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

Joerg Schlingemann[a,1,*], Michael J. Burns[b,1,*], David J. Ponting[b], Carolina Martins Avila[b,f], Naiffer E. Romero[c], Mrunal A. Jaywant[c], Graham F. Smith[d], Ian W. Ashworth[e], Stephanie Simon[a], Christoph Saal[a], Andrzej Wilk[c]

[a] *Merck KGaA, Frankfurter Str. 250, 64293 Darmstadt, Germany*
[b] *Lhasa Limited, Granary Wharf House, 2 Canal Wharf, Leeds, United Kingdom*
[c] *U.S. Pharmacopeia, 12601 Twinbrook Parkway, Rockville, MD, USA*
[d] *AstraZeneca, Data Science and AI, Clinical Pharmacology and Safety Sciences, R&D, Cambridge, United Kingdom*
[e] *Chemical Development, Pharmaceutical Technology & Development, Operations, AstraZeneca, Macclesfield, United Kingdom*
[f] *Current affiliation: Sai Life Sciences Limited, Basement A, Block 33, Alderley Park, Macclesfield, United Kingdom*

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ABSTRACT

This article reports the outcome of an *in silico* analysis of more than 12,000 small molecule drugs and drug impurities, identifying the nitrosatable structures, assessing their potential to form nitrosamines under relevant conditions and the challenges to determine compound-specific AIs based on data available or read-across approaches for these nitrosamines and their acceptance by health authorities. Our data indicate that the presence of nitrosamines in pharmaceuticals is likely more prevalent than originally expected. In total, 40.4 % of the analyzed APIs and 29.6 % of the API impurities are potential nitrosamine precursors. Most structures identified through our workflow could form complex API-related nitrosamines, so-called nitrosamine drug substance related impurities (NDSRIs), although we also found structures that could release the well-known small and potent nitrosamines NDMA, NDEA, and others. Due to common structural motifs including secondary or tertiary amine moieties, whole essential drug classes such as beta blockers and ACE inhibitors are at risk. To avoid the risk of drug shortages or even the complete loss of therapeutic options, it will be essential that the well-established ICH M7 principles remain applicable for nitrosamines and that that the industry and regulatory authorities keep an open communication not only about the science but also to make sure there is a good balance between risk and benefit to patients.



## Introduction

The recent discovery of small-molecule nitrosamine impurities in marketed drugs, starting with *N*-nitrosodimethylamine (NDMA) in batches of Valsartan in 2018, has led to significant regulatory response, including drug recalls and regulatory guidance that requires the re-evaluation of all synthetic and formulation routes for the potential presence of nitrosamine impurities.[1]

Due to the wide range of potential routes of formation for nitrosamines, many active pharmaceutical ingredients (APIs) and impurities are themselves liable to be nitrosated, either during the later stages of the synthetic process of the API, during drug product manufacturing, or in the finished and packaged drug product. Several recent drug recalls were conducted due to contamination with such API-derived complex nitrosamines, also called Nitrosamine Drug Substance Related Impurities (NDSRIs) (Fig. 1), e.g., Nitroso-Varenicline,[2,3] Nitroso-Propranolol,[4] Nitroso-Orphenadrine,[5] and Nitroso-Quinapril.[6] Nitrosamines are of concern as some of them have been reported to be potent rodent mutagens and carcinogens and have been categorized as probable or possible carcinogens by the WHO IARC. Because of this higher potency, nitrosamine impurities are considered to be members of the “cohort-of-concern” according to the ICH M7 guideline,[7] and need to be controlled at or below compound-specific limits. These might be much lower as compared to the limit of 1.5 $\mu$g/day acceptable intake (AI) for other potentially

* Corresponding authors.
*E-mail address:* joerg.schlingemann@merckgroup.com (J. Schlingemann).
[1] These authors contributed equally to this work

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**Nonstandard abbreviations**

| | |
|---|---|
| AI | Acceptable Intake |
| API | Active Pharmaceutical Ingredient |
| DMA | Dimethylamine |
| DP | Drug Product |
| EDQM | European Directorate for the Quality of Medicines |
| EMA | European Medicines Agency |
| EML | Essential Medicines List |
| FDA | U.S. Food and Drug Administration |
| GSRS | Global Substance Registration System |
| HA | Health Authority |
| HC | Health Canada |
| HSA | Health Sciences Authority Singapore |
| IARC | International Agency for Research on Cancer |
| ICH | International Council for Harmonisation of Technical Requirements for Pharmaceuticals for Human Use |
| MDD | Maximum Daily Dose |
| MeNP | 1-Methyl-4-nitrosopiperazine |
| MFDS | Ministry of Food and Drug Safety Korea |
| NCATS | National Center for Advancing Translation Science |
| NDEA | N-Nitrosodiethylamine |
| NDBA | N-Nitrosodibutylamine |
| NDMA | N-Nitrosodimethylamine |
| NDIPA | N-Nitrosodiisopropylamine |
| NDSRI | Nitrosamine Drug Substance Related Impurity |
| NEIPA | N-Nitrosoethylisopropylamine |
| NIH | National Institute of Health |
| NMBA | N-nitroso-N-methyl-4-aminobutyric acid |
| NMT | Not more than |
| Ph.Eur. | European Pharmacopoeia |
| RSD | Residual Standard Deviation |
| $S_N1$ | First order nucleophilic substitution |
| $S_N2$ | Second order nucleophilic substitution |
| $S_NAr$ | Nucleophilic aromatic substitution |
| SST | System Suitability Test |
| USP | United States Pharmacopoeia |
| WHO | World Health Organization |

mutagenic impurities which lack carcinogenicity data, as defined by ICH M7 if treatment lasts more than 10 years. Due to the lack of compound-specific limits for most of the complex, e.g., API-related, nitrosamines and the absence of an effective process to establish AIs for NDSRIs, a class-specific AI of 18 ng/day for nitrosamines is required by many Health Authorities as a precautionary approach.

This article reports the outcome of an *in silico* analysis of more than 12,000 small molecule drugs and drug impurities, identifying the nitrosatable structures, assessing their potential to form nitrosamines under relevant conditions and the challenges to determine compound-specific AIs based on data available or read-across approaches for these nitrosamines and their acceptance by health authorities.

*Amines as N-Nitrosamine Precursors*

In this section, we give a brief introduction to the different types of amines and discuss if they can act as nitrosamine precursors.

*Primary Amines*

Primary amines are no nitrosamine precursors. The nitrosation of an aliphatic primary amine yields an alkyl diazonium ion and water, not a nitrosamine. The alkyl diazonium ion is very reactive and will, e.g., form a hydroxyl compound and release $N_2$ in a reaction with water. The nitrosation of an aromatic primary amine yields an aryl diazonium ion, which is generally more stable than its alkyl counterpart and might be mutagenic. However, aryl diazonium ions are not part of the cohort of concern.

*Secondary Amines*

Secondary amines can be converted to nitrosamines following the reaction schema depicted inFig. 2. The reactivity depends on the basicity of the secondary amine. For simple aliphatic amines, nitrosation involves nucleophilic attack of the amine lone pair to the electrophilic nitrosating agent, thereby necessitating the free-base form of an amine to enable nitrosation to occur. This reaction cannot occur if the secondary amine is protonated as in this case the lone pair is not available for reaction with the nitrosating species. Given the acidic nature of conditions required to generate the active nitrosating species, the protonation state of the amine has an important bearing on the rate of nitrosation.[8] Less basic amines are easier to nitrosate as there will be a lower fraction in the protonated state at an acidic / neutral pH compared to more basic amines. In the case of secondary aromatic amines, the mechanism becomes more complicated, with $\pi$-orbital interactions suggested as alternative initiating event. Therefore, changes in steric and electronic properties of the aromatic ring may impact susceptibility to nitrosation.[9]

*Tertiary Amines*

Tertiary amines can undergo nitrosation as well. This reaction is referred to as nitrosative cleavage[8] or nitrosative dealkylation.[10] In principle, three different nitroso-compounds can be formed from each unsymmetrical tertiary amine. However, the reaction necessitates the presence of at least one proton in the alpha-position to the amine nitrogen to enable the dealkylation pathway via an iminium intermediate (Fig. 3).[8,11]

The kinetic data available for the nitrosative dealkylation of tertiary aliphatic amines suggest that the reaction rate is 2-3 orders of

