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Rapid Communication

# Revisiting the Landscape of Potential Small and Drug Substance Related Nitrosamines in Pharmaceuticals

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ABSTRACT

*N*-Nitrosamines are a class of indirect acting mutagens, as their metabolic degradation leads to the formation of the DNA-alkylating diazonium ion. Following up on the *in-silico* identification of thousands of nitrosamines that can potentially be derived from small molecule drugs and their known impurities described in a previous publication, we have now re-analyzed this dataset to apply EMA's Carcinogenic Potency Categorization Approach (CPCA) introduced with the 16th revision of their Q&A document for Marketing Authorization Holders. We find that the majority of potential nitrosamines from secondary amine precursors belongs to potency categories 4 and 5, corresponding to an acceptable daily intake of 1500 ng, whereas nitrosamines from tertiary amine precursors distribute more evenly among all categories, resulting in a substantial number of structures that are assigned the more challenging acceptable intakes of 18 ng/day and 100 ng/day for potency categories 1 and 2, respectively. However, the nitrosative dealkylation pathway for tertiary amine is generally far slower than the direct nitrosation on secondary amines, with a direct nitrosation mechanism suspected only for structures featuring electron-rich (hetero)aromatic substituents. This allows for greater focus towards those structures that require further review, and we demonstrate that their number is not substantial. In addition, we reflect on the nitrosamine risk posed by secondary amine API impurities and demonstrate that based on the ICH Q3A/B identification threshold unknown impurities may exist that could be transformed to relevant amounts of NA. We also demonstrate that the analytical sensitivity required for the quantification of high potency nitrosamines can be problematic especially for high dose APIs. In summary, the regulatory framework rolled out with the latest Q&A document represents a substantial improvement compared with the previous situation, but further refinement through interaction between manufacturers, regulators, not-for-profit and academic institutions will be required to ensure patient access to vital medicines without compromising safety.



*Abbreviations:* AI, Acceptable (Daily) Intake; API, Active Pharmaceutical Ingredient; CPCA, Carcinogenic Potency Categorization Approach; DP, Drug Product; EAT, Enhanced Ames Test; EFPIA, European Federation of Pharmaceutical Industries and Associations; EMA, European Medicines Agency; EMA-MutaMind, EMA-funded project lead by Fraunhofer Institute for Toxicology and Experimental Medicine to better understand the mutagenicity of N-nitrosamines; EML, Essential Medicines List; FDA, U.S. Food and Drug Administration; GSRS, Global Substance Registration System; HCTZ, Hydrochlorothiazide; HESI-GTTC, Genetic Toxicology Technical Committee of the Health and Environmental Sciences Institute; ICH, International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use; LOQ, Limit of Quantification; MDD, Maximum Daily Dose; NCATS, National Center for Advancing Translation Science; NDEA, *N*-Nitrosodiethylamine; NDMA, *N*-Nitrosodimethylamine; NDSRI, Nitrosamine Drug Substance Related Impurity; OECD, Organisation for Economic Co-operation and Development; Ph.Eur., European Pharmacopoeia; SAR, Structure Activity Relationship; TTC, Threshold of Toxicological Concern; USP, United States Pharmacopoeia; WHO, World Health Organization.

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## Introduction

*N*-Nitrosamines have been a matter of intense pharmaceutical research and regulatory debate since their unforeseen detection in AT1 receptor antagonists such as Valsartan in late 2018 [1], as they may act as DNA alkylating mutagens. Other drugs that faced nitrosamine-related recalls or suspensions include propranolol [2], varenicline [3,4], and ranitidine [5,6]. Nitrosamines can form when a nitrosatable amine group and nitrosating agents, frequently derived from inorganic nitrite, are combined under promoting conditions. In a recent publication we reported, in addition to extensive considerations about the chemical background of nitrosamine formation, the outcome of an *in silico* analysis of >12k small molecule drugs and impurities extracted from the Global Substance Registration System (GSRS) database with respect to their potential to form nitrosamines [7]. We showed that a large proportion of these molecules contain secondary or tertiary amine moieties as structural features that make them susceptible to nitrosation, i.e., 40.4 % of the analyzed APIs and 29.6 % of the API impurities, resulting in thousands of different potential nitrosamine impurities.

