SPECIFIC CONTRACT

No. 05 (SC05)

implementing framework contract No. EMA/2020/46/TDA/38

Deliverable 2 – Study protocol

Contractual Submission Date: 05/10/2025

Actual Submission Date: 06/10/2025

Responsible partner: Fraunhofer ITEM

Content

1. List of Abbreviations

AI	Acceptable Intake
API	Active Pharmaceutical Ingredient
AUC	Area Under Curve
BMD	Benchmark Dose
CAS	Chemical Abstracts Service
Cmax	Maximal Concentration
CPCA	Carcinogenic Potency Categorization Approach
CPDB	Carcinogenicity Potency Database
CYP450	Cytochrome P450
DMSO	Dimethyl sulfoxide
D	Deliverable
DNA	Deoxyribonucleic Acid
EAT	Enhanced Ames assay
E. coli	Escherichia coli
FDA	Food Drugs Administration
GSH	Glutathione
HPLC	High Performance Liquid Chromatography
LC/MS-ToF	Liquid Chromatography/Time-of-Flight/Mass Spectrometry
LC-MS/MS	Liquid Chromatography Tandem Mass Spectrometry
LTL	Less-than-Lifetime
MACCS	Molecular Access System
MRM	Multiple Reaction Monitoring
NAs	*N*-Nitrosamines
NDSRIs	*N*-Nitrosamine Drug Substance-related Impurities that share structural similarity to the API
NNK	4-(*N*-Nitrosomethylamino)-1-(3-pyridyl)-1-butanon
^{1}H NMR	Proton Nuclear Magnetic Resonance
^{13}C NMR	Carbon-13 Nuclear Magnetic Resonance
NNO	*N*-Nitroso group
PHH	Primary Human Hepatocytes
pKa	Negative logarithm of the acid dissociation constant (Ka)
OECD	Organisation for Economic Co-operation and Development
(Q)SAR	(Quantitative) Structure Activity Relationship

SMILES	Simplified Molecular Input Line Entry System
SOM	Site of metabolism
Synapt G2-Si q-ToF HRMS	Synapt G2-Si Quadrupole Time-of-Flight high-resolution mass spectrometer
S9 mix / homogenate	Supernatant fraction obtained from liver homogenate by centrifuging at 9000 g
TD50	Median Toxic Dose
TO	Target Organ
UPLC	Ultra Performance Liquid Chromatography

2. Introduction

The mutagenicity and carcinogenicity of *N*-Nitrosamines (NAs) are an important factor to consider in understanding their impact of human health. The presence of NAs in human medicinal products emerges as a significant concern due to their recognized toxic properties. Small NAs such as nitrosodiemthylamine (NDMA) can be detected as residual impurities carried over from the synthetic process alongside the active pharmaceutical ingredients (API) in medicines. A category of NAs which are derived from the reaction of some APIs in the finished products with nitrites present in excipients are referred to as *N*-nitrosamine drug substance-related impurities (NDSRIs).

. NAs need to be metabolically activated to express their mutagenic properties. The metabolic activation begins by an oxidation process, which involves the hydroxylation in the α-position adjacent to the NNO-group [17, 18] by cytochrome P450 enzymes. The resulting hydroxylated product can undergo further metabolic activation processes, which include the β-hydroxylation or denitrosation. Altogether, these series of activations lead to the formation of highly reactive and electrophilic diazonium ions, which can interact with the cellular molecules. Particularly of concern, this can lead to the covalent binding to the nucleophilic part of the DNA bases, forming DNA adducts. This binding can cause mutation, which may contribute to the development of cancer.

There is, however, a difference between mutagenic activity of various NDSRIs. This can be attributed e.g. to the variations of the chemical features of the NDSRIs, particularly the sites adjacent to the NNO group. These variations are assumed to contribute to their ability to be metabolically activated or for the activated species to bind to DNA. In contrast, alternative metabolic pathways may deactivate the NDSRI. Altogether, the understanding of the metabolic activation/deactivation of NDSRIs is of particular importance, considering their effects leading to the carcinogenicity.

The MUTAMIND SC05 project aims to investigate the major metabolic sites of the NDSRIs, which are presumed to impact the metabolic activations of these compounds. For this purpose, the MUTAMIND SC05 project will explore the (de)activation of NDSRIs using the *in vitro* human liver models. This aim to identify the major sites of the metabolic activation to study e.g. the hydroxylation sites.

