response. The use of PDE values assumes that exposure below the threshold dose does not pose a cancer risk. For example, DNA-reactive compounds must overwhelm a DNA repair mechanism prior to showing an effect.

To justify use of a permitted daily exposure (PDE) for regulation under M7, there is a requirement to define a threshold mechanism. Within the ICH M7 guideline, N-nitrosamines are defined as a Cohort of Concern and thus all are assumed not to display threshold effects. *N*-Nitrosamines are known to metabolize to DNA reactive mutagens that result in methylation and alkylation which can result in base-pair substitution during replication (Arimoto-Kobayashi et al. 1997). However, alkyl groups can be removed by AGT repairing the DNA residue. AGT's ability to restore the normal wild-type DNA sequence represents an error-free DNA damage response that can mechanistically account for a dose-response threshold [Fahrer et al. 2015; Johnson et al. 2021; Thomas et al. 2013] and lead to a non-linear dose-response or having a practical threshold [Johnson et al. 2021; Kobets & Williams, 2019). Mechanism of such thresholds, and their impacts on dose response, are part of the reflections of authoritative bodies when examining non-linearity and include considerations on areas such as secondary/indirect origins of the observed damage, enzyme interactions, detoxification capacity and DNA repair mechanisms (MacGregor et al. 2015; Kobets & Williams, 2019). The potency of *N*-nitrosamines depends on the specifics of metabolic activation and repair efficiency and capacity (EMA, 2024).

NMPEA is positive in the Ames test and hence a DNA mutagen (Andrews and Lijinsky, 1984; Kier et al. 1986). The dose-response curve for NMPEA was found to be non-linear with BMD curve fitting using the

exponential model providing the best fit (i.e., the highest weighted model). *N*-nitrosamine SAR considerations would indicate methyl and ethyl-phenyl DNA base adducts as the most common DNA adducts with AGT being its DNA repair mechanism.

Conclusion

Use of the BMD and PDE approach represents a valid and scientifically robust alternative to the AI, that relies more on dose-response and knowledge of threshold effects (such as DNA repair) than simple linear extrapolation. The BMD and PDE approach also account for factors such as interspecies and intraspecies variation and can compensates for database insufficiencies.

Updating the AI limit of NMPEA to 40.1 ng/day (or adopting a PDE) has ramifications when determining AI limits for marketed drugs similar in structure to NMPEA but not having carcinogenicity data. Such is the case for *N*-nitroso-nortriptyline whose current AI limit (8 ng/day) was established using NMPEA as a suitable analog. These AI limits would be expected to change with any updating of the NMPEA limit. Lastly, as NMPEA is the most potent *N*-nitrosamine in the CPDB database having carcinogenicity data, updating this limit would establish a new, more accurate baseline for the most severe level of carcinogenicity potency known for *N*-nitrosamines.

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David R. Woolley, Ph.D.: animal study toxicity analysis, writing – original draft, writing – review and editing; George E. Johnson, Ph.D.: benchmark dose analysis, writing – original draft; Kevin P. Cross, Ph.D.: Structure-Activity Relationship analysis, writing – original draft, editing, writing – review and editing, conceptualization.

Conflict of Interest and other Ethics Statements

There are no conflicts of interests by any of the authors.

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Figures

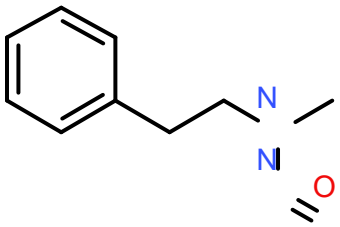

Figure 1. N-Methyl-N-nitrosophenethylamine (NMPEA; CAS No. 13256- 11-6)

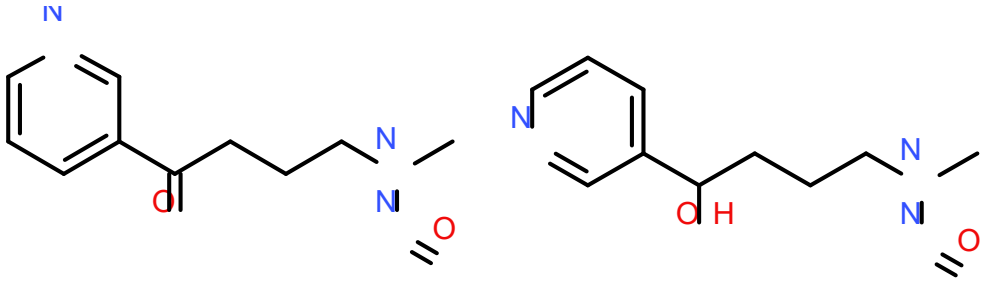

Figure 2. 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK; CAS No 64091-91-4; left) and 4-Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL; CAS No 76014-81-8; right)