As many nitrosamines, especially those of the small dialkyl type, are exceptionally genotoxic, they belong to the ICH M7 cohort of concern and cannot by default be controlled to the threshold of toxicological concern at 1.5 $\mu$g/day. For a long time this meant that, unless a substance specific limit could be established through carcinogenicity testing or read across to a structurally related surrogate substance (close structural analog) [8], one had to apply the default acceptable intake set by the European Medicines Agency (EMA) at 18 ng/day, calculated from the 5th percentile of $TD_{50}$ values for nitroso compounds from the Lhasa Carcinogenicity Database (LCDB) [9], linearly extrapolated to a theoretical cancer risk of $10^{-5}$. FDA set this limit at 26.5 ng/day, which is also the acceptable intake for NDEA.

On July 7th, 2023, EMA published a substantial revision of their Q&A document on nitrosamine impurities in human medicinal products that introduces a novel SAR-based approach for AI determination of nitrosamines, using five potency categories related to known nitrosamines with available carcinogenicity data [10]. Potency category 1 requires control to the default AI at 18 ng/day, whereas categories 2, 3, 4 and 5 implicate AIs of 100 ng/day, 400 ng/day, 1500 ng/day and again 1500 ng/day, respectively. Here we report the mapping of the previously identified potential nitrosamines to the EMA potency categories, applying the rules defined in Annex 2 of the Q&A document, revision 16. It is to be noted that this is a purely academic exercise; many of the potential nitrosamines, especially those derived from tertiary amines, may not form under the conditions of API synthesis, drug product manufacturing or drug product storage. Furthermore, for those that do form it may be possible to justify higher AIs than those proposed by the CPCA, based on read-across, or even control to ICH Q3A/B limits if non-mutagenicity can be demonstrated.

## Materials and Methods

Data for APIs and API impurities from the Global Substance Registration System (GSRS) were extracted and processed as previously described [7], to derive nitrosamine structures that could be formed from secondary and tertiary amine APIs and API impurities. These potential nitrosamines were then further evaluated and assigned to the potency categories defined in Annex 2 of the EMA Q&A document on nitrosamines, revision 16 [10]. The overall data flow is described in Fig. 1.

### *Nitrosamine Evaluation - Potency Categorization & Identification of Activated Tertiary Amines*

A version of the CPCA workflow was created in the KNIME Analytics Platform 4.6 [11] to evaluate and assign the potency categories for the dataset of potential nitrosamines previously identified [7]. Whilst a range of nodes are available to evaluate the molecular properties, in this instance the key substructures were assessed using nodes developed using Lhasa limited's internal chemical engine, which underpins Derek and Sarah Nexus [12]. Any nitrosamines which are included in the list of 83 published acceptable intakes in Appendix 1 of the EMA Q&A document [10] were not included for evaluation. The CPCA workflow calculates a Potency Score for a nitrosamine to assign a Potency Category (1 – 5) which ultimately dictates the recommended AI limit; Potency Score = $\alpha$-Hydrogen Score + Deactivating Feature Score + Activating Feature Score. Calculation of these three component scores is assigned by matching the features (e.g., count of hydrogen atoms on each $\alpha$-carbon, functional groups considered to deactivate or activate carcinogenic potency) present in the nitrosamine to the list of features in Appendix A [10] and assigning the appropriate score for each component. A set of structural patterns were created (using the same technology as Derek Nexus [12–14]) to represent these published features and the respective scores were assigned to any nitrosamine which matched a given pattern. Therefore, based on the collection of patterns matched, the KNIME workflow could apply a series of logic to calculate the final Potency Score and assign a Potency Category. Examples for representative structures with activating or deactivating features and particular hydrogen scores are provided in the EMA Q&A document [10]. Another publicly available resource is the USP Nitrosamine Exchange (https://nitrosamines.usp.org/) [15], where several users have posted visualizations of NDSRIs that belong to the respective CPCA categories.