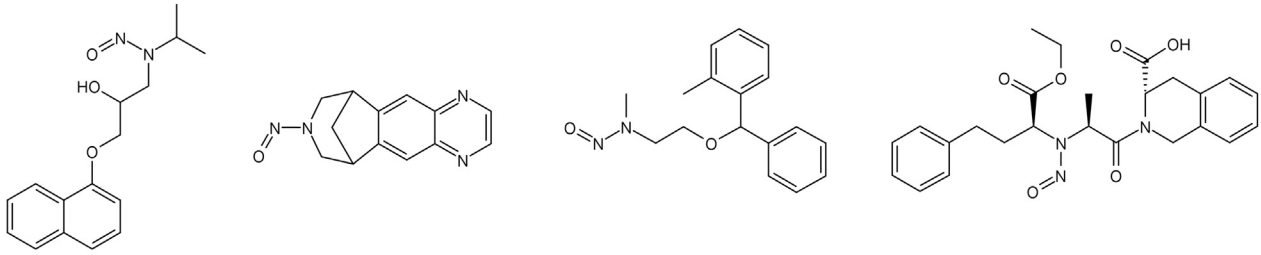



**Figure 1.** NDSRIs responsible for recent drug recalls.

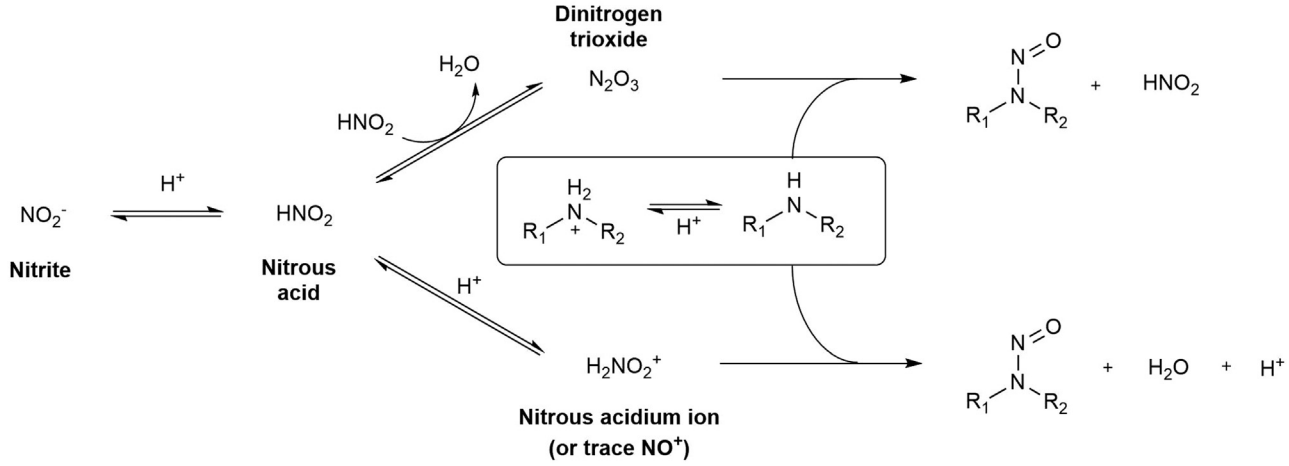



**Figure 2.** Nitrosation of a secondary amine.

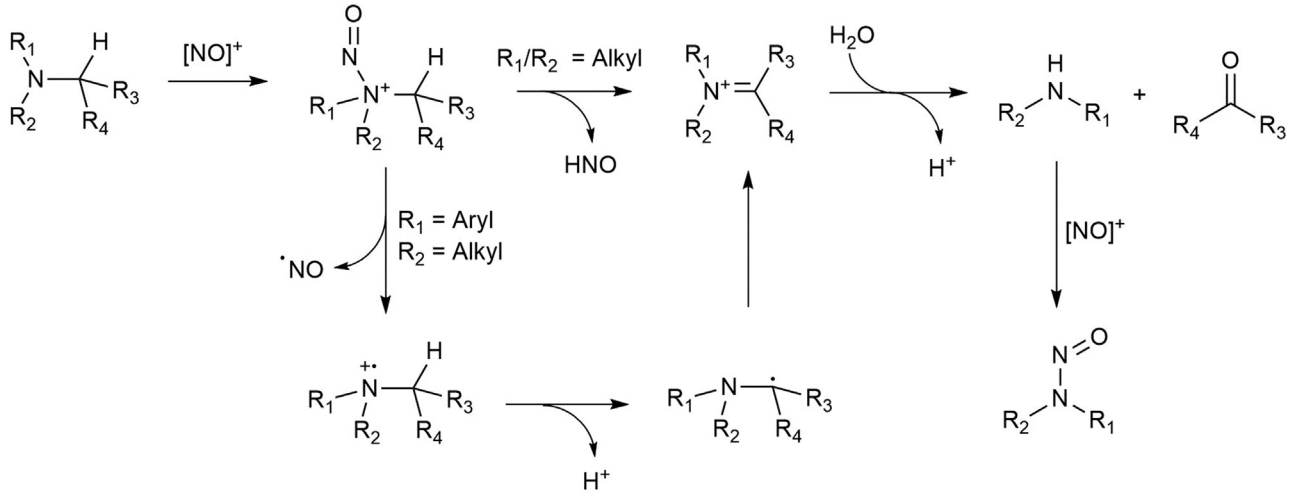



**Figure 3.** Nitrosative cleavage of a tertiary amines.

magnitude slower than nitrosation of secondary amines.[12] This reduces but does not exclude the risk of accumulating relevant amounts of nitrosamines derived from a tertiary amine. Additionally, dealkylation of dialkyl aromatic amines is faster than that of trialkyl amines,[13] and other structural features may enable release of a nitrosamine directly from the nitrosoammonium species (Fig. 4).[14] In any situation where electronic properties proximal to a nitrosoammonium could rationally describe such elimination of a nitrosamine, these structures would represent a different class of risk, as they only require one equivalent of nitrite, akin to secondary amines.

The nitrosative cleavage of a cyclic tertiary amine can result in the release of a cyclic amine with the non-connected residue released as an aldehyde or ketone, or in a “ring-opening”, with the aldehyde/ketone on either of the previously connected residues (Fig. 5).

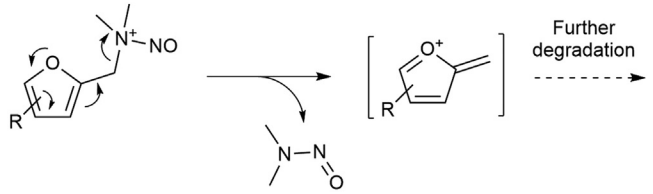



**Figure 4.** Direct release of nitrosamine from a (2-furanylmethyl)dimethylamine species.

*Tertiary Amines Containing a Nitro Group.* The presence of NDMA in some ranitidine products was shown to be linked to a slow degradation of the ranitidine molecule.[15] Whilst the tertiary amine would present a risk of nitrosamine formation in the presence of nitrite, in this case NDMA is formed directly from an intermolecular reaction of ranitidine hydrochloride without involvement of impurities or additional nitrosating agents. Ranitidine is a tertiary amine with a dimethylamine group at one position and a nitro group at another position of the molecule. The nitro group of one ranitidine molecule can act as nitrosating agent precursor, while the tertiary amine part of another molecule provides the dimethylamine for the formation of NDMA in an intermolecular reaction.[15] To assess compounds with similar risks, we have screened available small molecule drug data (see 2.1) for the presence of structures that contain both a tertiary amine functionality and a nitro group as the basis for a similar nitrite-independent N-nitrosamine formation.

*Quaternary Ammonium Salts*

Quaternary ammonium compounds are mentioned as potential nitrosamine precursors in the current regulatory guidance.[16,17]

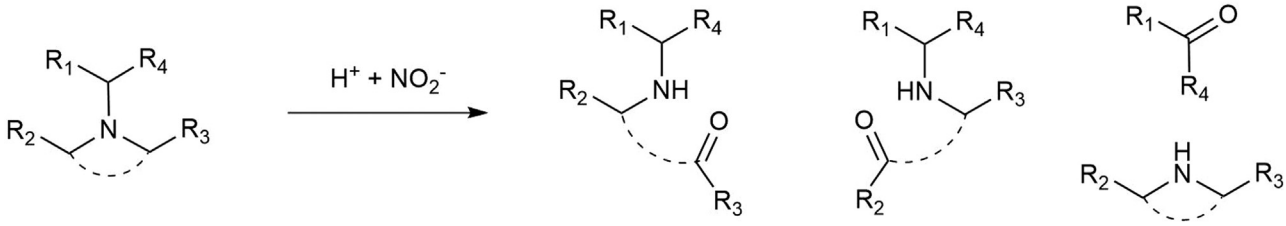



**Figure 5.** The nitrosative cleavage of a cyclic tertiary amine can result in the release of the non-connected residue as an aldehyde/ketone, or in a "ring-opening", with the aldehyde/ketone on either of the previously connected residues.

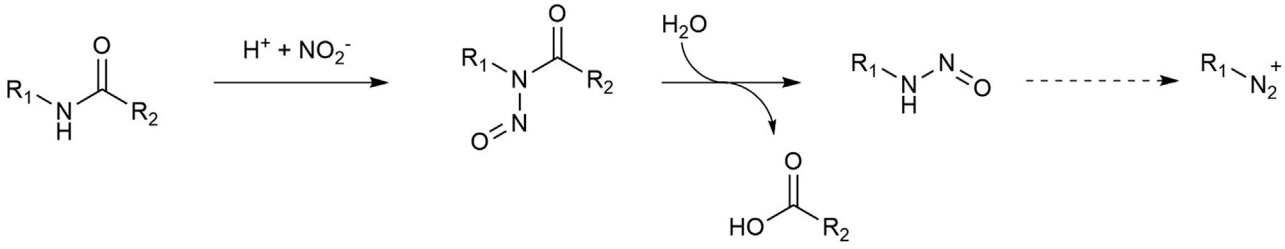



**Figure 6.** Nitrosation of a secondary amide yields an N-nitrosamide.

However, they cannot be nitrosated directly and would need to undergo non-nitrosative dealkylation to yield a nitrosatable tertiary amine which can yield nitrosamines as described in section 1.1.3. Since quaternary ammonium compounds are made from tertiary amine precursors, the latter could also be present as impurities. For this *in silico* exercise we rely on the fact that our data set (see 2.1) includes such nitrosatable tertiary amines as known impurities.

*Amides as* N-*Nitrosamine Precursors*

More than a third of the evaluated structures contain secondary and/or tertiary amides, most of them of the simple aliphatic or aromatic type. In addition, a wide variety of APIs are secondary or tertiary sulfonamides, e.g., sulfonamide antibiotics, anti-diabetic sulfonylureas, some diuretics such as hydrochlorothiazide, as well as many anti(retro) viral protease, transcriptase, or polymerase inhibitors. Secondary or tertiary amidine type amides are found in the biguanidine class of hypoglycemic agents, e.g., metformin. Thioamides such as thiamazole are used to treat hyperthyroidism. Where nitrosation occurs, the nitrosated amide may undergo hydrolysis to release a carboxylic acid and primary nitrosamine (Fig. 6). If this occurs *in vivo*, mutagenic diazonium ions ($R\text{-}N_2^+$) could form in proximity to DNA. However, *ex vivo* the primary nitrosamine is unlikely to persist due to poor stability. Additionally, secondary amides are not easily nitrosated, due to the electron withdrawing properties of the amide oxygen. We therefore decided not to consider this pathway in our evaluation and excluded secondary amides as nitrosamine precursors.

Tertiary amides cannot be nitrosated directly, requiring prior hydrolysis, which creates a carboxylic acid and a secondary amine (Fig. 7). The secondary amine would then be nitrosatable as per Fig. 2. However, amides are typically quite resistant to hydrolysis, which is only achieved by heating under acidic or basic conditions for extended periods. An exception are so-called twisted amides, in which the amide bond N-C=O is not fully planar and the bond stabilization by resonance is reduced.[18,19] Examples for twisted amides are beta-lactams (penicillin type antibiotics) and the quinuclidine moiety, which is part of numerous approved APIs such as quinine, palonosetron, cevimeline, benzoclidine, azasetron, solifenacin, and quinupramine.[20] For these structures our analysis relies on the assumption that for any easily hydrolysable tertiary amide the secondary amine hydrolysis product will be included as an impurity in the database we have used (see section 2.1).

*Structures Out of Scope of This Manuscript - Non-Basic and Basic Aromatic Rings*

Examples for non-basic and basic aromatic rings are provided in Fig. 8. Chemically, they are not amines as the nitrogen atom is

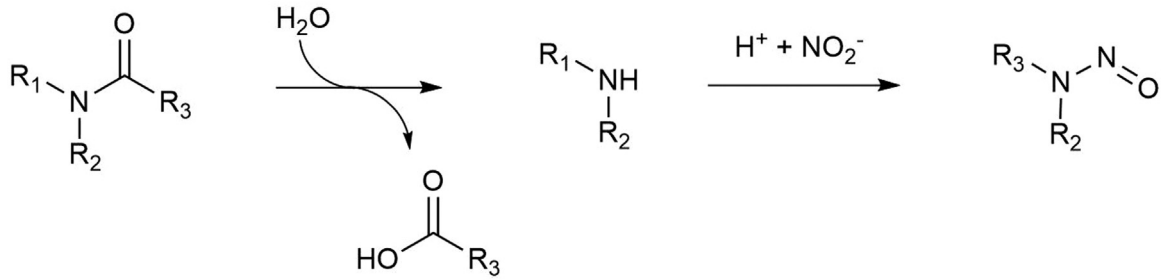



**Figure 7.** Tertiary amides could form nitrosamines via hydrolysis and subsequent nitrosation of the resulting secondary amine.

