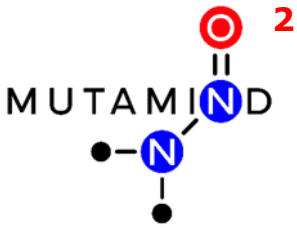
SPECIFIC CONTRACT

No. 04 (SC04)

implementing framework contract No. EMA/2020/46/L1.02



Deliverable 2 – Study Protocol

Study protocols for the bacterial mutagenicity assay based on the current version of the EAT protocol for testing N-nitrosamines, including phenotyping the activation methodology of commonly used positive controls and selected NDSRIs.

Contractual Submission Date: 05/04/2025

Actual Submission Date: 05/04/2025

Responsible partner: Fraunhofer ITEM

Content

List of Abbreviations

2-AA	2- aminoanthracene
API	Active Pharmaceutical Ingredient
CPCA	Carcinogenic Potency Categorization Approach
CPDB	Carcinogenicity potency database
CYP450	cytochrome P450
DMSO	Dimethyl sulfoxide
D	Deliverable
EAT	Enhanced Ames assay
E. coli	Escherichia coli
LC-MS/MS	Liquid chromatography - tandem mass spectrometry
NA	N-nitrosamine
NDSRIs	Nitrosamine Drug Substance-related Impurities that share structural similarity to the API
OECD	Organisation for Economic Co-operation and Development
S9	Supernatant fraction obtained from organ homogenate by centrifuging at 9000 g

Introduction and aims

Determination of the mutagenicity of small-molecule N-nitrosamine drug impurities and N-nitrosamine drug substance-related impurities (NDSRIs) is critical for risk assessment of N-nitrosamines (NAs) in human medicinal products. The Ames test is an integral part of the regulatory test strategy for the assessment of genotoxicity. The project MUTAMIND 1 aimed at generating a better understanding how the Ames test and the *in vitro* Comet assay can be methodologically optimized to reliably detect mutagenicity of different N-nitrosamines. The present project is thematically linked to and continues the above-mentioned question of an optimized test design for the AMES test including already collected knowledge of MUTAMIND 1 and based on the current version of the *Enhanced Ames test* protocol (EAT protocol).

In general, three bacterial tester strains detecting primarily base pair substitutions (TA 1535, TA 100, and EC WP2 *uvrA*) have been described as the most sensitive ones for the detection of genotoxicity induced by N-nitrosamines and NDSRIs and were used in MUTAMIND 1. However, there is lacking data on the sensitivity of the remaining two tester strains mentioned in the OECD TG 471 for the basic Ames test protocol (TA 1537, TA 98) over a broad range of compounds of this chemical class. The MUTAMIND 2 project will include these two tester strains and in sum a set of five tester strains will be used for the investigation of nitrosamine specific positive controls as they are part of the recommendation in the current EAT protocol. Therefore, in D1 extensive literature research was performed to identify relevant studies of NDSRIs, that can be further investigated as possible positive controls. Relevant parameters included in the research were metabolic activation via relevant CYP450 enzymes, in vivo and in vitro reference cancer and AMES data. The result of this approach was a candidate list identifying NDSRIs to be investigated as positive controls. Based on these candidate list in agreement with the EMA six NDRSIs were selected as N-nitrosamines of interest in the current research question.

The EAT protocol is mainly based on preincubation method and the supplementation with S9 liver homogenate from hamster. Although both aspects have to be proven in several publications it is not yet clear if the S9 homogenate should be obtained from non-induced or induced liver from hamster. Therefore, in the current project one batch of non-induced hamster S9 and two batches of induced hamster S9 will be used for a comparative approach. This is part of the investigation of the positive control and will be performed as pre-incubation assays Since Additionally, the influence of organic solvents and solvent concentrations on the sensitivity of the detection of N-nitrosamines and NDSRIs in the Ames test will be investigated in more detail using plate incorporation and pre-incubation method as well.

It is thus the aim of the SC04 study to propose NDSRI positive controls for the EAT, to assess the assay performance and sensitivity of the enhanced Ames test (EAT) for evaluating the mutagenicity of NDSRIs and to identify CYP450 enzymes responsible for the metabolic activation of NDSRIs. Deliverable 2 will set the basis by summarizing the current state of knowledge. In detail, Deliverable 2 pursues the following **objectives:**

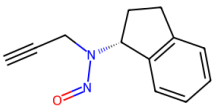
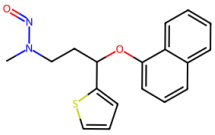
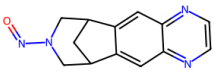
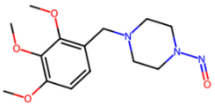
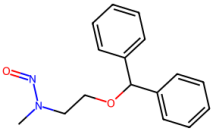
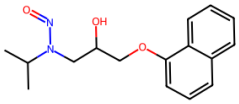
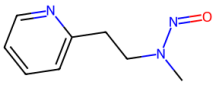
1. Detailed method description of the Ames test, including strains, S9 homogenates, assay types etc.
2. Detailed method description of phenotyping of NDSRI metabolization

Test compounds

Revised Compound selection based on D1 shortlist

Based on the extensive research described in D1 the following six N-nitrosamines (Table 1) have been selected to investigate their suitability as positive controls in the EAT protocol. In addition, N-nitroso-betahistine (32635-81-7) can be included for investigations of metabolism and phenotyping.

