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Are all nitrosamines concerning? A review of mutagenicity and carcinogenicity data

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ABSTRACT

The control of potentially mutagenic impurities in pharmaceutical products is of key importance in assessing carcinogenic risk to humans. The recent discovery of nitrosamine impurities in several marketed pharmaceuticals has increased interest in their mutagenic and carcinogenic potential. This chemical class is considered part of a 'cohort of concern', indicating that standard control protocols, such as the use of a threshold of toxicological concern (TTC), cannot be applied. Whilst some nitrosamines are known to be exceptionally potent carcinogens, it's not clear whether this is a property of all members of the class. To investigate the mutagenic and carcinogenic potential of nitrosamines, data was extracted from published literature to augment that already present in the Vitic and Lhasa Carcinogenicity Databases. This data was analysed to assess the application of the ICH M7 guideline to nitrosamine impurities, with respect to the predictivity of the Ames test for carcinogenic potential and the distribution of carcinogenic potency. It was found that 18% of nitrosamines were considered non-carcinogenic. Nitrosamines showed a greater correlation between mutagenicity and carcinogenicity compared to non-nitrosamine compounds. Whilst nitrosamines, in general, are more potent carcinogens than non-nitrosamines, there is a significant overlap between the two distributions of TD50s for each class.

1. Introduction

Mutagenic impurities are compounds which may react directly with DNA and potentially introduce genetic mutations, even at low concentrations, which can initiate tumour formation. The assessment and control of mutagenic impurities in pharmaceutical products with respect to their carcinogenic risk is outlined in the ICH M7 guideline. This guideline describes how theoretically acceptable levels of human exposure can be derived for mutagenic impurities lacking adequate experimental carcinogenicity data. For example, the threshold of toxicological concern (TTC) is a commonly used acceptable intake level derived by linear extrapolation from preclinical TD_{50} values (Cheeseman et al., 1999) (the dose at which the probability of remaining tumour-free after chronic administration for the standard lifespan would be halved), which are themselves derived from experimental tumour incidence data. In the context of ICH M7, the TTC-based approach applies a threshold of 1.5 μg/day as the level at which a compound with inadequate experimental carcinogenicity data can be considered as having a negligible carcinogenic risk in humans. Where adequate data is available, acceptable intakes can be derived on a compound- or class-specific basis (Bercu et al., 2018). In the absence of mutagenicity data for an impurity, the assessment of mutagenic potential may be made using *in silico* predictions from two complementary (quantitative) structural activity relationship ((Q)SAR) methodologies, one expert rule-based and one statistical-based (ICH, 2017).[1]

N-Nitrosamines (nitrosamines) [Fig. 1] are a class of compounds which humans are commonly exposed to in small doses via tobacco and food products (for example, cured meats). The recent discovery of nitrosamine impurities in several marketed pharmaceuticals has led to increased interest in their mutagenic and carcinogenic potential. Regulatory authorities have requested that marketing authorisation holders review the manufacturing process and finished products for all

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[1] Abbreviations: CPDB- Carcinogenic Potency Database; EMA- European Medicines Agency; LCDB- Lhasa Carcinogenicity Database; NDEA- N-nitrosodiethylamine; NDMA- N-nitrosodimethylamine; NPV- negative predictive value; NTP- National Toxicology Program; OECD- Organisation for Economic Cooperation and Development; PPV- positive predictive value; (Q)SAR- (quantitative) structural activity relationship; TTC- threshold of toxicological concern.

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Fig. 1. Substructure for N-nitrosamine compounds.

synthesised active pharmaceutical ingredients to identify the potential for the presence of nitrosamine impurities. This is to be done using a risk-based approach to prioritise evaluations and subsequent testing (EMA, 2019a; Health Canada, 2019; Swissmedic, 2019). Should a risk be identified, then confirmatory testing and a change to the manufacturing process would be required to remove the risk. The ICH M7 guideline includes nitrosamines in a 'cohort of concern', alongside potent carcinogens such as aflatoxin-like and alkyl-azoxy compounds. This means that acceptable levels of exposure are likely to be significantly lower than the TTC defined by the guideline. In this sense, the 1.5 μg/day threshold cannot be applied, and the presence of a nitrosamine must be controlled on a case-by-case basis using carcinogenicity data for closely related compounds.