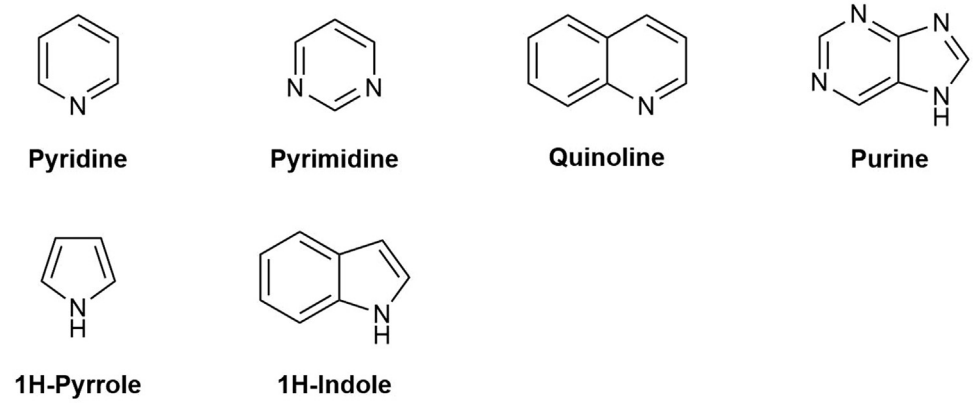



**Figure 8.** Examples for Nitrogen-containing aromatic rings. First row: basic aromatic rings*; these are not tertiary amines. Second row: non-basic aromatic rings. These are not secondary amines. *Purine has both a basic and a non-basic aromatic ring.

contained within the aromatic system and hence cannot form *nitrosamines*.

Where nitrosation occurs of non-basic aromatic rings, such as pyrrole, an *N*-nitroso-heteroaromatic compound is formed. Whilst these compounds may be mutagenic, the mechanism will not rely on $\alpha$-hydroxylation and C-N cleavage as this would require breaking the aromaticity of the ring. As such any toxicity is likely to occur through a non-cohort of concern mechanism with available data suggesting they may behave comparably to other *C*-nitroso aromatic compounds.

Nitrosation of basic aromatic rings can only be achieved by excess of the strong nitrosating agent nitrosonium tetrafluoroborate, whereas an excess of the ring leads to its cleavage.[21] As for nitrosated non-basic rings, potential mutagenicity cannot be due to alpha hydroxylation and C-N cleavage.

### *Estimating* N-*Nitrosamine Carcinogenic Potency Via Structure-Activity Relationships*

For most potential nitrosamines identified in the following no experimental carcinogenicity data is available, and what data exists is typically related to low molecular weight N-nitrosamines rather than the NDSRIs identified. As a result, the derivation of acceptable intake limits for these compounds cannot be performed without some method of extrapolation from the data that is available. As discussed, Health Authorities have set a class-specific limit of 18 ng/day for all N-nitrosamines; however, the general use of this limit for all NDSRIs sets significant analytical and practical challenges. As a result, the use of read-across – the assumption that the single most structurally-similar nitrosamine with reliable data is of similar potency and a limit comparable to that nitrosamine is protective – can be used. Understanding which structural features impact the potency of the nitrosamine requires a thorough analysis of the effects of the varying structural features.

To estimate their carcinogenic potency, we used structure-activity relationships, as previously described.[22] Briefly, the immediate vicinity of the *N*-nitroso substructure influences the chemical reactions that the nitrosamine undergoes in a biological context. In some cases, such effects can be strong enough to be statistically significant. The nitrosamine must be metabolically activated by cytochrome P450 enzymes – CYP2E1 for the smallest nitrosamines, such as NDMA and NDEA, but a wide variety of Cytochromes are involved even for relatively small nitrosamines.[22–24] Despite this wide variety of metabolizing enzymes, the biotransformation induced is the same; hydroxylation of the $\alpha$-carbon-atom, adjacent to the N-nitroso group, leading to a subsequent series of non-enzymatic reactions that lead, ultimately, to an alkyldiazonium ion. Alkyldiazonium ions can interact with DNA either via a $S_N1$ reaction (loss of nitrogen ($N_2$) and subsequent reaction of the carbocation with DNA) or a $S_N2$ reaction, direct displacement of nitrogen by a nucleophile on the DNA. Aryl diazonium ions cannot undergo either $S_N1$ or $S_N2$ and thus a diazo adduct is initially formed (C in Fig. 9), with potential subsequent rearrangement via $S_NAr$, and for cyclic diazonium ions an intramolecular rearrangement may result in the ultimate alkylating agent being an oxonium ion rather than diazonium or carbocation (B in Fig. 9). However, the metabolic activation step is consistent no matter the class of nitrosamine.

Since these two key steps can be described as covalent chemical reactions, the usual considerations of steric and electronic interactions can be addressed. Those functional groups that, through steric hindrance or electronic destabilization of intermediates and transition states, slow the rate of the activating reaction – for both metabolism and alkylation steps – lead ultimately to a reduction in carcinogenic potency. If the potency is sufficiently reduced, it cannot be measured anymore (see sections 2.4 and 3.3 for further discussion of these effects). On the other hand, groups that increase reaction rate via the stabilization of transition states, or particularly unhindered groups such as methyl, result in an increase in potency. As a result of these observations, derived from analysis of available carcinogenicity data, a series of structural features have been developed.[22,25] These can be used to support read-across and structure-activity relationships since they capture the effects of steric hindrance and electronic interaction described.

In a biological system, there are additional complications to be addressed. Critically, the nitrosamine must reach the cytochrome P450 enzymes discussed, and the formed intermediate must reach DNA in sufficient abundance. Even for potent nitrosamines, it is expected that the vast majority of diazonium ions generated are ultimately quenched by reaction with freely-available water yielding alcohols and nitrogen– not to induce mutations at a rate that exceeds the repair capacity of the cell. The latter is harder to quantify - a conservative assumption it is considered that, if a diazonium ion is formed, it will reach DNA. However, the distribution and metabolism do lead to significant effects on carcinogenic potential. Those nitrosamines which do not either penetrate cells or, *in vivo*, distribute to metabolically competent organs such as the liver, cannot be activated metabolically or react with DNA in the nucleus of a cell.

This biological complexity implies that several factors, in addition to the structural features alone, must be considered when selecting an analogue from which to derive an AI limit. These include both similarity of key physicochemical parameters (molecular weight, log P, solubility) that affect absorption, distribution and clearance and

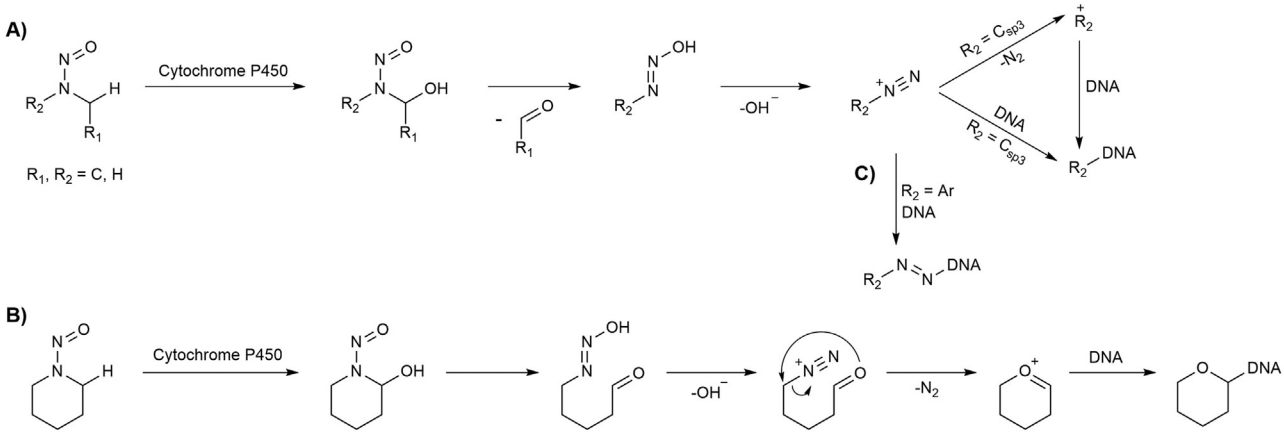



**Figure 9.** A) metabolic activation and DNA adduct formation of nitrosamines. B) potential alternate route for cyclic nitrosamines. C) Diazo adduct-forming reaction step for alkylaryl nitrosamines.

similarity of metabolic profile (predicted or experimentally-determined) for the molecule - if the parent drug is principally excreted unchanged or metabolized by non-CYP enzyme families, the $\alpha$-hydroxylation may be of lower concern.

## Materials and Methods

The overall data flow is described in Fig. 10.

*Small Molecule Drug Data from GSRS*

The Global Substance Registration System (GSRS) is a registration system for the ingredients in medicinal products.[26] The system was created in collaboration by FDA's health informatics team, NIH's National center for advancing translation science (NCATS) and the European Medicine Agency (EMA).[27] GSRS provides a system for the definition and identification of substances within medicinal products or substances used for medicinal purposes. The system also captures relationships between substances and references all captured data to a definitive source of information. The GSRS knowledge base makes it possible for substances to be defined by standardized, scientific descriptions. It classifies substances as chemical, protein, nucleic acid, polymer, structurally diverse, or mixture as detailed in the ISO 11238.[28]

To address emerging informatic needs, the United States Pharmacopoeia (USP) is collaborating with the FDA and NCATS to leverage the capabilities of the GSRS. Under a cooperative research and development agreement (CRADA) with the FDA, USP is contributing drug impurity information that can be leveraged by digital platforms to programmatically query impurity profiles for APIs. USP has installed an internal instance of the GSRS platform that can be used to provide a source of truth for common chemical data elements (molecular weight, structures, nomenclature, etc.) for USP publications, such as the USP Dictionary of USAN Names. A comprehensive list of small molecules structures was obtained from this USP's internal GSRS system.

In this study, the GSRS database was used for extracting APIs and API related impurities and degradants as reported to be linked to API in the database. This resulted in 12,175 structures which were evaluated. Some numerical results are also provided