Furthermore, the impact of the potential conjugation reactions, which may influence the reactivity of the NDSRIs and the general detoxification process will be addressed in *in vitro* studies. Additionally, *in silico* tools will be employed to predict the metabolites generated during this process. This will facilitate the evaluation of their applicability and predictivity for the upcoming projects related to NDSRIs.

This deliverable provides an overview of the approaches that will be employed throughout the course of the project. The project is categorized into two main objectives:

- Objective 1: Review of quantitative organ-specific carcinogenicity for *N*-nitrosamines.
- Objective 2: Mechanistic study on the metabolism of *N*-nitrosamines.

3. Review of quantitative organ-specific carcinogenicity for *N*-nitrosamines

This chapter focuses on the tissue-dependent carcinogenicity of the NAs using the existing scientific literature and databases to identify organ-specific TD50 values derived from robust carcinogenicity studies. This aims to guide the sampling of the relevant tissues for *in vivo* mutagenicity studies of NAs.

The review of quantitative organ-specific carcinogenicity for NAs (including NDSRIs if data is available) comprises four phases:

(i) Database development of tissue-specific TD50s for NAs
(ii) Analyse of the relationships between structure, functional groups and/or physicochemical properties and tissue-specific carcinogenicity
(iii) Analyse contributions of organ-specific metabolism and tissue distribution to tissue-specific carcinogenicity
(iv) Combine data from (i)-(iii) and find conclusions

The planned process will be described in the following sections.

3.1 Creation of a database of tissue-specific TD50s for N-nitrosamines.

The tissue specific tumor formation of NAs will be analyzed based on existing robust and reliable carcinogenicity studies. For this purpose, information will be integrated from three complementary sources:

1) The original CPDB created by Lois Gold and her team with the corresponding TD50 values (NIH, 2021. Download Carcinogenic Potency Database (CPDB) Data[1] [46].
2) An extended version of the CPDB[1] (CPDB, [47]), which includes all studies from the original CPDB plus additional studies being published at later time points from NTP[2], Repdose [48] and the COSMOS DB[3].
3) An extended version of the CPDB reanalyzed by Lhasa including the recalculated Lhasa TD50 based on the values provided by Gold et al. [46]. The methodology adopted for calculating TD50 values from experimental data is consistent with the CPDB [49]

Data for compounds including a *N*-nitroso group will be -extracted from the Lhasa database[4] and annotated to identify NAs. The annotation will include NDSRIs and other related structures like N--nitrosamides, N-nitrosoureas, N-nitrosoguanidines some of which do not require metabolic activation.

Database structure

The data will be stored in relational database, which comprises information on the chemical identity including CAS number, chemical name and SMILES notation. Each compound is linked to N-studies for which the study design is reported, e.g. species, number of tested animals, sex, route, study duration, and dosing regimen. The observed tumor incidence including the tissues and the tumor type, alongside the corresponding BMD or TD50 values will also be documented. This proposed data model will enable the integration of the data from different tables. An additional literature search will be carried out to identify *in vivo* carcinogenicity studies for NAs published after the Yang et al. update (2023) [47], which has already complemented the CPDB.

Study quality

To ensure that the carcinogenicity data for NAs are reliable and relevant, the quality of studies will be assessed using the Lhasa reliability framework as the primary basis. This framework considers key aspects such as:

- Dosing frequency: whether it was sufficient to allow for treatment-related tumour formation;

[1] https://www.nlm.nih.gov/databases/download/cpdb.html
[2] https://ntp.niehs.nih.gov/publications/reports/tr
[3] https://cosmostox.org/
[4] https://lcdb.lhasacloud.org/

- Experiment time: whether the study spanned approximately the full lifespan of the animals, acknowledging that any study showing a clear positive response may still be considered adequate;
- Route of administration: whether the chosen route was appropriate, with justification required for uncommon routes;
- Species selection: whether the use of non-rodent or non-standard species was scientifically justified.

In addition to applying the Lhasa criteria, we will complement this assessment with further quality checks, e.g. the number of dose groups, the number of animals per group, as well as the extent of study documentation accessible. As far as possible differences in scope of examination will be addressed. Moreover, the tumor types used to derive the TD50 values from the original CPDB database need to be considered regarding their relevance.

3.2 Analysis

Initial analyses will evaluate differences in target organs (TOs) and their reference values (like TD50 and BMD values) from the three different sources Gold et al. (1984), Yang et al. (2023) [46, 47] and Lhasa DB.