Table 1: NDSRI selected based on D1 candidate list (source as given the EMA (EMA, 2024) or FDA list (FDA, 2024). Pictures generated with rdkit 2023.3.2 (Python 3).

CAS	N-nitrosamine name	API/source	NDSRI structure
2470278-90-9	N-nitroso-rasigelene	Rasigelene	
2680527-91-5	N-nitroso-duloxetine	Duloxetine	
2755871-02-2	N-nitroso-varenicline	Varenicline	
92432-50-3	N-nitroso-trimetazidine	Trimetazidine	
55855-43-1	N-nitroso-desmethyl-diphenhydramine	Diphenhydramine	
84418-35-9	N-nitroso-propranolol	Propranolol	
**32635-81-7*	*N-nitroso-betahistine*	*Betahistine*	

*N-nitrosamine will not be tested in first instance, but is listed for sake of completeness

The most important substance information in terms of their Ames data and the enzymes involved in metabolic activation are summarized shortly as follows:

- **N-nitroso-rasagiline** - belongs to CPCA category 2 and induces a strong positive response in TA 100 and TA 1535 and low responses in E. coli WP2 *uvrA* (fold change: 2.2 in acetone and 3 in DMSO, MUTMAMIND 1) after activation with hamster S9 mix. Nothing is known about the CYPs involved in its metabolic activation.
- **N-nitroso-duloxetine** - No CPCA category. Induces a strong positive response in all three tester strains after activation with hamster S9 mix according to literature data. A low response was

observed in TA 100 and in E. coli WP2 *uvrA* in the MUTAMIND 1 project in both data sets. Metabolic activation involves CYP2B6, and CYP2C19.

- **N-nitroso-varenicline** – belongs to CPCA category 3. Although the compound is hardly soluble it was selected since it was found to be positive in strain TA 98 and TA 1537 according to literature data. In MUTAMIND 1 project strain TA 100 and TA 1535 responded well, WP2 *uvrA* showed the lowest fold change. There is no data regarding metabolic activation via CYP450 are available.
- **N-nitroso-trimetazidine**- belongs to CPCA category 3 and induces a strong positive response in TA 100 and TA 1535 and a low response in E.coli WP2 *uvrA* (fold change: 2.4 in acetone) as well as a negative response in E.coli WP2 *uvrA* when used in DMSO after activation with hamster S9 mix. Nothing is known about the CYPs involved in its metabolic activation. Also, solubility issues in DMSO were observed In the MUTAMIND 1 project.
- **N-nitroso-desmethyl-diphenhydramine** - belongs to CPCA category 1 and induces a strong positive response in all three tester strains after activation with hamster S9 mix. Metabolic activation involves human CYP2B6, and CYP2C19.
- **N-nitroso propranolol** – belongs to CPCA category 4 and induces responses in strain TA 98 according literature. Additionally positive responses were found in MUTAMIND 1 project in strain TA 1535 and TA 100. Metabolic activation involves human CYP2C19.
- **N-nitroso-betahistine** – belongs to CPCA category 1 and induces a strong positive response in all three tester strains in all tested solvents after activation with hamster S9 mix. Data from MUTMAMIND 1 suggest involvement of CYP2B6 and CYP2A6 in metabolic activation.

Sourcing and distribution of NA compounds

Based on the experience in MUTAMIND 1 sourcing of the NAs is difficult in terms of delivery times. The compounds were partly on stock available but for three of them delivery times are up to 11 weeks, since they were synthesized on request. A purity of about 95% was applied as requirement for the compounds, but false-positive test outcomes related to specific impurities then cannot be excluded and will have to be considered during data interpretation.

As the preferred method, compounds will be ordered by ICCR-Rossdorf GmbH. The compounds will then be provided to the other partner from the respective suppliers directly.

Pre-tests on solubility

The solubility of NAs, to be reconstituted/diluted by non-critical solvents for bacterial survival and/or enzyme stability, is crucial for all experiments. Highly concentrated stock solutions with completely dissolved compounds will be generated to define the minimal concentration of organic solvent required to ensure sufficient homogeneity and to avoid precipitation. The solvent concentration should be kept to a minimum to circumvent both non-specific cytotoxicity and inhibition of metabolic activation. Specific investigations evaluating potential solvent effects will be performed in objective 2.