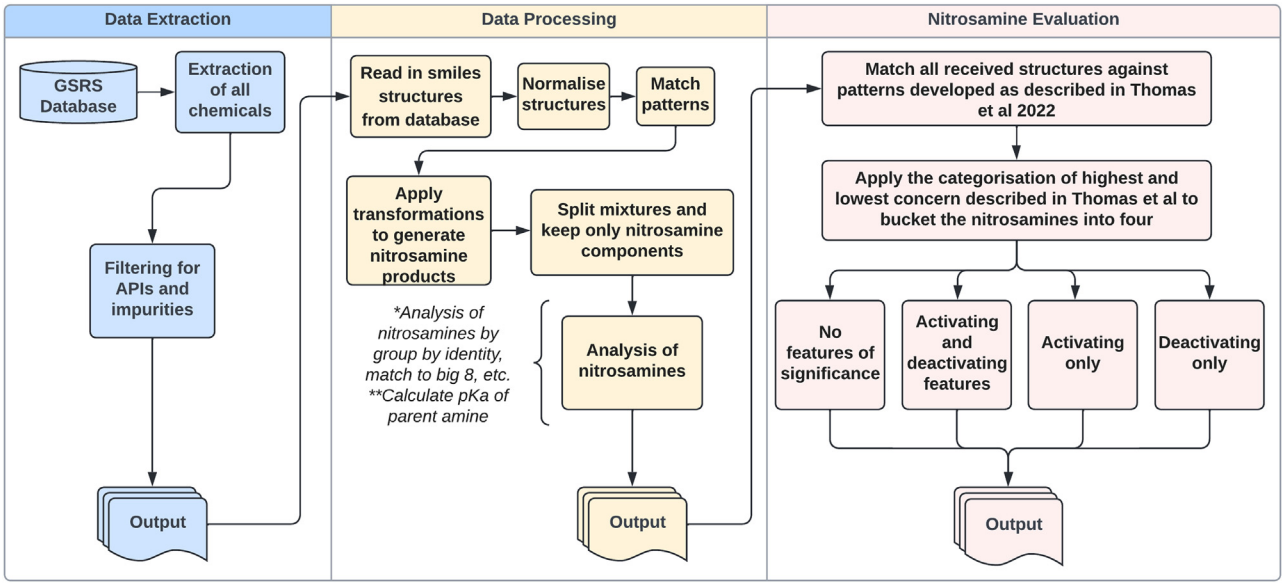



**Figure 10.** Data flow.

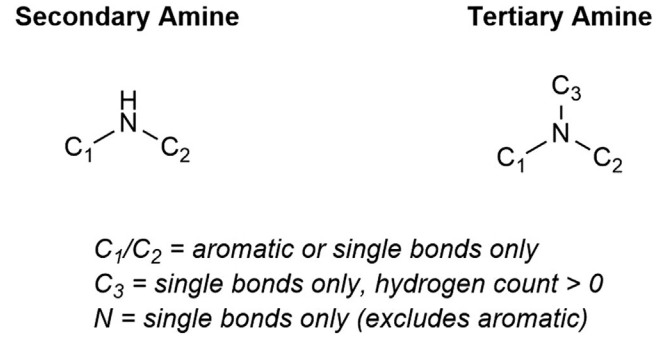



**Figure 11.** Rules applied for the identification and extraction of secondary and tertiary amines. For secondary amines, two carbons and one hydrogen must be bound to the amine nitrogen, and both carbons may only have single or aromatic bonds. This excludes enamines and amides. The bonds to amine nitrogen must be single bonds. This excludes nitrogen-containing aromatic rings (see Fig. 8). For tertiary amines, three carbons must be bound to the amine nitrogen. Two of them may only have single or aromatic bonds, while the third must only be single-bonded and be attached to at least one hydrogen. This hydrogen is required to allow for nitrosative cleavage of the residue (see Fig. 3).

for subgroups in the database, i.e., the FDA Orange Book of approved drugs as an example of an extensive formulary,[29] the WHO List of Essential Medicines,[30] and the US Top 200 small molecule drugs by sales in 2021.[31]

*Identification of Secondary and Tertiary Amines*

Secondary and tertiary amines as potential precursors of nitrosamines were extracted by matching substructure patterns in KNIME 4.5 (https://www.knime.com), using the module "Ceres" [32] according to the rules shown in Fig. 11. For this study these rules excluded enamine functionality for the amines due to the potential for tautomerization to form the imine.

Having identified the structures possessing a vulnerable amine, a transformation was applied to generate each nitrosamine that could be formed from the parent structure, correlating to the mechanisms of nitrosation and nitrosative dealkylation. Up to three different nitrosamines can be formed from each unsymmetrical tertiary amine, if all three residues are bound to at least one hydrogen. Where multiple vulnerable amines (secondary and/or tertiary) existed within the same structure, only first-generation nitrosamines were considered. The low concentration of nitrite makes multiple nitrosations highly unlikely, in the same way that nitrosative dealkylation of a tertiary amine has a significantly lower likelihood than nitrosation of a secondary amine.

*Calculation of pKaH Values*

The susceptibility of amines to nitrosation relates to the basicity of the amine nitrogen. Higher basicity means that the free electron pair more readily accepts a proton, preventing the nitrosation as described above. The basicity of an amine can be expressed as the pKa of its conjugate acid, i.e., the pKaH. The higher the pKaH, the weaker is the acid and stronger the conjugate amine base, i.e., the lower is its susceptibility to nitrosation. To understand trends in the pKaH of the parent secondary amine, the pKaH was calculated at the site of nitrosation, rather than the for the most basic sites within the molecule (Fig. 12).

In the case of tertiary amines, each possible dealkylation was applied and the pKaH of the resulting amines predicted. Whilst basicity will also affect propensity to dealkylate, these values were not calculated, with the focus instead on the key nitrosating step. For the calculation of pKaH values of the amine conjugate acids we used the method recently described by Plante *et al.*[33] The suitability of the approach was validated by comparison to published experimental data.[34–37]

The data shown in Table 1 indicates a good correlation between predicted and experimental values for a small subset of amines, with a tendency to underpredict for the simple aliphatic amines. Given an underestimation of the basicity corresponds to an overestimation of the nitrosation potential, we decided to accept this limitation and use the algorithm for the purpose of this in-silico exercise. Only in the case of bis(p-nitrophenyl)amine ($(p\text{-}NO_2\text{-}Ph)_2NH$) the pKaH is clearly overpredicted. However, since both experimental and predicted pKaH are strongly negative and only few amine precursors of nitrosamines with potency reducing features have pKaHs in this range this discrepancy is not relevant.

*Nitrosamine Evaluation - Potency Categorisation*

The substructural classes described earlier[22,38] have been implemented as Ceres patterns[32] and pattern matching was performed against these in KNIME (KNIME 4.5, www.knime.org) in a manner analogous to the matching performed for the nitrosamine formation described above. The dataset thus developed was analyzed to flag which of four categories the nitrosamine concerned could be fitted into:

- Nitrosamines with features that are expected to lead to increased potency (e.g., methyl/ethyl substituents and those such as beta-ketones that stabilize the transition state in the metabolic activation step[22,25,39]
- Nitrosamines with decreased potency (e.g., steric hindrance such as isopropyl and tert-butyl groups, or effects on DMPK properties such as carboxylic acids[22,25,39]
- Nitrosamines with no features from either list

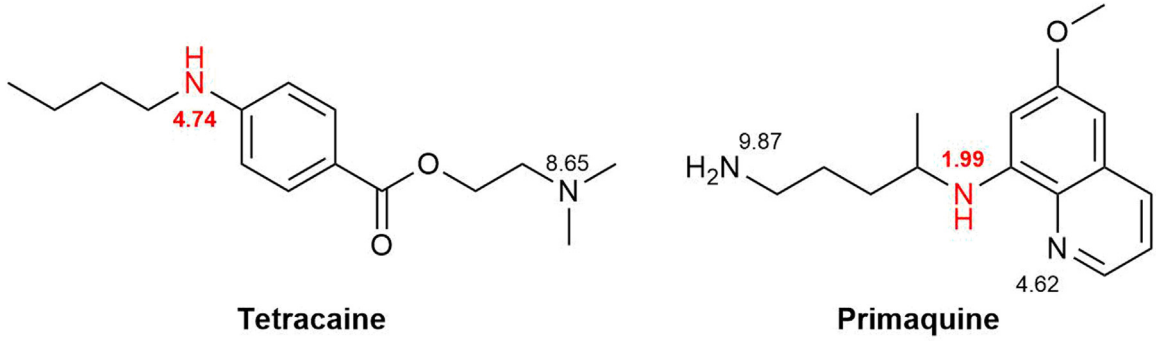



**Figure 12.** Representative examples of amine pKaH calculations in molecules with multiple basic sites.

**Table 1**
Predicted vs experimentally determined pKaH values for exemplary amines, spanning a range of -6.2 – 11.1.

| Amine | CAS No. | Predicted pKaH | Experimental pKaH | Δ pKaH (pred vs exp) |
|---|---|---|---|---|
| DIPA[34] | 108-18-9 | 10.6 | 11.1 | −0.6 |
| DEA[34] | 109-89-7 | 10.6 | 10.0 | −0.4 |
| DMA[34] | 124-40-3 | 10.1 | 10.6 | −0.5 |
| EIPA[35] | 19961-27-4 | 10.6 | 10.8 | −0.2 |
| 4-Me-Amino BA[35] | 1119-48-8 | 10.3 | 10.7 | −0.4 |
| Morpholine[34] | 110-91-8 | 8.1 | 8.4 | −0.3 |
| Ph(Me)NH[34] | 100-61-8 | 4.8 | 4.9 | −0.1 |
| $PhNH_2$[34] | 62-53-3 | 4.8 | 4.6 | +0.2 |
| $Ph_2NH$[36] | 122-39-4 | −0.6 | 0.8 | −1.4 |
| Ph(p-$NO_2$-Ph)NH[34] | 836-30-6 | −2.8 | −2.5 | −0.3 |
| (p-$NO_2$-Ph)$_2$NH[37] | 1821-27-8 | −4.9 | −6.2 | +1.3 |

- Nitrosamines with features from both lists – these may require additional review[25]

The latter two categories can be considered to be of medium potency, however those with features from both lists may require additional review for potency since they do contain a feature of high concern. A series of closely related structures can be used to demonstrate this: Nitrosodipropylamine contains no significant features. If one propyl group is swapped for an ethyl, this is a concerning feature and N-nitroso-N-ethyl propylamine would be in the 'increased potency' category. If instead N-nitroso-N-isopropyl propylamine is considered, the steric hindrance of that isopropyl group reduces the level of concern, and the compound would be in the 'decreased potency' category. Finally, N-nitroso-N-isopropyl ethylamine contains both the potency-increasing ethyl group and the potency-reducing isopropyl group, and (though potency would still be expected to be moderate[25]) the effects of these two would need to be reviewed and compared.

*Data Analysis and Visualization*

Statistical data analysis and visualization were done with the R language for statistical computing version 4.1.1[40] using the packages ggplot2 version 3.3.5,[41] patchwork version 1.1.1[42] and ggpmisc version 0.4.3.[43]

## Results and Discussion

*The Scope of the Problem*

Our analysis, which is summarized in Table 2, revealed that a substantial portion of the APIs (40.4 %) and API impurities (29.6 %) listed in the GSRS database are potential nitrosamine precursors. The same is true for each subset of APIs taken into consideration, i.e., the WHO list of essential medicines (24.9 %), the US top 200 drugs with respect to sales volume (34.3 %), and the Orange Book (29.6 %). There are more possible nitrosamines from secondary nitrosamines than structures featuring secondary amines because some of these structures have more than one secondary amine functionality and may be nitrosated at one or several positions. Likewise, there are more nitrosamines from tertiary nitrosamines than structures featuring tertiary amines. This is because up to three different nitrosamines can be formed from any tertiary amine, depending on which of the three residues is eliminated. Details on the scoring for individual structures are provided as online supplementary material. It is to be noted that our in silico workflow only identified hypothetical, potential nitrosamine impurities. Whether they are really formed in a particular API or drug product depends on suitable reaction conditions defined by e.g., pH, availability of nitrosating agents, temperature, formulation, particle size, presence of catalysts, packaging, etc. The interaction of all these factors is too complex to forecast if they allow for the formation of a nanogram amount of nitrosamine by the end of shelf life in a portion that represents a patient's maximum daily dose.