Whilst some nitrosamine compounds are exceptionally potent carcinogens, it is unclear whether this is a universal property of all members of this class. The European Medicines Agency have set interim acceptable limits for selected nitrosamine impurities to 26.5 ng/day or 96 ng/day, based on their similarity to N-nitrosodiethylamine (NDEA) or N-nitrosodimethylamine (NDMA), respectively (EMA, 2019b). The current work was undertaken to investigate how the mutagenic activity of nitrosamines is indicative of their carcinogenic potential and the degree to which tumourigenic potency varies across this chemical class. This may then inform the how theoretically safe levels of human exposure could vary depending on the structure of individual nitrosamine compounds. With regard to mutagenicity data, analysis focussed on the bacterial reverse mutation assay (Ames test), which is commonly used as an *in vitro* predictor of a compound's *in vivo* carcinogenic potential (ICH, 2017).

2. Method

2.1. Mutagenicity and carcinogenicity data

Vitic is a commercially available, structure-searchable database which contains curated toxicology data collected predominantly from public sources (Lhasa Limited, 2020). To expand the coverage of nitrosamines in Vitic, a series of literature searches were carried out using PubMed (U.S. National Library of Medicine (NLM)) (to provide a list of articles containing Ames test and/or rodent carcinogenicity data relating to nitrosamines. Publications containing nitrosamines that were absent from Vitic were prioritised for data extraction, with a view to expanding the chemical coverage of nitrosamines in the database.

Following data collection and its addition to the Vitic database, all Ames test and rodent carcinogenicity data contained within Vitic was reviewed. To compare the mutagenicity and carcinogenicity data for each compound, overall Ames and rodent carcinogenicity calls were derived following a previously defined workflow (Thresher, 2016) with minor modifications. This process consisted of the following steps:

1. Mutagenicity data was selected from the Vitic database where the test type was labelled as either 'Ames test' or 'bacterial reverse mutation assay' and the test species was given as either *Salmonella typhimurium* or *Escherichia coli*. Carcinogenicity data was selected from studies using rodent species.
2. Studies with a Klimisch score of 3 ('Not reliable') were removed from the data set.
3. Data records were grouped into subsets of similar studies. For Ames data this was done according to the bacterial strain, metabolic activation system and study protocol used (e.g. plate incorporation). For carcinogenicity data, subsets were defined according to the species and sex of the test subjects, the route of administration and the duration of exposure to the test substance.
4. For each subset, an activity call was generated based on the original author's call for each study. Subsets containing a mixture of positive and negative, or equivocal and negative studies were given an activity call of 'conflicted'.
5. Ames subsets were prioritised by preferentially selecting those containing well defined strain and metabolic activation information. Subsets where these values were not specified were only used to determine the overall call where no complete subsets where present. No prioritisation was carried out with the carcinogenicity subsets.
6. The most conservative subset call was taken as the overall compound call.

These calls were then used to examine the concurrence of mutagenic and carcinogenic activity for nitrosamines, both as a class and juxtaposed with the compounds in Vitic which do not contain the nitrosamine toxicophore (non-nitrosamines). As such, compounds resolving as either conflicted or equivocal were removed from the data set.

2.2. Carcinogenic potency

The Carcinogenic Potency Project (CPDB), project, originally created by Gold et al. (1984), was a database of long-term carcinogenicity study data gathered from public literature and the U.S. National Toxicology Program (NTP) and included calculated TD_{50} values. The data in the CPDB was last updated in 2007, and so the Lhasa Limited Carcinogenicity Database, (LCDB) (Lhasa Limited) was created to safeguard the CPDB data. The freely available LCDB contains both the original CPDB TD_{50} values together with Lhasa-generated TD_{50} values created using a script based on the original CPDB methodology (Thresher et al., 2019). This data was examined in relation to nitrosamines, both to clarify the correlation between CPDB- and Lhasa-generated values specifically for this class of compounds and to examine how the tumourigenic potency distribution compares to the remaining non-nitrosamine carcinogens.

3. Results

3.1. Assessment of mutagenicity and carcinogenicity data

The Vitic database (Vitic, 2020.1) now contains a total of 479 nitrosamines: 381 with Ames test data and 228 with rodent carcinogenicity data, of which 178 of these contain both Ames test and rodent carcinogenicity data. The increase in data compared to the previous version of the Vitic database (Vitic, 2018.1) is shown in Fig. 2. The number of nitrosamines in the database increased by 24%, with a 35% increase in those with Ames test data, 55% increase with rodent carcinogenicity data and 105% increase in those with both Ames test and rodent carcinogenicity data. In addition to novel nitrosamines, Ames and carcinogenicity data for 150 nitrosamines already in Vitic was expanded to include broader information on the range of strains and study protocols.