Since the focus of the present study lies on DMSO as solvent, test for solubility will be done creating stock solutions of 50 mg/ml as a starting point. To keep the DMSO concentration in the final mixture as low as possible and for the approach described in objective 2, solubility tests will be performed up to a maximum concentration of 714 mg/mL, considering that the maximum dose required by OECD

471 is 5000 µg/plate and the DMSO concentration will be approximately 1.15% in the final treatment mixture.

In case of insufficient solubility at ambient temperature, solubility will be re-tested at 37°C and at lower concentrations. Optionally, solubility might also be enhanced by vortexing or an ultrasound treatment. All samples will be evaluated by considering opacity, re-precipitation of the solution, and color change. Collected information on solubility of the selected compounds during the course of MUTAMIND 1 are given in the table below (Table 2). Based on that, for already known compounds the solubility trial can be shortened. For unknown compounds the trial will be performed as described above. For N-nitroso-varenicline a further solubility trial should be done including warming of the stock solution.

Table 2: Solubility of NAs (data base MUTAMIND 1)

CAS	**N-nitrosamine name**	**Highest concentration in DMSO**	**Additional treatments**	**Further solvent**
2470278-90-9	N-nitroso-rasigelene	714 mg/mL	no	Acetone, methanol (714 mg/mL)
2680527-91-5	N-nitroso-duloxetine	714 mg/mL	ultrasound treatment & vortexing	Acetone (167 mg/mL)
2755871-02-2	N-nitroso-varenicline	167 mg/mL (suspension)	ultrasound treatment	no
92432-50-3	N-nitroso-trimetazidine	230 mg/mL	vortexing	Acetone (714 mg/mL)
55855-43-1	N-nitroso-desmethyl-diphenhydramine	Not tested	/	/
84418-35-9	N-nitroso-propranolol	714 mg/mL	Vortexing	Acetone (714 mg/mL)
**32635-81-7*	*N-nitroso-betahistine*	*167 mg/mL*		*deion. Water (50 mg/mL)*

*Nitrosamine will not be tested in first instance, but is listed for sake of completeness

If not mentioned otherwise a clear solution was achieved at the indicated concentrations in the specific solvent.

Metabolism and Phenotyping

Relevant Enzymes

To gather background information on enzymes involved in the metabolic activation of N-nitrosamines (NAs), including nitrosamine drug substance-related impurities (NDSRIs) and their respective active pharmaceutical ingredients (APIs), a comprehensive literature review was conducted. Relevant data on selected NAs and API-related compounds were systematically compiled. Regarding metabolic activation, CYP2E1 has been well-documented as a key enzyme in the metabolism of smaller nitrosamines, such as N-nitroso-dimethylamine (NDMA) and N-nitrosodiethylamine (NDEA). In contrast, the metabolism of the more structurally complex nitrosamine 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK) involves multiple cytochrome P450 (CYP) subfamilies. In humans, these include CYP1A2, CYP2A6, CYP3A4, CYP2D6, CYP2F1, CYP3A5, CYP2B6, CYP2B7, CYP2E1, and CYP2C8, while in rats, the implicated enzymes are CYP1A, CYP1A2, CYP2A4, CYP2A13, CYP2B1, and CYP3A. Furthermore, the metabolism of 4-(methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) is primarily mediated by CYP2A6 and CYP2A13. Findings from the MUTAMIND 1 study (publication in preparation), along with data reported by Li et al., provide evidence for the involvement of CYP2B6, CYP2C19, CYP2A6, and CYP3A4 in the metabolism of NDSRIs. Identifying additional enzymes responsible for NDSRI degradation remains a critical focus of this study. A comprehensive summary of cytochrome P450 enzymes involved in the metabolism of NAs, APIs, and NDSRIs is presented with references in **Table 3 and Table 4.**

Table 3: Cytochrome P450 (CYP450) enzymes relevant for the metabolic activation of selected NAs, with predominant CYP450 enzymes highlighted in bold