*Simple Potent Nitrosamines that Could form by Nitrosative Dealkylation of Tertiary Amines*

Most of the simple dialkyl / alkyl-aryl nitrosamines that we identified originate from tertiary amine sources. Frequently, two of the residues of tertiary amine APIs and impurities are simple alkyl and/or aryl chain, while the third residue is more complex. A typical example is a dimethyl group. Alkyl chains may also be connected to form morpholines, pyrrolidines, piperidines, piperazines etc. In such cases, if the complex residue were eliminated by nitrosative dealkylation, this would create some of the well-known simple potent nitrosamines that were listed in the regulatory guidance documents from the beginning.[16,17] Table 3 shows the top 20 occurrences of nitrosamines that could be formed by nitrosative dealkylation of the small

**Table 2**
Summary of potential nitrosamines that may be formed. The different sources are not mutually exclusive, with some overlap observed. Limited overlap is observed between the USP API and impurity datasets.

| Source | Number of Entries | Structures featuring [Absolute (%)] | | | Possible nitrosamines | | |
|---|---|---|---|---|---|---|---|
| | | 2° amine | 3° amine | 2° and/or 3° amine | Total | from 2° amine | from 3° amine |
| USP – APIs | 8611 | 1268 (14.7) | 2517 (29.2) | 3536 (41.4) | 7895 | 2375 | 5525 |
| USP – Imp | 3564 | 459 (12.8) | 681 (19.1) | 1077 (30.2) | 2213 | 720 | 1493 |
| WHO EML | 563 | 58 (10.3) | 95 (16.9) | 140 (24.9) | 287 | 79 | 208 |
| Top 200 | 210 | 41 (19.5) | 43 (20.5) | 71 (33.8) | 170 | 81 | 89 |
| Orange Book | 2211 | 296 (13.4) | 526 (23.8) | 757 (34.3) | 1739 | 512 | 1227 |

molecule API and impurity structures from the GSRS database. NDMA and NDEA are at the top of the list, but it also includes unexpected structures that have not attracted scientific focus yet, such as *N*-Nitrosoiminodiacetic acid (NIDA; rank 7), 1-(2-Methoxyphenyl)-4-nitrosopiperazine (rank 8), 2-(4-Nitrosopiperazin-1-yl)ethanol (rank 9) and 10-Nitroso-10H-phenothiazine (rank 10).

When comparing the occurrence rates of the 'big 8' nitrosamines – the set union of the small volatile and semi-volatile compounds referenced in the FDA guidance document "Control of Nitrosamine Impurities in Human Drugs"[17] and the EMA assessment report "Nitrosamine impurities in human medicinal products" [16]- across the various databases (Table 4), NDMA and NDEA continue to be the main source of risk due to their frequency of occurrence, followed by MeNP. Perhaps more surprisingly given their inclusion within the original 'big 8' is the absence of any possible formation of NMPA, NEIPA or NMBA. Whilst NEIPA and NMBA have been previously identified in drug product samples (NMPA has only been hypothesized),[44,45] it demonstrates that these structures are likely formed within the API synthesis itself rather than within the drug product. For example, DIPEA is a common tertiary amine base used in synthesis, with NDIPA and NEIPA as possible nitrosamines that could be formed from it, though absent as a declared impurity within the USP impurities database. A similar analysis had previousy been reported by Kao et al.[46] although it was based on a smaller subset of approved small molecule drugs from DrugBank.[47]

### *Potential Nitrosamines from Tertiary Amines Containing a Nitro Group*

We have identified a total of 10 structures that are tertiary amines, and additionally carry a vinylic or aliphatic nitro group. Seven of them are ranitidine related (including salt forms). The remainder are Nitromifene, Niperotidine and Nizatidine which represent potential risks of NDMA and NPYR formation from dimethylamino and pyrollidino groups respectively. A further 44 structures feature aromatic nitro groups; however this functional group would require a highly electron deficient aromatic ring to enable loss of nitrite via an $S_NAr$ mechanism. The full list is available as online supplementary material.

### *Estimation of Potency/Mutagenicity of the Potential Nitrosamines*

The classification methods described previously in chapter 2.4 result in a split of the nitrosamines which may potentially be formed into four categories. Table 5 shows the distribution of the nitrosamines the different datasets described into these categories. Most of the nitrosamines proposed are API-like, complex nitrosamines (NDSRIs) without available carcinogenicity data. A small number could be removed from the lists of 'nitrosamines which need assessment of potential potency' since potency data in the form of reliable $TD_{50}$ values exist, and a small fraction further have available positive/negative classification data for carcinogenicity and/or mutagenicity. However, these high-level classifications may be the best available prediction for many compounds, unless structurally close analogues are available from which read-across may be used to set the limits.

Of the nitrosamines listed in Table 5, the majority (e.g., 5749 of the 6401 unique nitrosamines in the USP-API dataset) have at least one $\alpha$-CH2 group. That being stated, the most common structural feature by far is steric hindrance at the $\alpha$-position (aromatic (1345/6401) or isopropyl (1702/6401) substituents; *tert*-butyl are rarer (192/6401)) which would place a nitrosamine into either the potency-reducing or 'both lists' categories. On the other side of the equation, methyl and ethyl substituents are also common (1564/6401), and nitrosamines with these can be expected to be potent (unless also containing a deactivating group). Other common features are benzylic, allylic, or propargylic substitution (922/6401) – potency-increasing via stabilization of the transition state – and the presence of a carboxylic acid group (603/6401) – potency-reducing since it reduces the need for phase I metabolism.

The distribution of the nitrosamines into these categories also allows analysis of how many nitrosamines are susceptible to which of the three activation mechanisms described by Dobo *et al*[48] and shown in Fig. 9. The 1345 nitrosamines with aromatic substituents are expected to follow pathway C in Fig. 9, the 1714 cyclic nitrosamines to follow pathway B (or optionally A should the ring-closing step be prevented by the conformation of the ring) and the remaining 3342 can be expected to follow pathway A, forming diazonium ions (unless metabolic activation is prevented).

Numbers in this section refer to the totals in the USP-API dataset; the trends described are comparable across all datasets.

### *Theoretical Background of Nitrosamine Formation*

For nitrosation to occur, an amine must be present in its unprotonated form. The concentration of the free amine can be calculated using a derivative of the Hendersson-Hasselbalch equation.

$$[free\ amine] = [amine] * \frac{10^{pH-pKaH}}{1+10^{pH-pKaH}}$$

At the same time, nitrite must be present in its protonated form $HNO_2$. The concentration of $HNO_2$ is also calculated according to the Hendersson-Hasselbalch equation with pKa $HNO_2$ = 3.29.[49]

$$[HNO_2] = \frac{[NO_2-]}{1+10^{pH-pKa}}$$

Two nitrosation pathways are considered, one that involves dinitrogen trioxide ($N_2O_3$) and the other with the nitrous acidium ion ($H_2NO_2^+$) or the nitrosonium ion ($NO^+$) as the nitrosating species.[12] $N_2O_3$ is formed from two $HNO_2$ molecules, so $[HNO_2]^2$ must be considered for the calculation of the reaction rate of the formation of nitrosamines. $H_2NO_2^+$ has been calculated to have a pKa of -10,[50] which suggests that very low levels will be formed under moderately acidic to basic conditions. This value is consistent with the experimentally observed rate law for this pathway that shows a dependence upon [HNO2] and [H+].[12]

The overall reaction rate will be

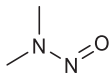
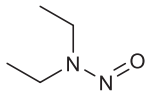
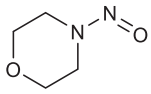
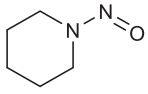
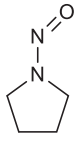
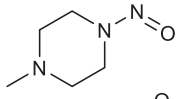
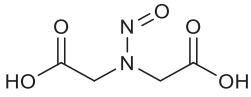
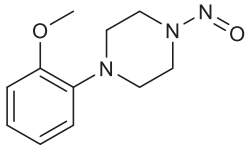
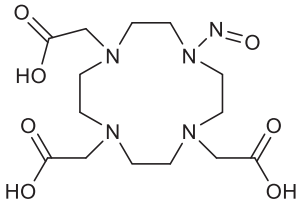
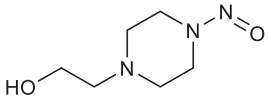
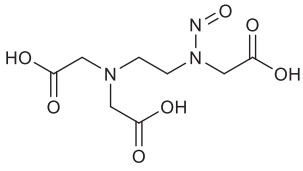
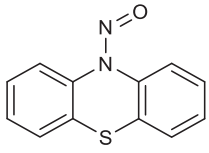
$$rate = k * [free\ amine] * [HNO_2]^2 + k' * [free\ amine] * [HNO_2] * [H+]$$

The first term of the sum describes the nitrosation rate from dinitrogen trioxide ($N_2O_3$), which is formed in a bimolecular reaction from nitrous acid ($HNO_2$). The second term describes the nitrosation rate from the nitrous acidium ion ($H_2NO_2^+$) and the nitrosonium ion ($NO^+$).

As rate constant k we used 9.3E+05 $M^{-2}{\cdot}s^{-1}$, a composite value based on the highest reported rate constant 3.1E+08 $M^{-1}{\cdot}s^{-1}$ for the reaction of a dialkylamine with $N_2O_3$ (ethylbenzylamine (pKaH 9.68))[51] and the dimerization constant of $HNO_2$ to form $N_2O_3$ of 3.1E-03 $M^{-1}$[52] The rate constant k' was set at 7.0E+03 $M^{-2}{\cdot}s^{-1}$ based on the limiting value observed for neutral nucleophiles[12] In the pH range 0 - 14 and the pKaH range -10 - 10 pathway 1 nitrosation exceeds pathway 2 nitrosation 133-fold to 259000-fold (data not shown), i.e., most of the nitrosation is due to $N_2O_3$.

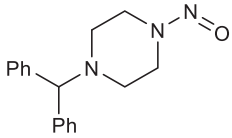
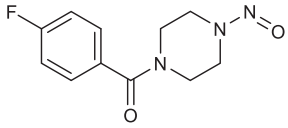
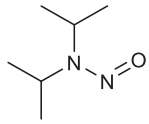
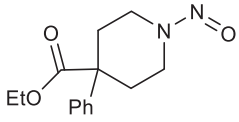
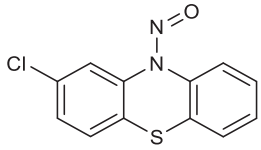
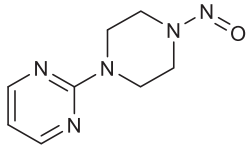
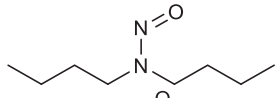
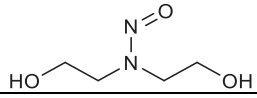
Fig. 13 illustrates the overall relative reaction rate (normalized to a maximum of 1 for each sub-figure A and B), which are clearly higher the lower the amine pKaH and the lower the pH. However, this does not imply that the risk for nitrosamine formation is negligible for higher pKaH and/or pH, demonstrated by the pKaHs of the parent

**Table 3**
Top 20 occurrences of nitrosamines that could be formed by nitrosative dealkylation of APIs with tertiary amine functionality within the USP API data set.