Compilation the structural, physico-chemical and ADME parameters

The analysis aims to identify relationships between structural properties, functional groups or physicochemical-parameter and tissue specific carcinogenicity. For this purpose, a representative canonical SMILES code will be used. In case of missing information, publicly available databases e.g. CompTox dashboard[5] will be employed to query the SMILES code.

Additionally, software such as ADMET Predictor (version 12.0.0.6)[6] will be used to predict physico-chemical properties, e.g. vapor pressure, Henry constant, water solubility, molecular weight, pka values etc.). The project will also compute the respective CPCA potency category and descriptors, e.g. number of hydrogens, activating and deactivating features. The functional groups per NAs/NDSRIs will either be identified using predefined or hashed fingerprints like MACCS or MORGAN fingerprints respectively. The similarity between compounds will be calculated using e.g. the global or local similarity scores developed in the MUTAMIND SC01 (QSAR for Nitrosamines EUPAS 46057; EMA/2020/46/L1.02; [3a]) project.

Evaluation of in vivo data

The project will identify the most frequently observed target sites of tumor findings of NAs/NDSRIs as well as their sensitivity regarding tumor formation.

For each NA with more than one relevant study the concordance in TO will be investigated taking account differences in study design, like species, route, dose and study duration. For seldomly observed TO, the original publication will be reviewed to also explore differences in study scope.

Then, the parameters which might influence the occurrence of the specific tumor types will be evaluated. This analysis will also evaluate the impact of study design differences including species, route, dosing and study duration.

[5] https://comptox.epa.gov/dashboard/
[6] https://www.simulations-plus.com/software/admetpredictor/

Another question is, whether NAs induce tumor types in TO, that differ significantly from those of other carcinogenic compounds. The significance of the NA targets will be compared to other compound classes requiring metabolic activation (e.g. aromatic amines), or all remaining compounds in the database using appropriate statistical methods (e.g. Fisher's exact test).

We will further analyse, if similar NAs, e.g. falling into similar CPCA categories, or sharing similar or physico-chemical features will induce the same tumor pattern.

Evaluation of organ-specific metabolism and tissue distribution

NAs require metabolic activation to generate reactive and instable carbenium intermediates, which then have the ability to bind to nucleophilic atoms e.g. of DNA bases. The activation is driven by hydroxylation. Therefore, the project will explore the occurrence and (if known) activity of CYP450 isoform in the main TOs of NAs in rodents, identified in the previous task. Occurrence data might rely on transcriptomic, or proteomic data. We will use Literature search to compile these data for rats and humans, as well as well-known repositories and databases like e.g. the human protein atlas[7].

3.3 Conclusion of generated data

Results from task 3.1 and 3.2 will be used to find conclusions on the implications the results for tissue sampling and analysis requirements for *in vivo* mutagenicity studies of NAs.

4. Mechanistic study on the metabolism of *N*-nitrosamines

The second part of the project comprises experimental and *in silico* approaches. This creates an overlap that interlocks and complements each other. In total four steps are included:

(i) Selection of NDSRIs
(ii) Pre-experimental *in silico-based* generation of metabolites from its structure
(iii) Experimental phase to assess metabolism of NDSRIs and corresponding active substances
(iv) *In silico-in vitro* comparison

The planned process will be described in the following sections.

4.1 Selection of NDSRIs for empirical evaluation

Literature search was done to identify relevant studies related to NDSRIs. Therefore, these steps were followed:

1. Gather information and available carcinogenic potency categorization approach (CPCA) classification of NDSRIs and their APIs. The results of *in vitro* mutagenicity from MUTAMIND SC01 [3a] were compiled and considered for the substance selection.
2. Gather information on human Cytochrome P450 (CYP450) enzymes involved in the metabolic activation of NDSRIs. The existing data on the CYP450 enzymes from MUTAMIND SC01 [3a] and MUTAMIND SC04 [3c] were incorporated in this project. The data for remaining substances are extracted and reviewed from e.g. open-source literature.

The process for the literature search focused on metabolic activation of targeted parent substances. Initially, summary information was gathered from Drugbank [4], providing foundational data on the

[7] https://www.proteinatlas.org/

substances of interest. This was followed by a review of primary literature, including studies from the MUTAMIND1 (SC01 – 02; [3a,b]) and MUTAMIND4 (SC04; [3c]) projects, as well as results from the AMES tests.