In total, Ames activity calls were generated for 381 nitrosamines and 14,193 non-nitrosamines, with carcinogenicity calls generated for 228 nitrosamines and 2913 non-nitrosamines. In all four data sets the number of conflicted or equivocal calls was negligible (Fig. 3). The highest number of conflicted and equivocal calls was 5% in the non-nitrosamine Ames calls and 3% in the non-nitrosamine carcinogenicity calls, respectively.

The nitrosamines showed an almost identical proportion of positive and negative calls in the Ames and carcinogenicity studies. A greater divergence was observed in the non-nitrosamines, with a higher

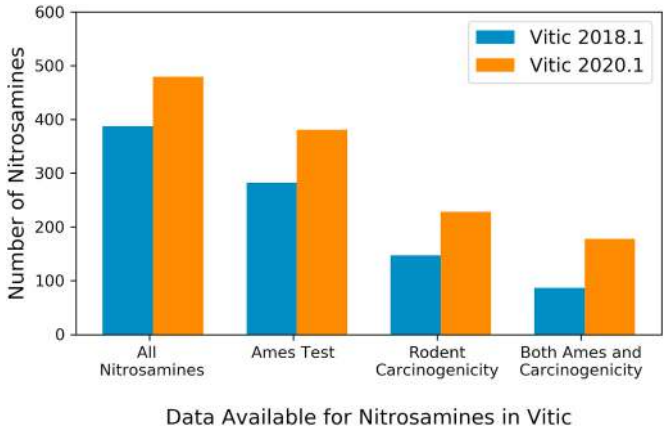

Fig. 2. Number of nitrosamines with Ames and/or rodent carcinogenicity data in Vitic 2018.1 and 2020.1.

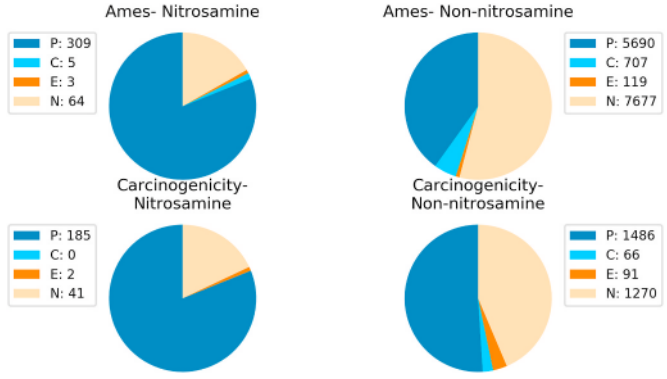

Fig. 3. Proportion of Ames and carcinogenicity activity calls for the nitrosamines and non-nitrosamines in Vitic 2020.1. P= Positive; C= Conflicted; E = Equivocal; N= Negative.

proportion being carcinogenic than mutagenic. While it is unsurprising that a much higher proportion of nitrosamines are mutagenic or carcinogenic compared to non-nitrosamines, given the presence of nitrosamines in the 'cohort of concern', it is surprising is that approximately 18% of nitrosamines appear to be non-carcinogenic.

3.2. Predictivity of Ames test for carcinogenic activity

After removal of compounds with either a conflicted or equivocal call, a total of 171 nitrosamines and 1862 non-nitrosamines containing both Ames and carcinogenicity calls remained. For each compound, these calls were compared to give an indication of the predictivity of the Ames test for carcinogenicity [Supplementary Table 1, Fig. 4].

The nitrosamines show a high balanced accuracy, sensitivity, positive predictive value (PPV) and negative predictive value (NPV), indicating that the Ames test can predict the carcinogenic potential of nitrosamines. Indeed, there was a greater correlation between the Ames and carcinogenicity calls than for non-nitrosamine compounds. The high specificity and comparatively low sensitivity of the nitrosamines (compared to the non-nitrosamines) could indicate lack of negative predictivity by the Ames test for this class. However, the number of non-carcinogenic nitrosamines is too low to make a definitive conclusion.