| Rank | APIs | IUPAC name or common name | Synonym | Structure |
|---|---|---|---|---|
| ***1*** | 373 | *N*-Nitrosodimethylamine | NDMA |  |
| ***2*** | 175 | *N*-Nitrosodiethylamine | NDEA |  |
| ***3*** | 89 | *N*-Nitrosomorpholine | NMOR |  |
| ***4*** | 88 | *N*-Nitrosopiperidine | NPIP |  |
| ***5*** | 54 | *N*-Nitrosopyrrolidine | NPYR |  |
| ***6*** | 36 | 1-Methyl-4-Nitrosopiperazine | MeNP |  |
| ***7*** | 26 | *N*-Nitrosoiminodiacetic acid | NIDA |  |
| ***8*** | 19 | 1-(2-Methoxyphenyl)-4-nitrosopiperazine | - |  |
| ***9*** | 16 | 2-[4,7-Bis(carboxymethyl)-10-nitroso-1,4,7,10-tetraazacyclododecan-1-yl]acetic acid | - |  |
| ***10*** | 17 | 2-(4-Nitrosopiperazin-1-yl)ethanol | - |  |
| ***11*** | 15 | 2-({2-[Bis(carboxymethyl)amino]ethyl}(nitroso)amino)acetic acid | - |  |
| ***12*** | 16 | 10-Nitroso-*10H*-phenothiazine | - |  |

(*continued*)

**Table 3** (*Continued*)

| Rank | APIs | IUPAC name or common name | Synonym | Structure |
|---|---|---|---|---|
| ***13*** | 16 | 1-Benzhydryl-4-nitrosopiperazine | - |  |
| ***14*** | 15 | (4-Fluorophenyl)(4-nitrosopiperazin-1-yl)methanone | - |  |
| ***15*** | 14 | *N*-Nitrosodiisopropylamine | NDIPA |  |
| ***16*** | 12 | Ethyl 1-nitroso-4-phenylpiperidine-4-carboxylate | - |  |
| ***17*** | 11 | 2-Chloro-10-nitroso-10*H*-phenothiazine | - |  |
| ***18*** | 11 | 2-(4-Nitrosopiperazin-1-yl)pyrimidine | - |  |
| ***19*** | 11 | *N*-Nitrosodibutylamine | NDBA |  |
| ***20*** | 10 | N-Nitrosodiethanolamine | NDELA |  |

amines for NDSRIs shown in Fig. 1, which ranged from 5.25 (Quinapril) to 9.39 (Propranolol). Fig. 14.

*Risk Matrix Likelihood of Formation vs Estimated Potency*

There is a clear difference in the distribution of pKaH values between the four potency categories (Fig. 14). The pKaH distribution of the structures with potency-increasing features is narrow and shifted towards higher values >>pKaH 5. The pKaH distribution of the structures with potency-reducing features is broader, with a first maximum around pKaH 8 incorporating amines which also feature distal potency-reducing features such as carboxylic acids, and a second maximum around pKaH 0 extending downwards beyond pKaH -5, broadly corresponding to diaryl amines and electron deficient secondary aryl amines. The category featuring both potency-increasing and potency-reducing features has a lower pKaH maxima and a broader distribution than category with potency-increasing features, but not as distinct as the category with only potency-reducing features.

Considering that the rate of nitrosation is higher for amines with lower pKaH, this means that it's more likely to find a low-potency nitrosamine at high concentration than a high-potency nitrosamine. Furthermore, in situations where multiple sites of nitrosation can occur, competition is likely to favor the formation of the lower potency nitrosamine.

*Molecular Weight Distribution Among the Potential Nitrosamines*

Molecular weight is another important property that is likely to impact the mutagenic potency of a nitrosamine. Nitrosamines are direct acting mutagens they require metabolic activation by specific enzymes of the cytochrome P450 complex (see chapter 1.4 and Fig. 9). Molecules with higher MW are likely to be more bulky and hence not as easily accessible for these enzymes. Secondly, the density of nitroso groups is lower in larger nitrosamines and as such the mutagenic potential per mass unit is lower. Furthermore, for larger molecules there are more likely multiple competing metabolic clearance pathways not all of them leading to species capable of DNA alkylation.

The distribution of molecular weight is similar between the four potency categories, with a maximum around 400 and few structures

**Table 4**
Occurrences of "Big 8" nitrosamines which may be formed from each dataset.

| Nitrosamine | USP (APIs) | USP (Impurities) | WHO EML | US top 200 drugs | FDA Orange book |
|---|---|---|---|---|---|
| NDMA | 369 | 135 | 26 | 6 | 117 |
| NDEA | 174 | 16 | 8 | 1 | 28 |
| MeNP | 36 | 3 | 4 | 5 | 24 |
| NDBA | 11 | 1 | 1 | 1 | 3 |
| NDIPA | 14 | 0 | 0 | 0 | 3 |
| NMPA | 0 | 1 | 0 | 0 | 0 |
| NEIPA | 0 | 0 | 0 | 0 | 0 |
| NMBA | 0 | 0 | 0 | 0 | 0 |

**Table 5**
Distribution of the potential nitrosamines from the datasets into the various potency categories. It is important to note that the numbers in this paragraph should not be expected to add up to the counts in table 5, since it is possible for a nitrosamine to have multiple potency-reducing (or indeed potency-increasing) features; for example N-nitrosoquinapril which has isopropyl groups on both sides of the nitrosamine (though one substituent on each of those is a carboxyl-derived EWG (ester and amide, respectively), a feature expected to increase potency) and a carboxylic acid (potency-reducing) elsewhere in the molecule. This combination of features places N-nitrosoquinapril squarely into the 'both lists' category but is both one of the 1702 with isopropyl groups and the 603 with carboxylic acids.

| Source | Distribution of NAs | | | |
|---|---|---|---|---|
| | *Potentcy increasing features* | *Both potency increasing and reducing features* | *No relevant features* | *Potency-reducing features* |
| USP – APIs | 1360 | 1327 | 1749 | 1965 |
| USP – Imp | 378 | 478 | 406 | 533 |
| WHO EML | 58 | 71 | 54 | 80 |
| Top 200 | 25 | 23 | 41 | 58 |
| Orange Book | 254 | 344 | 269 | 389 |

only below 200 and above 1000. The median MW of the potent category is slightly lower than those of the other categories. A density plot is available as online supplementary material.

*Examples of API Classes at Risk*

Many drug classes which share a common pharmacological target and similar target pharmacophores will carry a similar inherent nitrosamine formation risk. Thus, we note that due to their pharmacophore alone the following drug classes will tend to have a high risk of containing significant API related nitrosamine levels as both API as well as impurities and degradation products from API and drug product are under risk to yield nitrosamines. Respective drug classes are summarized in Table 6.

It should be noted that several adrenalin-like stimulants such as ephedrine or methylphenidate are also secondary amines and as such at risk of forming N-nitroso derivatives (Fig. 15).

*Intersection with 2020 top 200 Drugs and the WHO List of Essential Medicines*

Individual potential nitrosamines derived from APIs of the Top 200 drugs (US sales in 2020) or their impurities, as well as nitrosamines derived from the 2021 WHO list of essential medicines and the Orange Book are detailed in the online supplementary material.

*Availability of Reference Standards and Analytical Methods*

We have identified a substantial number of potential nitrosamine impurities that could be present in medicines that contain the APIs and their respective impurities subject to our analysis (see chapter 3.1.). These could be created through nitrosation of secondary amine moieties or nitrosative cleavage of tertiary amine moieties. To assess whether unacceptable amounts are present in a specific product, quantitative analytical testing is required. A prerequisite for this is the availability of a suitable analytical method, which can only be developed if a reference standard for the respective nitrosamine is available.

*Reference Standards*

Reference standards are readily available for the small and potent standard nitrosamines, e.g., the "big 8"; for the latter, even compendial reference standards are available from USP and/or Ph. Eur./EDQM. For NDSRIs, the situation is different. So far, the Pharmacopoeias do not offer any NDSRI compendial standards. The coverage with commercial standards has increased over the past months as suppliers have recognized the demand and the business opportunity, but it is far from complete. Furthermore, some of the offered compounds are not in stock but will only be (tried to be) synthesized upon request. We have estimated that maximum 300-500 different NDSRI standards are actually in stock with the main commercial suppliers. That is less than 5 % of all potential NDSRIs we have identified.

If a reference standard is commercially not available, it needs to be obtained by custom synthesis. However, some compounds may be impossible to make, either due to lack of reactivity, chemical instability and/or preference of competing reactions. Such outcome indicates that the likelihood of presence in the relevant API or drug product is negligible, because the nitrosamine either does not exist or because there is no risk of it being formed, as stated in the EMA Q&A document on nitrosamines.[53]

*Analytical Methods*

Quantification of nitrosamine impurities is commonly done by mass spectrometry and requires the respective complex and expensive analytical equipment as well as specialized personnel. National regulatory agencies (e.g., FDA, EMA, HAS, MFDS) and Pharmacopeias (e.g., USP, Ph.Eur.) have published referential analytical methods for the analysis of the small and potent nitrosamine impurities that were discovered first. USP has also built a Nitrosamine knowledge virtual community ('Nitrosamines Exchange') for supplemental discussions and knowledge exchange in all-things nitrosamines.[54]

**Table 6**
Representative scaffolds of drug classes at risk of forming NDSRIs, based on common structural motives including secondary and/or tertiary amines. The list is most likely non-exhaustive.

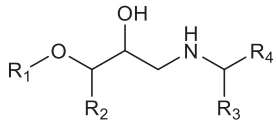
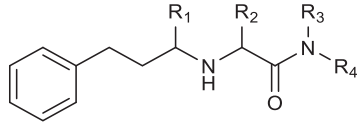
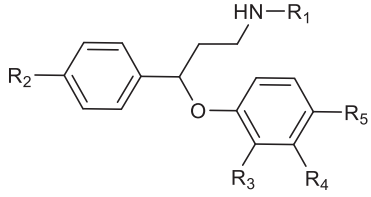
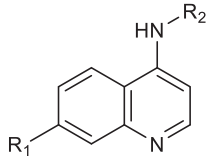
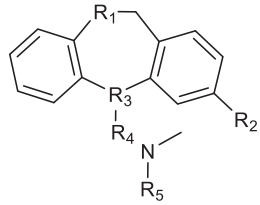
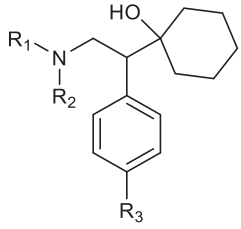
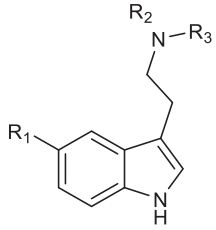
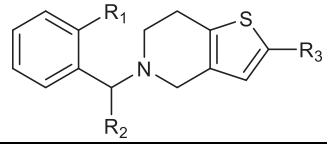
| Drug class | Amine | Examples | Representative scaffold |
|---|---|---|---|
| Beta adrenoreceptor blockers and beta agonists | secondary | propranolol, atenolol, bisoprolol, metoprolol |  |
| ACE inhibitors | secondary | enalapril, ramipril, quinapril, lisinopril |  |
| SNRIs with aryloxy-propanamine scaffold | secondary | fluoxetine, paroxetine, duloxetine |  |
| Aminoquinoline antimalarial drugs | secondary | chloroquine, primaquine, amodiaquine |  |
| Tricyclic antidepressants | tertiary | amoxapine, desipramine, doxepin, imipramine |  |
| SNRIs, Cycloalkanol ethylamine scaffold | tertiary | venlafaxine, desvenlafaxine |  |
| Triptans | tertiary | sumatriptan, rizatriptan, naratriptan, almotriptan |  |
| Thienopyridines | tertiary | clopidogrel, prasugrel, ticlopidine |  |