MUTAMIND SC05 selected seven NDSRIs (**Table 1**) according to the following criteria:

i) cover a broad structural diversity of the NAs to evaluate the relationship between structural features as well as their potential hindrance on metabolic activation and mutagenicity.
ii) include compounds with a wide range of mutagenic potency
iii) include compounds which showed an unexpected high mutagenic activity in *in vivo* mutagenicity like *N*-nitroso-ketamine
iv) include compounds for which positive Ames results are available

and ideally those

v) for which relevant metabolites are commercially available to quantify their formation.

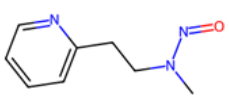
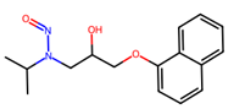
The five candidates (NNO-betahistine, NNO-propranolol, NNO-sertraline, NNO-varenicline, NNO-ketamine) were selected for testing, alongside two additional candidates (NNO-trimetazidine, NNO-duloxetine).

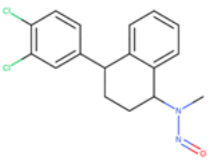
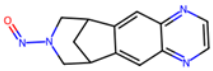
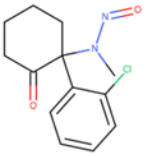
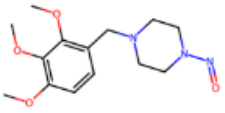
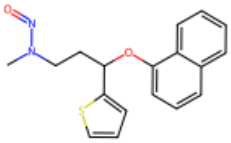
They are structurally diverse, with some featuring a terminal nitroso-methyl groups (*N*-nitroso-betahistine, *N*-nitroso-sertraline, *N*-nitroso-ketamine, *N*-nitroso-duloxetine), as well as some with embedded nitroso-methyl groups (*N*-nitroso-propranolol) etc. These structural variations will allow the assessment of the structural hindrance on the metabolic activation and genotoxic potential.

In addition, most of them have been tested in AMES assays and showed positive responses, confirming their mutagenic potential. According to their structure, they are classified into CPCA classes 1 to 5, covering a range of different potencies.

For some of them also the CYP450 enzymes are known, which are responsible for the metabolic activation (**Table 2**). Most often detected enzymes for NDSRIs are in decreasing order: 2B6=2C19 > 2A6.

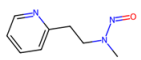
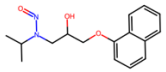
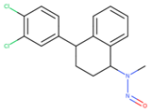
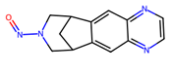
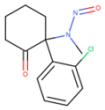
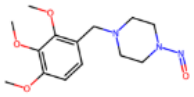
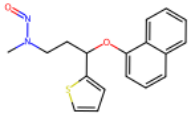
Table 1: Overview on selected NDSRIs - CAS, structure and CPCA [1] are provided. Ames test results were compiled from the previous MUTAMIND project SC02 [3b] and literature. Pictures generated with rdkit 2023.3.2 (Python 3) [45].

CAS	Name	Structure	API/source	CPCA	Mut. *In vitro*
32635-81-7	*N*-nitroso-betahistine		Betahistine	1*	Pos[3b]
84418-35-9	*N*-nitroso-propranolol		Propranolol	4	Pos[3b]

noCas	*N*-nitroso-sertraline		Sertraline	2*	Pos.[6]
2755871-02-2	*N*-nitroso-varenicline		Varenicline	3	Pos.[3b]
86144-35-6	*N*-nitroso-ketamine		Ketamine	5**	n.d.
92432-50-3	*N*-nitroso-trimetazidine		Trimetazidine	3*	Pos.[3b]
2680527-91-5	*N*-nitroso-duloxetine		Duloxetine	1***	Pos.[3b]

* CPCA derived AI as substance tested positive in *in vivo* mutagenicity study.
** CPCA derived AI as substance tested positive in *in vivo* mutagenicity study. LTL cannot be applied and the AI will be capped at 1500ng.
*** Limit derived using structure-activity-relationship (SAR)/read-across approach using the TD50 of NNK as point of departure as substance tested positive in *in vivo* mutagenicity study.

Table 2: Overview on known human CYP450 isoenzymes involved in the bioactivation of the selected seven NDSRIs. These data are compiled from literature in vitro studies and previous MUTAMIND project SC01 [3a]. Also, human CYPs for the respective APIs are given.