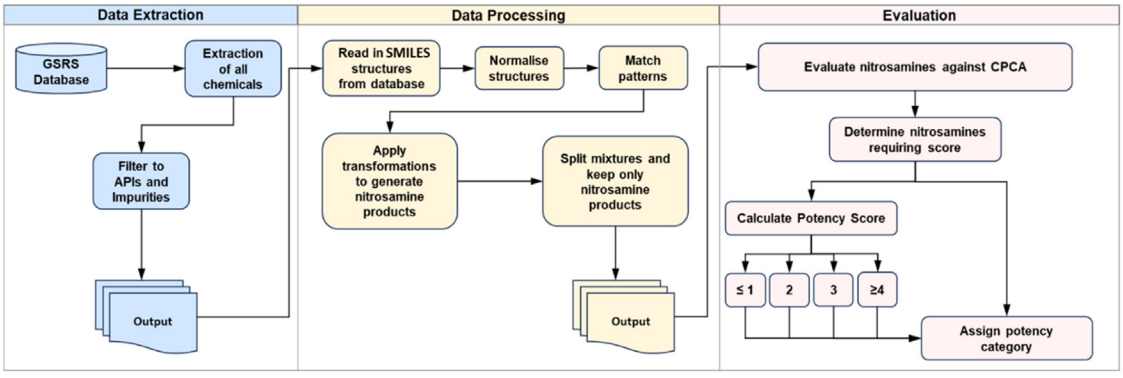


**Fig. 1.** Data flow.

**Table 1**
Distribution of the potential nitrosamines from the datasets into the various potency categories.

| Source | | Distribution of NAs | | | | | |
|---|---|---|---|---|---|---|---|
| Dataset | | *Compound Specific AI* | *Category 1 (18 ng)* | *Category 2 (100 ng)* | *Category 3 (400 ng)* | *Category 4 (1500 ng)* | *Category 5 (1500 ng)* |
| USP APIs | 2° | 24 | 90 | 118 | 225 | 381 | 526 |
| | 3° | 28 | 1269 | 961 | 1280 | 1261 | 276 |
| USP Imp. | 2° | 15 | 49 | 36 | 62 | 138 | 188 |
| | 3° | 16 | 320 | 207 | 349 | 397 | 55 |
| Orange Book | 2° | 19 | 17 | 25 | 37 | 74 | 111 |
| | 3° | 17 | 231 | 142 | 227 | 314 | 52 |
| WHO EML | 2° | 11 | 0 | 3 | 9 | 19 | 17 |
| | 3° | 10 | 49 | 26 | 44 | 59 | 15 |
| Top 200 | 2° | 7 | 1 | 0 | 8 | 8 | 14 |
| | 3° | 5 | 18 | 15 | 26 | 32 | 6 |

To explore the occurrence of tertiary amines with activating features (section 3.2; note that in this case 'activation' refers to the susceptibility to nitrosation, not to the mutagenic potency as in the previous paragraph), respective patterns were created in KNIME, again using nodes based on proprietary and internal Lhasa software. The workflow was then applied to all tertiary amine APIs and impurities previously identified in GSRS [7].

*Data analysis and Visualization*

Statistical data analysis and visualization were principally done with the R language for statistical computing version 4.1.1 [16] using the package ggplot2 version 3.3.5 [17]. Fig. 7 was generated in Python version 3.9.7 [18] using the package matplotlib version 3.4.3 [19]

## Results and Discussion

*Estimation of Potency/Mutagenicity of the Potential Nitrosamines*

Our re-analysis of the available data, which is summarized in Table 1 and Fig. 2, highlights the clear trend for complex nitrosamines derived directly from secondary amines to be of significantly reduced concern. Across each of the datasets analyzed the number of theoretical nitrosamines assigned to categories 1 and 2 can be seen to represent a small fraction of the total number. Across the USP dataset of 8611 APIs, only 99 structures featuring a secondary amine pose the risk of generating a nitrosamine which would require controlling to 18 ng, with ~2/3 of all the datasets categorized in the highest AI categories (Fig. 2). This is a significant change from the previous landscape, with the majority of predicted structures now possible to be controlled to the standard mutagenic TTC.