**Table 7**
Parameters that (may) impact the nitrosamine content of a finished drug product. Depending on when and where nitrosamines are formed, modification of one or several of these factors can be effective.

| | Nitrosamine forms in the API (process) | Nitrosamine forms during DP manufacture | Nitrosamine forms during DP storage |
|---|---|---|---|
| **Law of mass action** | | | |
| Content of vulnerable amine | X | X | X |
| Content of nitrosating agent | X | X | X |
| **Process** | | | |
| Purification steps | X | X | X |
| Chemical degradation | X | | |
| Process parameters | X | X | |
| Unit operations | X | X | |
| **Formulation** | | | |
| pH | | X | X |
| Water content | | X | X |
| PSD | | X | X |
| Formulation | | X | X |
| Addition of scavenger | | X | X |
| **Storage** | | | |
| Primary packaging | | | X |
| Storage conditions | | | X |
| Shelf-life | | | X |

For most NDSRIs, analytical methods are not yet available, so manufacturers will need to develop and qualify a dedicated analytical method that is suitable for their sample matrix and specific for the nitrosamine in question. Accurate mass approaches (QToF- or Orbitrap-based HRMS instead of QqQ tandem MS) can be a means to avoid false positive results due to incomplete chromatographic separation of the analyte from isobaric impurities, as reported for DMF and NDMA by Yang et al.[55]

Analytical methods must be sensitive enough to quantify the nitrosamine in question down to a level that corresponds to 10% of the applicable AI.[44] This level can be calculated as follows:

$$LOQ\ \left[\frac{ng}{g}product\right] = \frac{AI\ [ng]\ x\ DML\ x\ 100}{MDD\ [mg]}$$

*AI Acceptable Intake per day, lifetime daily exposure [ng]*
*MDD Maximum Daily Dose (MDD) of API*
*DML Drug/Mass Load of highest dose strength = Dose strength API [mg] / Dose mass [mg]*

Fig. 16 illustrates this relation for samples with a drug load of 1 %, 5 %, 25 % and 100 %. Logically, the required analytical sensitivity is higher the larger the MDD and the smaller the AI. Some combinations of large MDD and small AI call for LOQs beyond current technical feasibility, which can be expected to be in the range of 5-50 ppb, depending on the analyte and the product matrix.

If the presence of a postulated nitrosamine is confirmed, knowledge of its toxicological potential is essential to decide at which level it needs to be controlled.

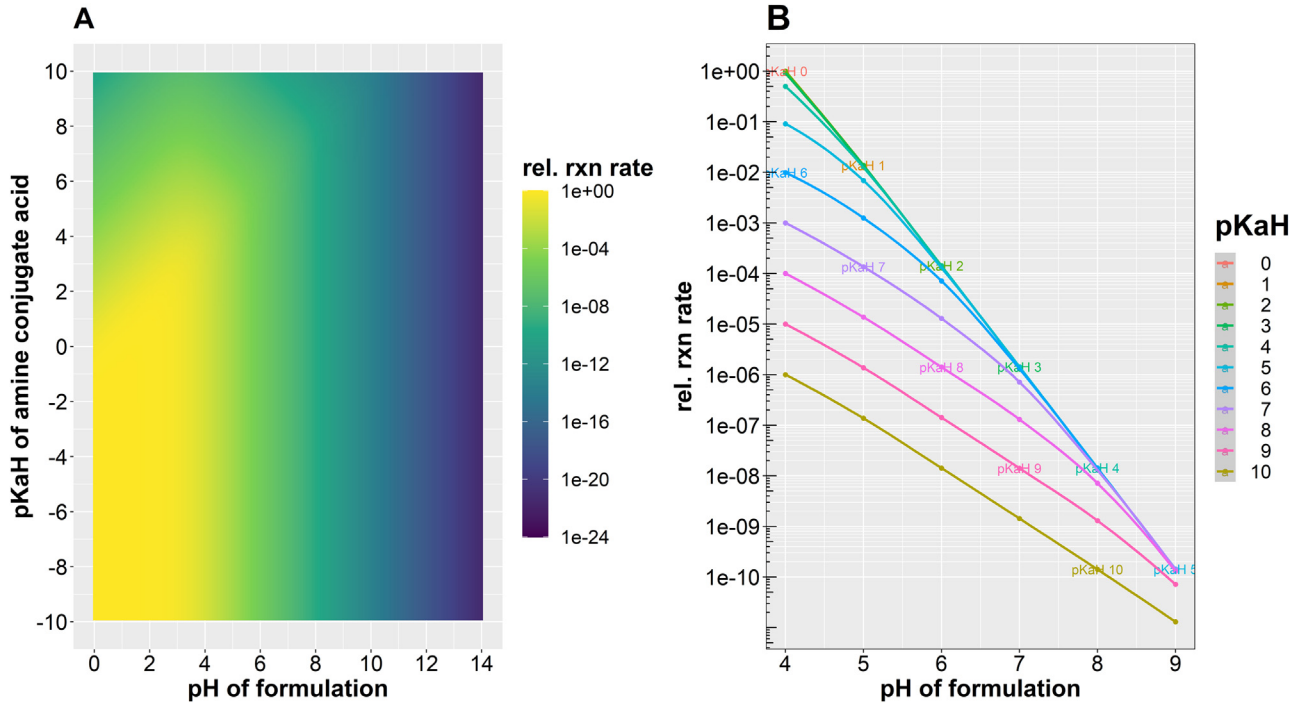



**Figure 13.** A) Heatmap of relative reaction rate (normalized to a maximum of 1) in relation to formulation pH and pKaH of the amine conjugate acid. B) Relative reaction rate (normalized to a maximum of 1) in relation to realistic ranges of formulation pH and pKaH of the amine conjugate acid.

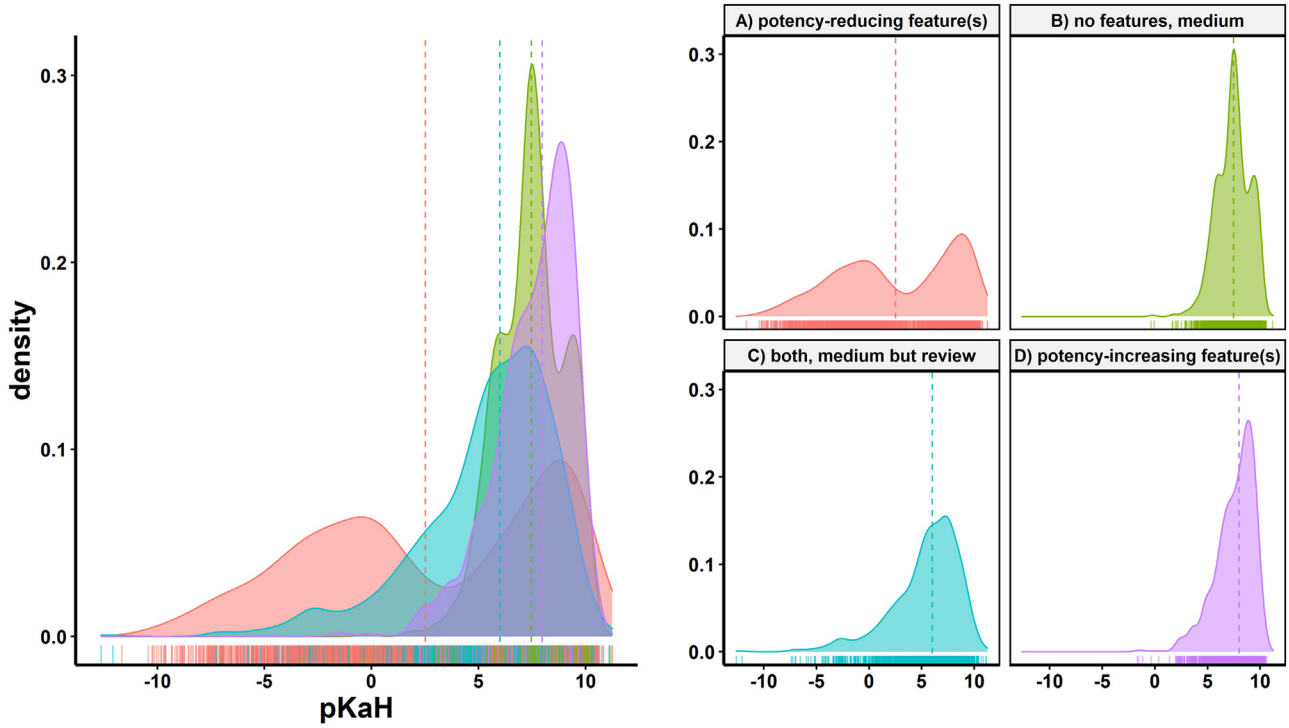



**Figure 14.** Density plot of amine pKaH values. The pKaH values calculated for secondary and tertiary amines identified in the GSRS dataset were binned into four potency groups for the respective N-nitrosamine derivatives. The y axis represents the probability density, i.e., the probability per unit on the x axis. Vertical dashed lines represent the median pKaH values for each group.

*Mutagenicity Testing of NDSRIs and Challenges of Setting Limits*

While mutagenicity and even carcinogenicity data are commonly available for simple nitrosamines, this is not the case for NDSRIs, with few but increasing exceptions. In the absence of such data, in silico systems are usually used to predict the mutagenicity of impurities, as per guideline ICH M7. However, if the potential mutagenicity of an impurity cannot be mitigated with *in silico* approaches (which is currently true for most nitrosamines), the impurity needs to be controlled at or below an appropriate limit (TTC, threshold of toxicological concern) or a bacterial reverse mutation assay ("Ames test") can be performed to prove or to disprove mutagenicity. A negative outcome in the Ames test would warrant to control the impurity according to guidelines ICH Q3A (for APIs) or ICH Q3B (for drug products). ICH M7 also describes a group called "cohort of concern" - some structural classes for which the TTC cannot be applied, because they are generally more potent than the majority of carcinogens. This is the case of N-nitroso, aflatoxin-like and alkyl azoxy compounds. Nonetheless, all the ICH M7 principles should also apply to cohort of concern compounds, except the TTC.