CAS	Name	Structure	CYP450 (NDSRI)	API/source	Stereoisomer/API	CYP450 (API)
32635-81-7	*N*-nitroso-betahistine		2B6[3a], 2A6[3a]	Betahistine	no chiral centre	n.d.
84418-35-9	*N*-nitroso-propranolol		1A1[30], 2C19[30]	Propranolol	API is racemic[3]	1A1[4,31], 1A2[4,9,31,32], 2D6[4,9,32], 2C19[4,31], 3A5[4,33], 3A4[4,32], 3A7[4,33]
No CAS	*N*-nitroso-sertraline		2B6[29], 2C19[29]	Sertraline	(1S,4S)-form[3]	3A4[4,14,34], 2D6[4,14,34], 2C19[4,14,34], 2C9[4,14,34], 2B6[4,14,34], 2E1[4,34]
2755871-02-2	*N*-nitroso-varenicline		2B6[29], CYP3A4[29]*	Varenicline	(1S,12R)-form[3]	n.d.
86144-35-6	*N*-nitroso-ketamine		n.d.	Ketamine	API is racemic[5]	3A4[4,37], 2C9[4,37], 2C8[4], 2B6[4,34,35]
92432-50-3	*N*-nitroso-trimetazidine		n.d.	Trimetazidine	no chiral centre	n.d.
2680527-91-5	*N*-nitroso-duloxetine		2B6[29], 2C19[29]	Duloxetine	S-form[4]	2C9[4,33], 2B6[4,33], 2C19[4,33], 3A4[4,33], 1A2[4,33], 2D6[4,33]

**: out of 9 human CYP450 (1A1, 2A6, 2B6, 2C8, 2C9, 2C19, 2E1, 3A4).*

4.1.1 Sourcing and distribution of NDSRI compounds

Some of the selected compounds, namely *N*-Nitroso-duloxetine, *N*-Nitroso-betahistine and *N*-Nitroso-trimetazidine, are planned to be shared between the MUTAMIND SC04 and SC05 projects. The four remaining NDSRIs (*N*-nitroso-propranolol, *N*-nitroso-sertraline, *N*-nitroso-varenicline, *N*-nitroso-ketamine) can be synthetized by Lios. Synthesis routes towards selected NDSRIs and tentatively identified metabolites will be developed based on SciFinder data. Synthesised substances will be characterised by both proton (^{1}H) and Carbon-13 (^{13}C) nuclear magnetic resonance (NMR) as well as necessary 2D spectra prior to use as reference compounds. Purity will be assessed by HPLC and/or LC-MS.

4.2 Pre-experimental *in silico*-based generation of metabolites from its structure

Different *in silico* prediction tools will be employed for the selected NDSRIs (see Table 1) to provide insight into:

- Likely sites of metabolism (SoMs)
- Structures of primary, secondary and other metabolites of the NAs
- Competing detoxification vs. activation pathways

The following *in silico* tools among others will be applied:

- Nexus Meteor[8], a rule-based prediction software using empirically-derived rules to predict biotransformations for a given molecule
- Metasite [50] includes an algorithm that is not training set dependent and therefore exhibits improved predictive performance for novel chemical domains. Docking-based approaches
- Leadscope includes models to identify sites of metabolic liability and predict reversible and irreversible enzyme inhibition, supporting drug-drug interaction assessments in line with FDA guidelines.
- META Ultra from MultiCase[9], a data-driven approach to analyze the atomic environment around each site of metabolism (SOM) up to a depth of 3 bonds. The probability of the formation of Phase I and Phase II metabolites are predicted using (Q)SAR.

In addition, docking and Quanto-mechanic predictions will be performed using Prime/Glide (Schrödinger)[10] and Gold *et al* [46] for the selected NDSRIs. It is also planned to use up to six most relevant CYP450 enzymes based on literature search (see Table 2). The predicted data will be compared to the measured data from the experimental phase to assess their predictivity and usefulness (see also 4.4). Where appropriate, all data are planned to be shared with the consortium partners and analysed using e.g. a consensus approach.

Based on initial results and availability, additional methods and tools may also be used where deemed appropriate and will be documented in study files.

4.3 Experimental phase to assess metabolism of NDSRIs and corresponding active substances

Metabolic transformations of NDSRIs in the presence of glutathione (GSH) as a scavenger of electrophiles (carbocations).

[8] https://www.lhasalimited.org/solutions/metabolite-identification-and-analysis/
[9] https://multicase.com/meta-ultra/
[10] https://www.schrodinger.com/platform/products/prime/

NDSRIs are known to undergo oxidative transformations mediated by cytochrome P450 and lead to highly reactive species –diazonium salts, as shown in the scheme below.