Furthermore, within the top 200 drugs by sales, only *N*-Nitrosovonoprazan was identified as a category 1 nitrosamine derived from the secondary amine of the drug itself, which would require controlling to 450 ppb within the DS and even lower in the DP where the API may represent a small fraction of the total mass (Fig. 3). In the case of *N*-nitrosovonoprazan, this structure was identified within some batches made by Phathom Pharmaceuticals and was subsequently reformulated to adhere to an FDA-defined 96 ng/day [21]. It is important therefore to reflect that while 18 ng may be a default value to start from, read across and other forms of data appropriate to limit setting may result in amended values. The assessment of the essential medicines list identified no nitrosamines within category 1 to be formed from secondary amines in the API structures themselves, however 3 structures were identified in category 2, including *N*-nitrososertraline and *N*-nitrosoepinephrine (Fig. 3). In the case of *N*-nitrosoepinephrine a 100 ng AI in the drug product (a 0.1 mg/ml solution of API) correlates to a concentration of just 1 ppb. At present the CPCA framework does not incorporate the less than lifetime approach, however this scenario, though certainly an extreme case,

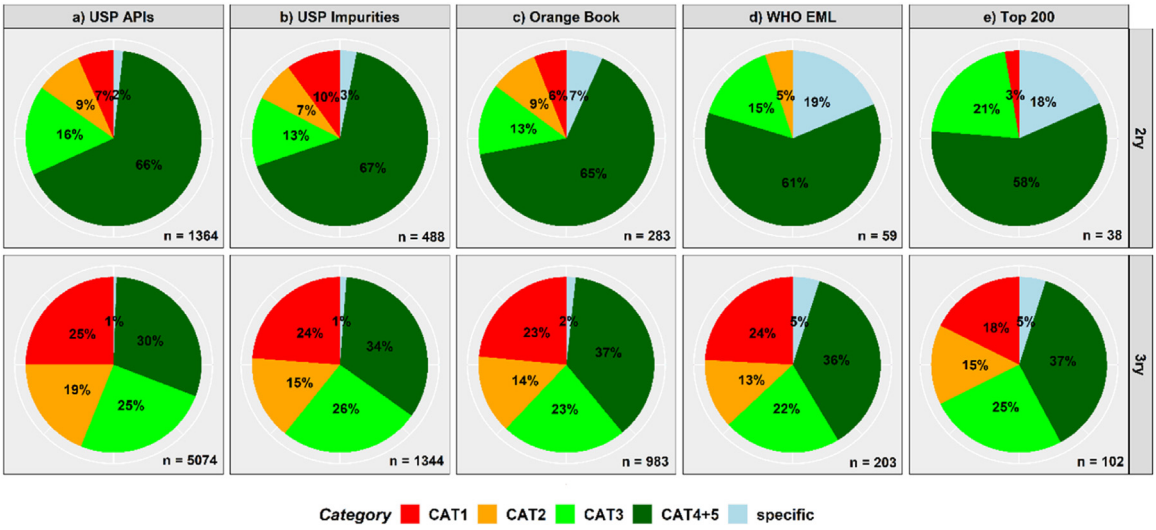


**Fig. 2.** Distribution of potential nitrosamines of small molecule drugs from GSRS to the CPCA potency categories. The numbers for categories 4 and 5 were combined as both categories call for the same AI of 1500 ng/day, which is the lifetime daily limit for regular mutagens defined in the ICH M7 guideline [20].