For nitrosamines however, in contrast to what is recommended by ICH M7, concerns have been raised on the acceptability of a negative Ames test alone for de-risking their mutagenicity, but there is also uncertainty as to which data would be considered adequate. Currently, there is no agreed and harmonized process for following up Ames negatives, thus industry is working on optimization of available *in vitro* and *in vivo* assays (e.g., Ames, *in vitro* Comet) and also using new methods like duplex sequencing to add supporting data. Recently, OECD guideline 470 on the *in vivo* mammalian erythrocyte *Pig-A* gene mutation assay was published.[56] Despite the fact that mutagenicity endpoints can be combined in one study (e.g., Micronucleus/Comet assay or gene mutation assays in transgenic rodents (OECD 488[57])/Pig-A), these studies require considerable money and time, and the testing capacities worldwide are limited. The requirement and the appropriateness of *in vivo* follow-up studies for Ames negative compounds are currently controversially discussed between

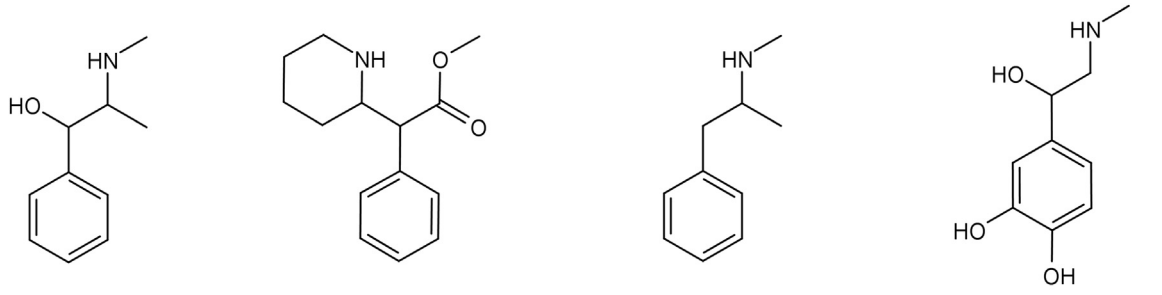



**Figure 15.** Adrenaline-like stimulants that are secondary amines.

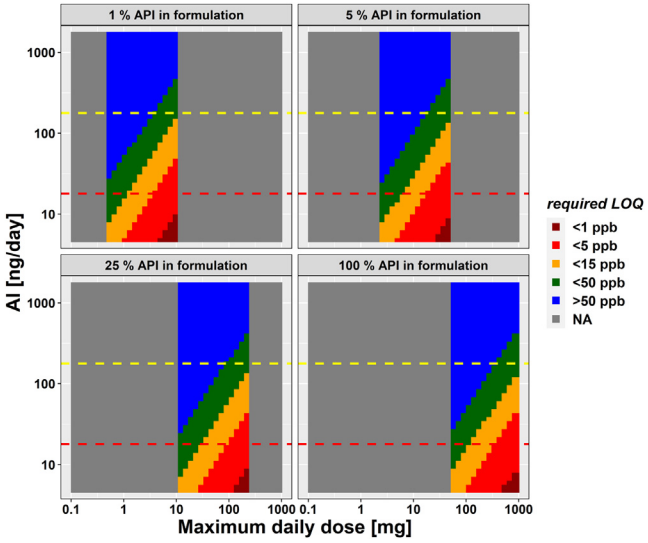



**Figure 16.** Correlation of required analytical sensitivity (LOQ) with the nitrosamine AI and the MDD of the API in question. The color coding represents the indicated bins of required LOQ. Grey areas correspond to AI/MDD combinations that would lead to a total weight of the to be administered product outside of a realistic range of 50 mg - 1100 mg. Red and yellow dashed horizontal bars represent AIs of 18 ng/day and 178 ng/day, respectively. Note that the zigzag appearance of the boundaries between the LOQ bins is a technical artefact.

regulators and industry, with regards to the ICH M7 procedure that seems to be no longer followed for nitrosamines, but also in the light of the 3R principles (refine, reduce, replace) and the efforts to reduce animal testing. Glowienke *et al.* recently presented data for a nitrosated valsartan impurity, in which the negative outcome of an Ames test was confirmed by a transgenic gene mutation assay in Muta™Mice.[58] The lead time for such endeavor is significant, due to limited global resources in combination with increased demand due to the regulatory scrutiny of nitrosamines. The cost, lead-time and time required for *in vivo* mutagenicity testing boost the value of previously generated data available in databases such as CPDB or LCDB. They have also led to the initiation of a respective data sharing initiative for complex nitrosamine related mutagenicity data hosted by Lhasa Limited.[59] Other international working groups are also exploring this topic, such as the Health and Environmental Sciences Institute (HESI).

If mutagenicity is confirmed, or if the available data showing non-mutagenicity don't find regulatory acceptance, a compound-specific acceptable intake (AI) needs to be derived. Generally, an AI can be extrapolated from a $TD_{50}$ value derived from carcinogenicity studies. The AI corresponds to a theoretical 1:100,000 risk to develop cancer during a lifetime. However, for most of the NDSRI's, there is no carcinogenicity data available. For nitrosamines without or with insufficient carcinogenicity data, some Health Authorities have set a class-specific limit of 18 ng/day (e.g., EMA,[53] HC[60]), derived from the 5$^{th}$ percentile of known nitrosamine $TD_{50}$ values (since the latest update of their Q&A document published 10$^{th}$ October 2022, EMA allows an interim AI of 178 ng/day based on the 33$^{rd}$ percentile of known $TD_{50}$ values, until a substance specific AI is established) which is a very conservative approach, considering that not all nitrosamines have the same high potency as the well-known small nitrosamines as NDEA.[61] Hence, the industry is working with high pressure on generating data to justify higher limits for NDRSI's. A grouping of nitrosamines into 13 classes of structural similarities was recently proposed by Dobo *et al.*[48] and Cross and Ponting provided a categorization of nitrosamines according to their structure activity relationship.[22] For a classical read-across exercise, $TD_{50}$ data for a closely related surrogate substance is required. Currently, considerable work is ongoing to define criteria what a "similar" molecule is. One source of $TD_{50}$ values is the Lhasa Carcinogenicity Database (LCDB), which is a widely used, free resource containing the results of animal cancer tests. So far, it includes carcinogenicity data for 138 nitrosamines, although only 3 of those are considered complex nitrosamines (e.g., nitrosocimetidine), and a further 2 are nitrosated drugs but are relatively simple molecules (e.g., nitrosoephedrine) (NDSRIs). A statistical evaluation of the data from LCDB augmented with other published data revealed that 18 % of the tested nitrosamines were non-carcinogenic, and that there was greater correlation between mutagenicity and carcinogenicity for nitrosamines than for non-nitrosamine compounds.[62]

However, both approaches – justifying a nitrosamine is not mutagenic or using read across to establish their limits— need industry to work closely with their respective regulatory agencies for the acceptance of the proposed limit or to agree on a testing strategy.

### *How to Avoid Nitrosamine Formation*

There are numerous factors that can affect nitrosamine content in a drug product (Table 7). Nitrosamines may form anywhere along the process chain from API synthesis, drug product manufacturing and storage of the finished product. They may form at one or several stages, depending on where the conditions for their formation are favorable. Most obviously, based on the law of mass action, a reduction in the content of the vulnerable amine and/or the nitrosating agent will reduce the nitrosamine load, as previously demonstrated.[63] Reduction of the vulnerable amine is certainly not feasible if it affects the API itself. Reduction of nitrosating agent or vulnerable amine may be achieved by switching the supplier of the contaminated material.[63] If the API synthesis is concerned and under control of the MAH, the contaminant (secondary amine or nitrosating agent) or the nitrosamine formed can be purged by extended/additional purification steps, or, with respect to the nitrosamine only, be degraded based on its susceptibility to reductive, oxidative, electrophilic, nucleophilic, and radical chemistry.[64] However, a supplier switch might not be feasible due to lack of alternatives, and profound changes to the API synthesis are complex and time consuming for approved commercial processes and even processes under development.

On the level of drug product formulation, nitrosamine formation may be reduced by increasing the pH, modulation of the water content or particle sizes,[65] or addition of inhibitors.[66] Again, none of these measures can be used as a quick fix for existing commercial or late-stage development products, and any modifications to the formulation must be carefully evaluated against potential unwanted side effects beyond nitrosamines, such as altered physicochemical properties, manufacturability, and stability. Modification of process parameters or unit operations affecting the exposure to heat and moisture could be worth consideration, if technically feasible.

On the level of drug product storage, nitrosamine formation could be reduced by requiring more stringent storage conditions and/or the application of more protective primary packaging. A reduction of the product shelf-life may be another viable option. On the downside, cold storage may be less- or even unavailable in the least developed and developing countries especially in private homes; more protective packaging comes at a higher price, and shorter shelf-life is not only less convenient for the patient and the MAH alike, up to a point where it becomes unsustainable, but also additional burden for the supply continuity and ecobalance.

It must be noted that even the combination of several of the above measures within the boundaries of technical feasibility may not suffice to decrease nitrosamine content below the limits as per current regulatory expectations, and the majority will require extensive development efforts and lengthy regulatory approvals. Therefore,

given the scale and complexity of these issues, it is important to establish a clear risk/benefit framework to ensure continued supply of drugs whilst these issues are dealt with. At present there have been instances of differing regulatory positions on acceptability of risk, with some favouring a 'no-risk endpoint', which looks increasingly unsustainable given the breadth of potential sources.[67]

## Conclusion

Our *in-silico* analysis of public structural information on APIs and known API impurities indicates that the presence of nitrosamines in pharmaceuticals is likely more prevalent than originally expected. In total, 41.4 % of the APIs and 30.2 % of the API impurities listed in the GSRS database are potential nitrosamine precursors. If one excludes tertiary amines as precursors and only considers the structures that contain the more reactive secondary amine moiety, it is still 14.7 % and 12.8 %, respectively. Most structures identified through our workflow could form complex API-related nitrosamines (NDSRIs), although we also found structures that could release the well-known small and potent nitrosamines NDMA, NDEA, and others. Due to common structural motifs including secondary or tertiary amine moieties, whole essential drug classes such as beta blockers and ACE inhibitors are at risk.

Analytical standards that would allow for a quantification in the pharmaceuticals concerned are currently only available for less than 5 % of all potential NDSRIs. Likewise, there is currently almost no *in vivo* mutagenicity or carcinogenicity data available that would allow for the deduction of realistic PDE or AI-based content limits. Harmonisation and discussion around the selection of suitable AI limits between pharmaceutical manufacturers and Health Authorities are important, since the determination of an AI limit lower than expected may result in additional costs and [temporary] drug shortages while manufacturing processes are further refined. This discussion should include greater clarity around the read-across methodology being used by Health Authorities to determine suitable analogues, e.g., the case of nitroso-varenicline, where Regulators and the MAH had different views of the correct read across approach.[68] Non-acceptance of control proposals based on actual product use (i.e., LTL durational limits), although recently demonstrated by Bercu et al.[69] to be protective for potential carcinogenic risk, further complicates the situation for pharmaceutical manufacturers. At the same time, some Health Authorities have practically removed the possibility to de-risk these impurities in a timely manner via the bacterial reverse mutation assay.[53]

For NDSRIs, if the nitrosamine precursor is the API molecule itself as in most cases we have identified, the only feasible remediation to avoid nitrosamine formation would be a reformulation with low-nitrite excipients and/or the addition of a scavenger or antioxidant such as $\alpha$-tocopherol.[66,70] Considering the required development activities and the complexity of rolling out such change to a global supply network under the current regulatory framework, the lead time for such endeavor would be in the range of 2-5 years. If a company's portfolio contains several products that require mitigation, this may lead to further delay due to overburdening of the available resources.

To avoid the risk of drug shortages or even the complete loss of therapeutic options, close cooperation between Regulatory Authorities and industry is of high importance to align on risk/benefit-based expectations.

## Online Supplementary Material

1. Database overlaps
2. Nitrocontaining structures
3. USP -API summary (David)
4. USP -Imps
5. Top200
6. Orange book
7. Who EML
8. Mass distribution image (Figure 16)

## 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.

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## Supplementary Materials

Supplementary material associated with this article can be found in the online version at doi:10.1016/j.xphs.2022.11.013.

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