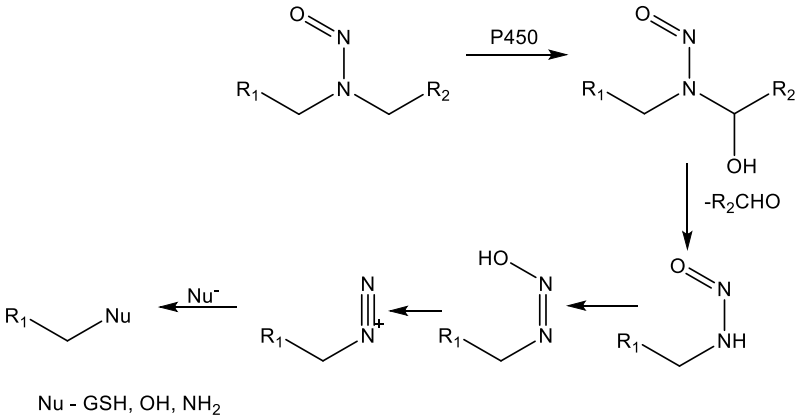


Figure 1 Interaction of NDSRIs with cytochrome P450. Modified from [51].

Diazonium salts may form carbocations and interact with nucleophiles, including DNA, causing mutations. The second step of the study aims to use GSH to scavenge the reactive species to identify the degradation pathways. It is expected to help select potential metabolites for the synthesis step.

Five NDSRIs will be incubated with microsomes in the presence of GSH. Liquid Chromatography/Time-of-Flight/Mass Spectrometry (LC/MS-ToF) will be used to profile the resulting product mixture.

Nitroso derivatives of betahistine, sertraline and ketamine possess a methyl group; thus, the formation of S-methylglutathione could be expected. S-methylglutathione is commercially available and can be used for quantitative assessment of the carbenium ion involving the degradation pathway. Alternatively, the methyl group can be split off as formaldehyde (**Figure 1**) and a larger carbocation can be formed, which further interacts with GSH. Both pathways are shown in **Figure 2** below.

Chemically, NAs are known to undergo denitrosation as shown in **Figure 3** [51]. Although this degradation pathway is not specifically related to metabolism, it should not be excluded, and the appearance of the parent drug can be expected.

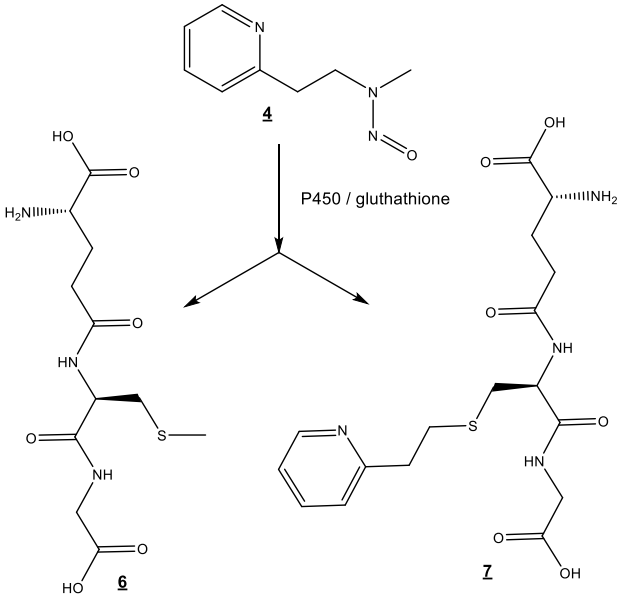


Figure 2 Possible competing N-nitrosobetahistine degradation pathways. Modified from [51].

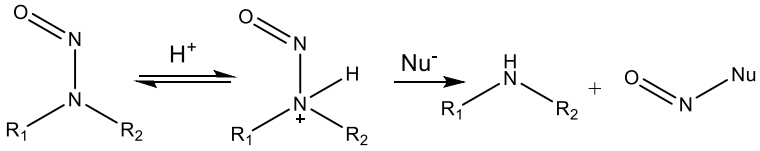


Figure 3 Chemical denitrosation pathway of NDSRIs. Modified from [51].

Based on initial results and availability, additional methods and tools may also be used where deemed appropriate and will be documented in study files.

4.1 Experimental plan

The work will be performed in two phases. Firstly, the clearance of the selected NDSRIs will be evaluated in the liver model to determine the rate of metabolism. Quantitative LC-MS/MS assays will be used to measure the concentration of selected compounds in MRM mode. In case the compounds are metabolised, an LC-MS-TOF assay will be performed to detect possible metabolites and to calculate their molecular formulae. Tentative identification of metabolites by MetaboLynx software, and the data will be compared to prediction results obtained by in-silico predictions.