The underlying reason for the high number of "false negatives" in the non-nitrosamine data is unclear; however, the possibility that several of these are either non-genotoxic carcinogens or active through a genotoxic mechanism not detected by the Ames test is consistent with the higher proportion of carcinogens compared to mutagens in this data set.

In the nitrosamines data set, 8 compounds were described as "false negatives", i.e. they had a negative Ames call and positive carcinogenicity call. A review of the study information available for these compounds shows that only 2 have been tested in 5 bacterial strains, both in the presence and absence of a metabolic activation system, as described in the current Organisation for Economic Co-operation and Development (OECD, 1997) test guideline 471 [Supplementary Table 2]. This indicates that the remaining studies may not offer a robust assessment of the mutagenic potential of these nitrosamines and further testing under OECD guideline 471 compliant conditions would be advisable to confirm them as non-mutagens.

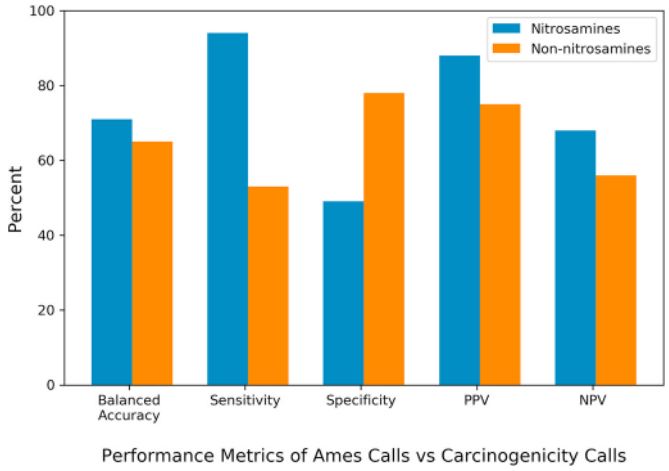

Fig. 4. Performance metrics for the comparison of Ames and carcinogenicity calls derived from the Vitic 2020.1 data.

Within the nitrosamine Ames test data set approximately 34% of the subsets defined during step 3 of the summary workflow did not conform to the current OECD 471 guideline. However, the removal of these subsets from the summary workflow resulted in only a small decrease in the total number of nitrosamines with summary Ames calls (8%), as many compounds contain data from multiple subsets. Of the remaining nitrosamines, the absence of the non-OECD compliant subsets did not significantly alter the summary calls generated, remaining identical in 92% of cases. Regarding the predictive performance of the Ames test for carcinogenic potential, removal of the non-OECD compliant Ames studies had a minimal impact on the correlation between Ames test and carcinogenicity summary calls. This resulted in a balanced accuracy of 68.9, compared to 69.4 when including the non-OECD compliant Ames data for the same compounds.

3.3. Evaluation of carcinogenic potency

The LCDB contains a total of 137 nitrosamines, of which 117 were considered carcinogenic by the original study authors. Of these, 46 contain both Lhasa and CPDB TD_{50} values. Previous work has demonstrated a high correlation between the original CPDB TD_{50} values and the Lhasa-calculated TD_{50} values (Thresher et al., 2019). The distribution of log Lhasa TD_{50} and log CPDB TD_{50} values for both nitrosamines and non-nitrosamines within the LCDB reiterates the high correlation between the CPDB- and Lhasa-calculated values, especially for the nitrosamines.

Nitrosamines are typically more potent carcinogens compared to the non-nitrosamine carcinogens in this data set, in that the log Lhasa TD_{50} values are generally lower for these compounds. While there is substantial overlap in potency between the two data sets, the mean nitrosamine log Lhasa TD_{50} value (−0.433) is considerably lower than that of the non-nitrosamines (1.418) [Fig. 5].