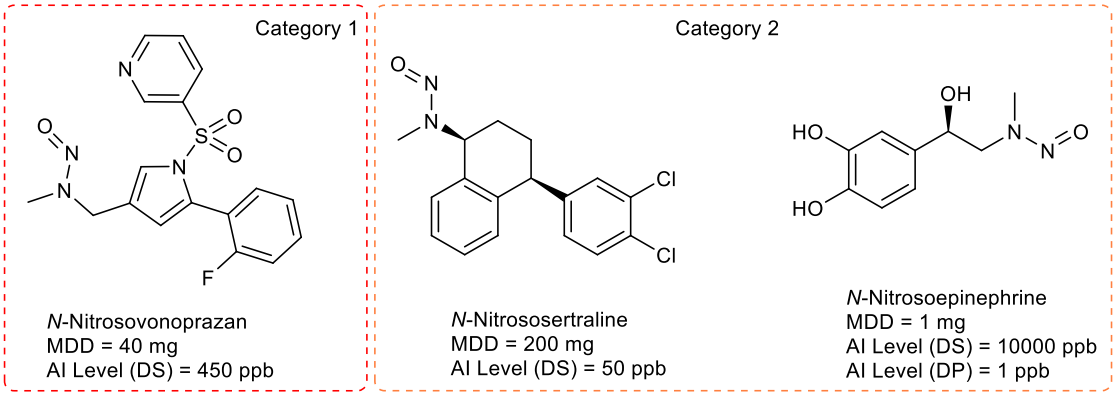


**Fig. 3.** Nitrosamines belonging to CPCA categories 1 and 2, derived from secondary amine APIs in the Top 200 drugs by sales (Vonoprazan) and WHO EML (Sertraline, Epinephrine).

clearly indicates the need to consider impact of attainable levels of control/detection for a 1 in 100,000 lifetime risk of cancer versus the acute treatment benefits.

Conversely, those NDSRIs derived from tertiary amines represent the majority of potential structures coupled with high levels of category 1 and 2 categorization. The reason for this can be attributed to the occurrence of small units such as $NMe_2$ and $NEt_2$ which may theoretically lose one of these groups to create a secondary amine possessing a methyl/ethyl group in addition to the other group. This orientation in many cases leads to an $\alpha$-hydrogen score of 1 which subsequently corresponds to a high potency in the absence of additional deactivating features.

### *Relevance of Potential Nitrosamines from Tertiary Amines*

It was recently demonstrated, both experimentally [22] and theoretically [23], that simple trialkyl amines pose a much lower nitrosamine risk than secondary amines, due to their differing reaction mechanisms. The reason is that secondary amines react directly with the nitrosating agent, whereas simple trialkyl amines require prior removal (dealkylation) of one of the residues on the amine, which takes place at a much lower reaction rate than the subsequent nitrosation. Exceptions to this include aromatic amines, which have been observed to dealkylate substantially faster than simple trialkyl amines [22]. It is also important to note that not all tertiary amines require initial dealkylation to generate a nitrosamine, with a direct nitrosation mechanism being proposed for structures featuring electron-rich (hetero)aromatic substituents, such as Ranitidine [24] and Gramine [25]. Therefore, there is an increased risk of nitrosamine formation compared to simple trialkyl amines for tertiary amines containing these activating features, i.e., one or two aryl substituents on the amine nitrogen or the presence of electron-rich (hetero)aromatic substituents (Fig. 4).

Reanalysis of the tertiary amines in the USP-API and USP impurities datasets revealed that only a few contain the relevant sub-structures that entail an increased nitrosation risk, and the majority of derived nitrosamines would fall under potency categories 3-5 (Fig. 5). A caveat to these data is that the features determining activation of benzyl-type tertiary amines are still relatively poorly understood, and hence the presented results only intend to be indicative of their frequency. The ability to donate electrons into the alpha position will be crucial, and therefore additional features such as aromatic ring fusions and other functionality affecting the electronics of the (hetero)aromatic ring will need, and indeed are being studied. If questions exist relating to the risk posed by a particular tertiary amine, where it is not clear if it is an activated tertiary amine or not, these may be resolved by a carefully conducted nitrosation experiment using sub stoichiometric nitrite [26]. Profiling of the reaction with a suitable detection limit makes it possible to determine that the reaction is >500 fold slower than would be expected for a dialkylamine such as diethylamine under the same conditions [22].