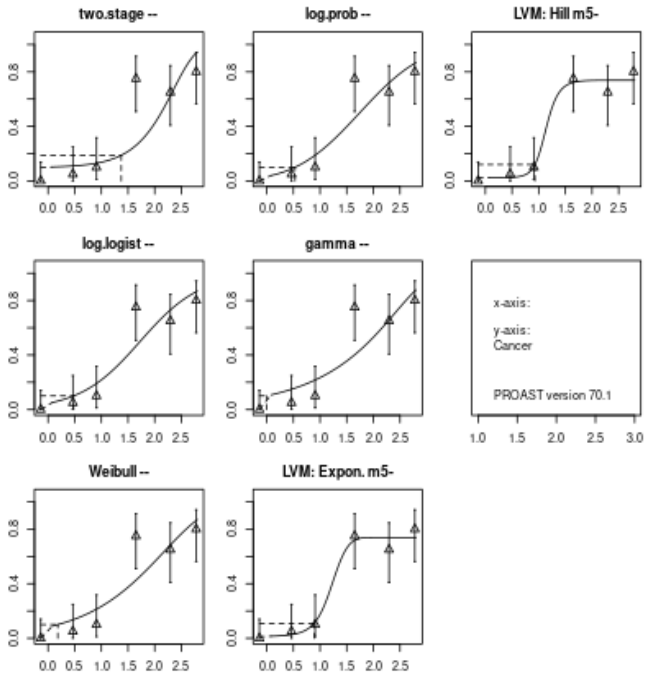

Figure 3: Individual model BMD analysis of NMPEA oesophagus study data

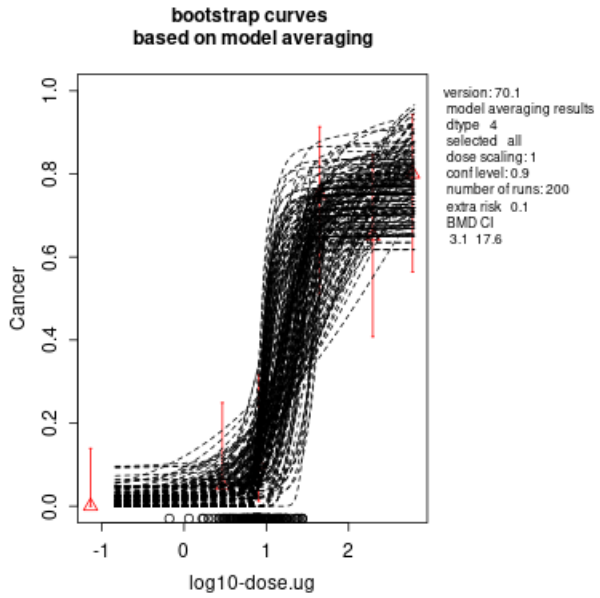

Figure 4: Model averaging BMD analysis of NMPEA oesophagus study data using a critical effect size (CES) of 10%. Model weightings were two stage=0; log logistic=0.0293; Weibull=0.0067; log probit=0.0324; gamma=0.0026; exponential=0.5176; and Hill=0.4113; and 200 bootstraps were used as default. $BMDL_{10}$: 3.06 µg/kg/day and $BMDU_{10}$: 17.6 µg/kg/day

Tables

Organ	Call according to author (if available; p Value)	Gold derived TD_{50} (mg/kg-bw/day)	Dose (µg/kg-bw/day) and Incidence*					
			0	2.92	8.10	44.6	197†	600‡
Forestomach: Carcinoma – basal cell	Positive (≤0.216)	2.57	0/20	4/20	7/20	15/20	4/20	9/20
Forestomach: Papilloma – basal cell	No opinion given (≤0.349)	2.21	0/20	6/20	1/20	1/20	4/20	(5/20)
Oesophagus: multiple tumor types	Positive (≤0.0005)	0.0401	0/20	1/20	2/20	15/20	(13/20)	(16/20)
Gastrointestinal tract-upper§ : Multiple tumour types	Positive (≤0.0005)	0.00788	0/20	9/20	11/20	(16/20)	(19/20)	(19/20)
Liver: Nodule-hyperplastic	No opinion given (1.0)	Not derived	0/20	0/20	5/20	0/20	0/20	0/20
Multiple sites: Leukemia - monocytic	No opinion given (1.0)	Not derived	9/20	16/20	13/20	9/20	0/20	0/20
Tongue: Carcinoma basal cell	Positive (1.0)	Not derived	0/20	0/20	0/20	6/20	0/20	0/20
N.B.: As per Gold et al., 1984 "if the departure from linearity in such cases was downward, then this fact is indicated by parentheses around the group that was excluded from the TD_{50} calculation."								

Table 1. Summary of selected data (TD_{50}, dose, incidence and tumor organ location) from Lijinsky *et al* and Gold *et al*

* The highest dose group was excluded from the CPDB calculations

† Survival times significantly reduced (proportion surviving reached 0 at approximately 44 weeks)

‡ Survival times significantly reduced (proportion surviving reached 0 at approximately 40 weeks), animals dosed for only 30 weeks compared with 33 week dosing for all other treatment groups.

§ Examines one tumor in any of the following organs: oesophagus, forestomach, tongue, or nasal cavity