NDEA is the most potent nitrosamine for which carcinogenicity data is available. Although it is commonly used to illustrate the carcinogenic potential of nitrosamines, NDEA is exceptionally potent compared to

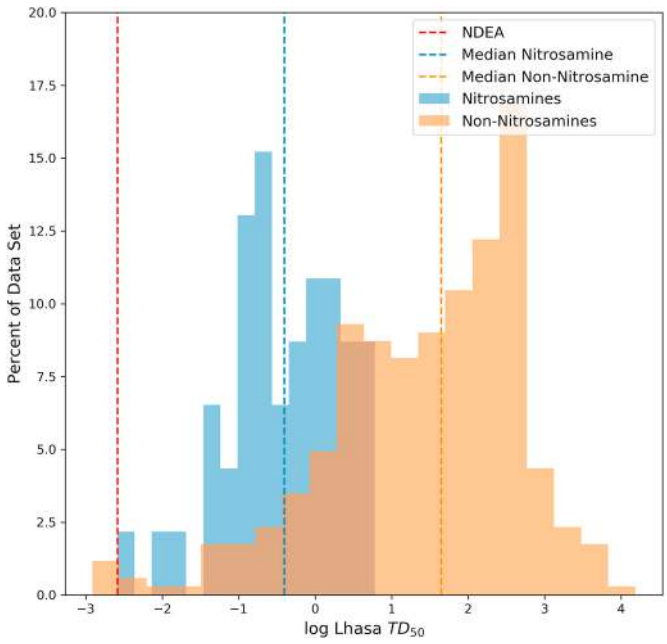

Fig. 5. Distribution of log Lhasa TD50 values for nitrosamine and non-nitrosamine compounds as a proportion of the respective data sets within the LCDB.

most other nitrosamines [Fig. 5]. The log Lhasa TD_{50} value for NDEA (−2.585) is considerably lower than the class mean (−0.433), with only N-nitroso-N-methyl-2-phenylethylamine (−2.10) displaying a similar value. The potency of NDEA is more comparable to aflatoxin B1 (−2.458), in the non-nitrosamine data set, which is also present in the 'cohort of concern'.

4. Discussion

A considerable amount of mutagenicity and carcinogenicity data for nitrosamines has been added to that already available in Vitic and the LCDB. This has shown that, despite the majority of nitrosamines being carcinogenic, approximately 18% of the data set resolved as non-carcinogenic based on the expanded data in Vitic. However, consideration should be given to assessing the study protocol used, specifically regarding the duration of exposure and highest tested doses, when regarding the validity of study results. Comparison of the activity calls generated from the Vitic data showed a strong correlation between the Ames and carcinogenicity results. This correlation proved to be greater than that observed for the non-nitrosamine compounds in Vitic, indicating that the Ames test is highly predictive of carcinogenic potential for this chemical class. The presence of 'false negative' Ames test results in the data set may be due to a lack of studies covering an adequate range of bacterial strains. While the standard assay conditions throughout industry often consist of the use of rat S9 under a plate incorporation protocol, the use of hamster S9 and/or a preincubation protocol is consistent with the current OECD 471 guideline. Removing non-OECD compliant subsets from the Ames summary call workflow did not significantly alter the final calls generated. Therefore, the use of the ICH M7 guideline in assigning an impurities classification based on adequate mutagenicity data is appropriate for this class of compounds.

Nitrosamines are specifically listed in ICH M7 within the 'cohort of concern' along with other highly potent carcinogens, such as aflatoxins, as they are considered to pose a significant risk to human health at doses below the standard acceptable intakes defined in the guideline. Instead, an assessment of the carcinogenic risk must be made for nitrosamines on a case-by-case basis. In response to the discovery of nitrosamine impurities in marketed pharmaceuticals, the EMA has imposed interim acceptable limits of 26.5 ng/day for selected nitrosamines based on their similarity to NDEA (EMA, 2019b). Although carcinogenic nitrosamines, as a class, are typically more potent than other carcinogens, they exhibit a wide distribution of log TD_{50} values, from NDEA at −2.585 to 1-nitrosopiperazine at 0.781. This distribution overlaps with that of the non-nitrosamine carcinogens, including some not present in the 'cohort of concern'. The mean log Lhasa TD_{50} value of −0.433 suggests NDEA may not be an exemplar of the carcinogenic potency of this chemical class.

5. Conclusion

The available data shows that not all nitrosamine compounds are concerning. The high correlation between the Vitic Ames test and carcinogenicity data shows that the Ames test, as described in the OECD 471 guideline, can be used as an indicator of carcinogenic potential for nitrosamines. While carcinogenic nitrosamines display a more potent TD_{50} distribution when compared to non-nitrosamine carcinogens in the LCDB, this potency does vary within the class. Further analysis of the structures of nitrosamines is required to determine what common features influence carcinogenic potency.

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Declaration of competing interest

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

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.yrtph.2020.104749.

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