### *Relevance of Secondary Amine Impurities*

The nitrosative dealkylation of tertiary amine APIs within drug products is now believed to be a significantly smaller concern compared to secondary amines [23,27]. Whilst the nitrosative dealkylation pathway is far slower than previously feared, the presence of more reactive secondary amine impurities from API degradation or precursor chemicals will remain a risk. The identification threshold for impurities is at 0.1 % (1000 ppm, i.e., 1000 $\mu$g/g API) and 0.2 % of the API in API [28] and drug product [29], for maximum daily doses up to 2 g and 1 g, respectively. Therefore, unknown secondary amine impurities could be present in tertiary amine APIs with a content up to 2000 ppm. Fig. 6 evaluates the amount of nitrosamine that could be formed from 10-1000 ppm of secondary amine impurity in the API,

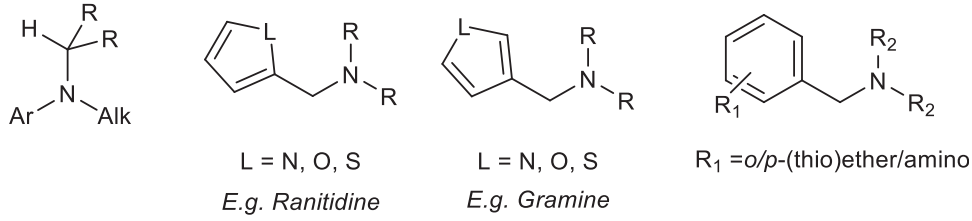


**Fig. 4.** Representative tertiary amine sub-structures considered more at-risk of nitrosation.

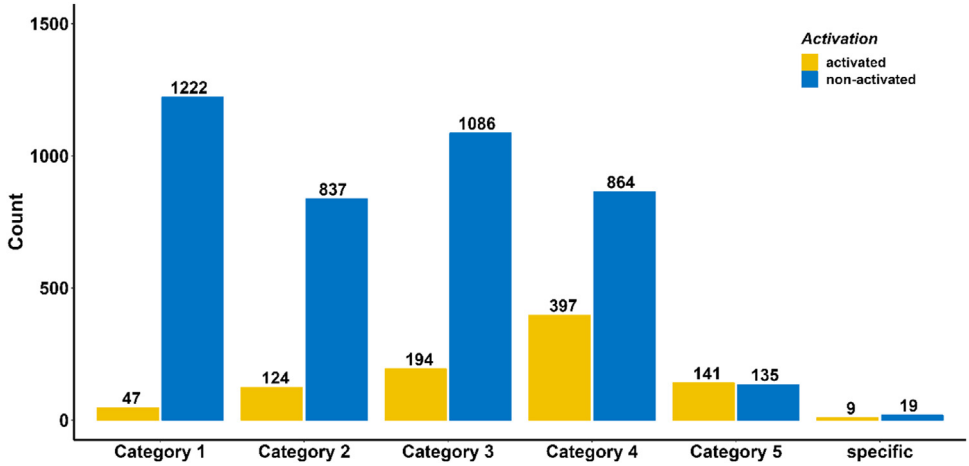


**Fig. 5.** Identification of Nitrosamines that could form from USP APIs containing tertiary amine sub-structures considered more at-risk of nitrosation.

considering 1 %, 10 % and 100 % conversion and maximum daily doses of 1 mg, 10 mg, 100 mg, and 1000 mg, respectively. Especially the higher API doses could contain relevant amounts of nitrosamine even if the content of the secondary amine is low and only a small fraction of it is transformed. For these reasons tertiary amine APIs should not by default be ruled out as a nitrosamine risk unless the presence of their secondary amine impurities has been disproved.

*Analytical Challenges*

To prove the absence of an NDSRI, analytical methods are required to have an LOQ at 10 % of the content limit derived from the NDSRI's acceptable intake and the API's maximum daily dose [10,30]. Quantification of an NDSRI has been estimated to be routinely achievable at levels down to 50-100 ppb (ng/g drug product; grey area in Fig. 7). However, in the case of NDSRIs for which control in categories 1-3 is required by the CPCA, this may not be sufficient, as the low acceptable intakes - in combination with even moderate allowed daily API doses - may result in LOQ requirements beyond technical feasibility. The analytical challenge may be exacerbated if the NDSRI co-elutes with the API due to high physicochemical similarity, or by the lack of robust reference standards. Where these do exist and co-elution with the API is not a concern, more sensitive results may be achieved; for example, it has been reported that NDMA can be quantified even