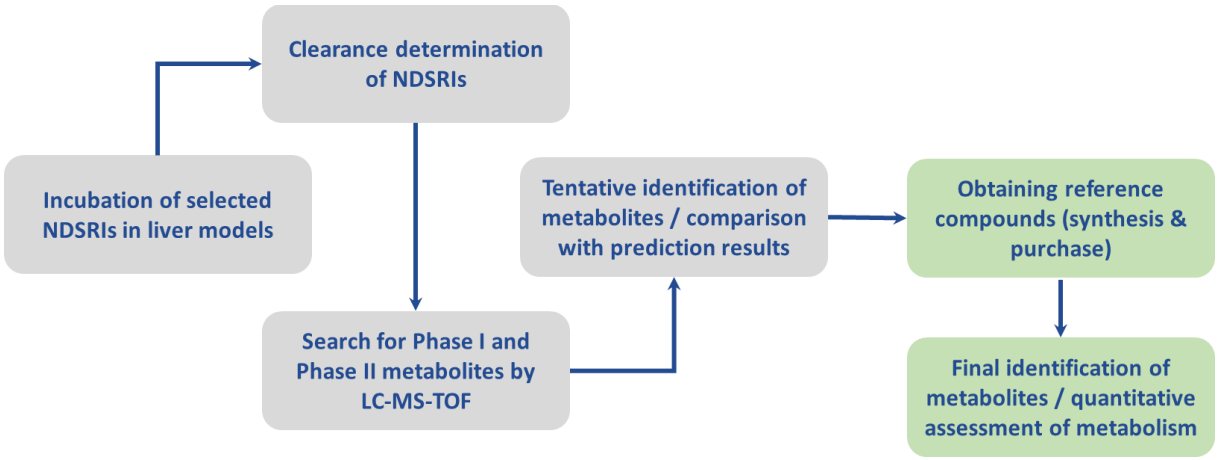


Figure 4 Study plan to assess metabolites and (de)activation of NDSRIs using relevant human liver models.

The second phase of the work will include the selection of compounds for either chemical synthesis or purchase from commercial vendors to obtain reference substances. This will lead to an unambiguous identification of the metabolites and yield a possibility to perform quantitative assessment of the metabolism by specific Multiple Reaction Monitoring (MRM) assays. Ultra-performance liquid chromatography-tandem mass spectrometry will be used for quantitative analysis of both NDSRIs and their metabolites. Specific MRM assays will be developed for each of the compounds.

4.1.1 LC-MS-TOF instruments and conditions

UPLC separation of tested compounds will be performed on a Waters Acquity UPLC system. Synapt G2-Si q-ToF HRMS system (Waters) in electrospray mode will be used for the acquisition of the mass spectra. Based on exact mass data, molecular formulae of the metabolites will be calculated.

MetaboLynx software will be used for tentative structure prediction of metabolites. Compounds that will be tentatively identified during the second step of the study will be synthesised to validate identification results and to enable quantitative assessment of metabolism and/or degradation. Additionally, the glucuronide of N-nitrosopropranolol will be obtained as glucuronidation is known as the dominant metabolic pathway of propranolol [51, 53].

4.1.2 LC-MS/MS Instruments and conditions

Quantitative measurement of metabolites will be performed by LC-MS/MS assays. UPLC separation of tested compounds will be performed using a Waters Acquity UPLC system. Tandem mass-spectrometer Xevo TQ-S micro (Waters) in electrospray mode will be used for quantification. Multiple reaction monitoring (MRM) parameters will be optimised for each test compound. Specific LC-MS/MS settings will be provided in the corresponding method file for each compound.

4.1.3 Incubation of *N*-nitrosamines in S9 liver fractions and hepatocytes

The metabolite formation can be analysed in different human and rat systems, e.g. human S9 homogenate, induced rat liver S9 homogenate and human/rat microsomes beside primary human hepatocytes (PHH). Optionally, rat primary hepatocytes can be used for specific research questions.

Each system has its own advantages; for example, an induced rat liver S9 liver homogenate is widely used in the Ames test to evaluate NA mutagenicity. Human liver preparations S9 and PHH have greater translation potential to humans.