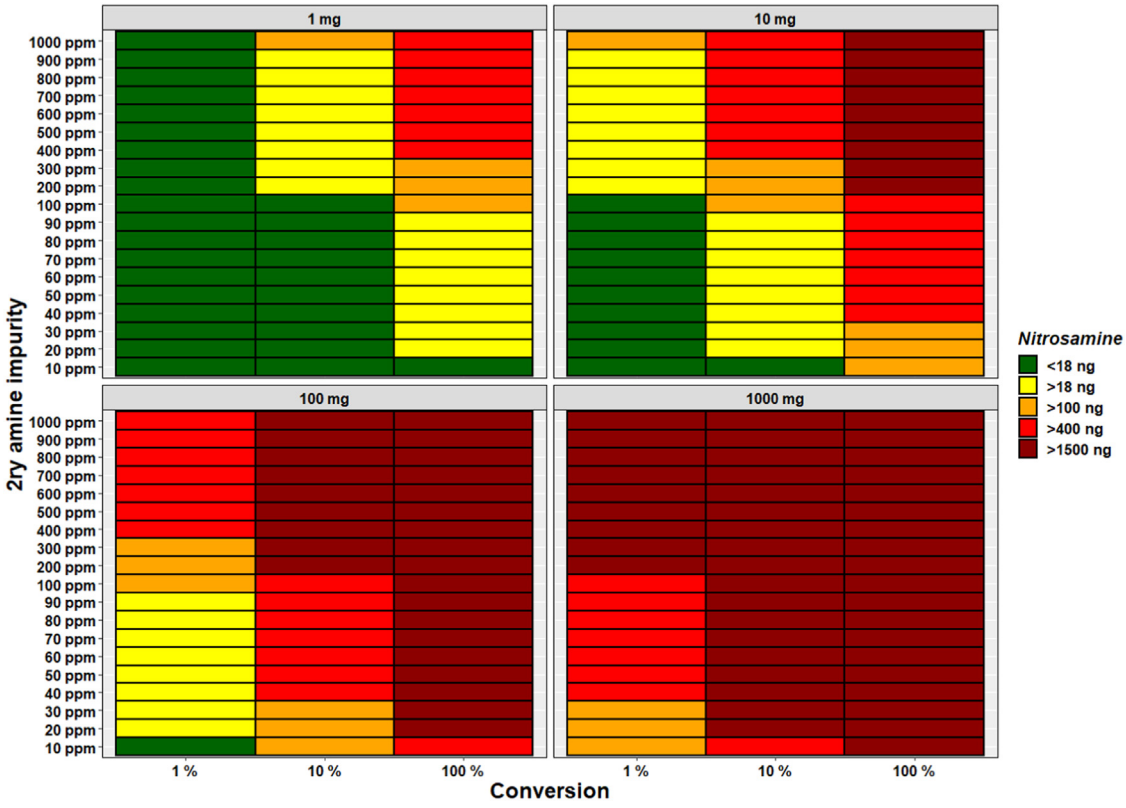


**Fig. 6.** Potential nitrosamine formation from secondary amine impurities. "2ry amine impurity" denotes the assumed content of the impurity in the API in $\mu$g/g. "Conversion" denotes the assumed percentage of the secondary amine being nitrosated. The calculations were performed for maximum daily API doses of 1 mg, 10 mg, 100 mg, and 1000 mg.

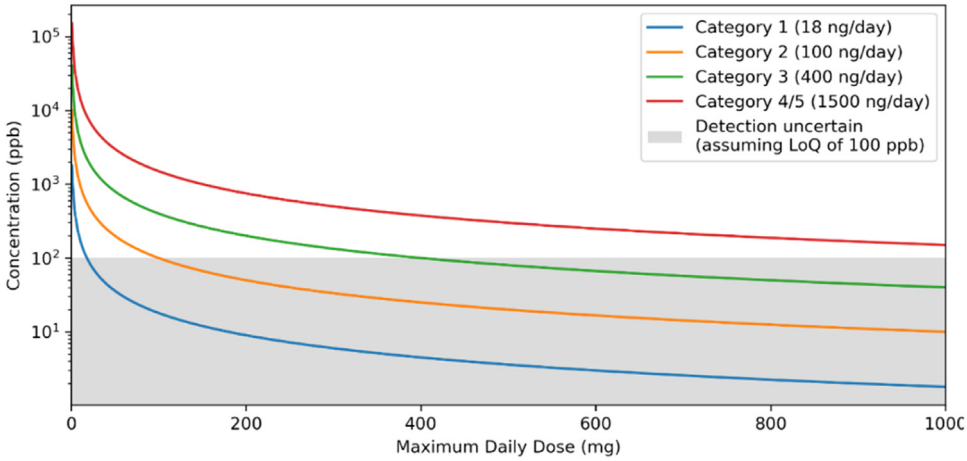


**Fig. 7.** Detection threshold (10 % AI) for Nitrosamine categories in APIs based on MDD of the API.

below 1 ppb [31], however this is emphatically an outlier, and impractical to reproduce on a daily basis in a routine QC laboratory.

## Conclusion

The latest update (Rev. 16) of the EMA Q&A document, which aligns with other Health Authorities, has substantially improved the nitrosamine situation for pharmaceutical manufacturers, as CPCA allows for the fast and straight forward derivation of acceptable intake limits for unknown nitrosamines without the need to perform *in vivo* carcinogenicity studies or wait for HA endorsement of read-across arguments. In addition, the list of established acceptable intakes was substantially extended including various Nitrosamines of calcium channel blockers, $\beta$-blockers and $\beta$-agonists, ACE inhibitors and nitroso HCTZ as proposed by EFPIA [32–35]. Looking at the sheer quantity of potential nitrosamines, it is unrealistic that Health Authorities could deduce and publish official AIs for all of them in a reasonable time frame. Furthermore, a negative result in an Enhanced Ames Test (EAT) now allows for control to the general threshold of toxicological concern (TTC) at 1500 ng/day (currently at least under EMA administration) – a true milestone considering the previous reservation towards the Ames test and its applicability to mutagenicity assessments for nitrosamines [8]. The CPCA framework in combination with EAT will allow the industry to prioritize the remediation of products based on the biggest risk to patients. Whilst secondary amines pose the highest risk of formation, they are more likely to lead to a higher CPCA category enabling easier control. Tertiary amines on the other hand are far less likely to nitrosate, yet account for the vast majority of potential CPCA category 1&2 compounds. It will be important to consider them but based on the data and rationales determining activating features, currently being explored/developed across industry.

There are, however, unresolved issues that still need to be addressed. A substantial number of potential nitrosamines are assigned to the lowest potency categories 1 and 2 at levels which may mean their parent APIs need to be removed from the market unless limits derived from other approaches (e.g., read-across) can be derived, as control to the required low levels could be extremely difficult. Further to the above observations there are also a number of other areas which remain unresolved with respect to the EMA Q&A, such as the introduction of the EAT creates uncertainty regarding the usability of preexisting Ames data created under non-EAT conditions. The current OECD protocol does not foresee the inclusion of nitrosamine positive controls, furthermore the use of induced hamster S9 may be detrimental for some compounds. The recommended EAT protocol conditions were mainly informed by work conducted by FDA's National Center for Toxicological Research [36]. However, several international working groups (e.g., HESI-GTTC, EMA-MutaMind) and industry are still working on optimizing the Ames protocol aiming to identify the most robust conditions allowing a further refinement of the EAT protocol.

It is clear that much of the framework has been built upon the vast amounts of research that have taken place over the last five years to gain understanding of many aspects of nitrosamines, across both toxicological and chemical domains [8,37–39]. It is inevitable that differences of opinion will remain and positions evolve as further studies are conducted, but it is testament to those involved that the channels of scientific discourse between regulatory, industrial, not-for-profit and academic institutions have enabled such a significant step forwards.

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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.

## CRediT authorship contribution statement

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