Parent NDSRIs will be incubated with induced rat liver S9 or human liver S9 fraction, and both positive and negative controls will be applied. Additionally, pooled cryopreserved PHHs will be used for testing

according to standard protocol. Compound stability in S9 will be performed according to general protocol. Test compounds and liver S9 fraction will be incubated in potassium phosphate buffer at 37°C. Reaction mixtures contain appropriate concentration of test compound, ≤1% DMSO, liver fraction and necessary cofactors. Concentrations of both parent NDSRIs and identified metabolites will be measured at up to five time points. Typically, reactions are terminated at 0, 5, 15, 30, 45, and 60 minutes (can be extended up to 120 minutes for S9 fraction and up to 240 min for PHH) by protein precipitation or by liquid-liquid extraction and analysed by UPLC–MS/MS.

The precise conditions, test substance concentrations and test systems to be used will be agreed with EMA and documented in a file note to the study.

Based on initial results and availability, additional methods and tools may also be used where deemed appropriate and will be documented in study files.

4.4 *In silico-in vitro* comparison

For each NDSRI all experimentally and predicted metabolites will be used to generate a detailed map. The concordance between the silico tools and between the *in silico* tools and the experimental data of the seven NDSRIs will be evaluated.

Conclusions about the applicability of the *in silico* tools to predict phase 1 and phase 2 metabolism will be provided.

Together with the detected amounts of metabolites, a weight of evidence analysis will be conducted to assess, whether the knowledge on (de)activation of NDSIRs can be used to assess differences in their mutagenic potency.

4.5 Schematic overview on study strategy and interrelationships

The overall study strategy explained in the chapter 4 (Mechanistic study on the metabolism of N-nitrosamines) are illustrated in **Figure 5**. This depicts the connection between *in silico* and experimental works in the course of this project.

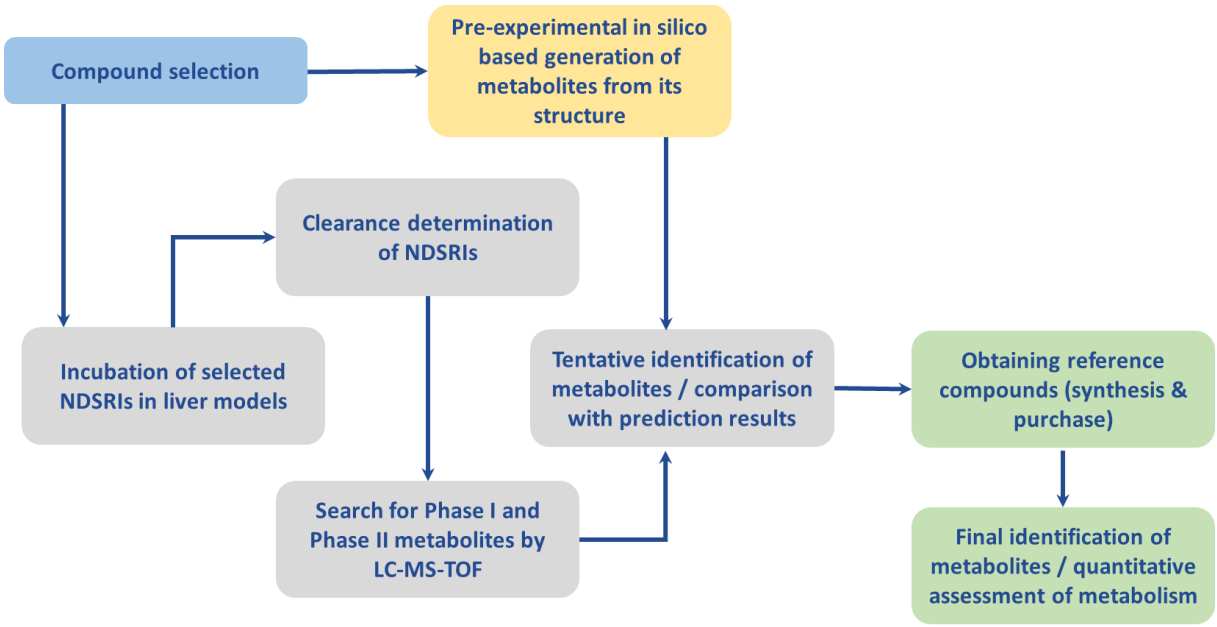


Figure 5 Overall schematic overview on study strategy and interrelationships. Blue: Selection of NDSRIs for empirical evaluation (see 4.1), yellow: (see 4.2) grey: (see 4.3, phase 1) green: (see 4.3, phase 2).

5. Protocol deviations

Minor deviations to the study protocol will be communicated to EMA and documented in the study files.

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