Compound	CAS No.	CYP450 enzymes relevant for metabolic activation of NAs	References
N-Nitrosodimethylamine	62-75-9	**2E1 isoenzyme**	(ATSDR, 2022; George, Tsuchishima, & Tsutsumi, 2019; Kay et al., 2021; Y. Li & Hecht, 2022)
N-Nitrosodiethanolamine	1116-54-7	**2E1 (hamster)**	(Frederick Peter Guengerich & Avadhani, 2018; Iarc, 2000; Y. Li & Hecht, 2022; Liu & Glatt, 2008; Loeppky, 2007)
4-(Methylnitrosamino)-1-(3-pyridyl)-1-(butanone) – NNK	64091-91-4	**1A (rat), 1A2 (human, rat), 2A1 (rat), 2A6 (human), 2A13**, **2B1 (rat)**, **3A (rat)**, **3A4 (human)**, 2A, 2D6, 2F1, 3A5, 2B6, 2B7, 2E1, 2C8	(Carlson, 2019; Hecht, 1998)
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol – NNAL	76014-81-8	**2A6**, **2A13**	(Carlson, 2019)
N-Nitrosodiphenylamine	86-30-6	**CYP450 (rat, mouse)**	(Appel et al., 1979; Appel, Rühl, & Hildebrandt, 1985; Appel, Ruhl, & Spiegelhalder, 1984; Schrenk, Schwarz, & Tennekes, 1982; Wakabayashi, Nagao, Kawachi, & Sugimura, 1982)
N-Nitrosomethylaniline	614-00-6	2B subfamily, 2A subfamily	(ATSDR, 2017; Sulc, Hodek, & Stiborova, 2010)

Table 4: CYP450 isoforms responsible for the degradation of APIs, with predominant CYP450 enzymes shown in bold

Compound	CAS No.	Main degradation pathway of non-nitrosated API	References for API metabolism
N-Nitroso-varenicline	2755871-02-2	Varenicline undergoes minimal metabolism with 92% excreted unchanged in the urine, 2-hydroxyvarenicline (only human 2.9% of dosage)	https://www.accessdata.fda.gov/drugsatfda_docs/label/2008/021928s008lbl.pdf ; Kassem et al., 2012
N-Nitroso-duloxetine	2680527-91-5	**1A2, 2D6**, (other) various hydroxy, dihydroxy metabolites which are further conjugated with glucuronic acid	Lanz et al. 2003; Drugbank, 2025
N-Nitroso-lorcaserin	2724616-80-0	1A1, 1A2, 2A6, 2B6, 2C19, 2D6, 2E1, 3A4, FMO1 Lorcaserin has extensive hepatic metabolism producing inactive compounds. Lorcaserin sulfamate (M1) is the major metabolite circulating in the plasma, and N-carbamoyl glucuronide lorcaserin (M5) is the major metabolite in urine. Other minor metabolites that are both excreted in urine are glucuronide or sulfate conjugates. Various hydroxy products shown in Cusack et al. (2013)	Gustafson, King, & Rey, 2013; Cusack et al., 2013
N-Nitroso-nortriptyline	55855-42-0	**2D6**, 1A2, 2C19, 3A4 Mainly demethylation and hydroxylation followed by glucuronidation	Drugbank, 2025; *PharmGKB*[(A)] https://www.pharmgkb.org/pathway/PA166163647
N-Nitroso-propranolol	84418-35-9	**1A2, 2D6** 4-hydroxypropranolol and N-desisopropylpropranolol. The latter reacts further to Naphthoxylactic acid. Also sulfation and glucuronidation	Routledge et al., 1929; *PharmGKB*[(A)] https://www.pharmgkb.org/pathway/PA166183426; Paterson, Conolly, Dollery (1970)
N-Nitroso-rasagiline	2470278-90-9	**1A2**, others 1-R-aminoindane (major metabolite), 3-hydroxy-N-propargyl-1-aminoindan, and 3-hydroxy-aminoindan	Wang et al., 2016; Lecht, Haroutiunian, Hoffman, Lazarovici (2007); Drugbank, 2025

N-Nitroso-trimetazidine	92432-50-3	No direct information about CYP450 specificity 60% are excreted unaffected. Trimetazidine can be oxidized at the piperazine ring to form trimetazidine ketopiperazine. Trimetazidine can also be N-formylated, N-acetylated, or N-methylated at the piperazine ring to form N-formyltrimetazidine, N-acetyltrimetazidine, and N-methyltrimetazidine respectively. Alternatively, trimetazidine can be demethylated at the 2, 3, or 4 position of the 2,3,4-trimethoxybenzyl moiety to form 2-desmethyltrimetazidine, 3-desmethyltrimetazidine, or 4-desmethyltrimetazidine. The desmethyltrimetazidine metabolites can undergo sulfate conjugation or glucuronidation prior to elimination.	Jackson et al., 1996; Drugbank, 2025
N-Nitroso-desmethyl-diphenhydramin	55855-43-1	2D6, (1A2, 2C9, and 2C19) Mainly N-Demethylation	Akutsu et al., 2007; Drugbank, 2025
N-Nitroso-betahistine	32635-81-7	MAO	Ihler et al., 2021; Val et al., 2010; Drugbank, 2025
1-Cyclopentyl-4-nitrosopiperazine	61379-66-6	No direct information Various hydroxyproducts conceivable	

Drug-metabolizing enzyme isoforms, their expression patterns, and activity levels can vary significantly across species, including humans, rodents, and hamsters. These interspecies differences are particularly relevant for the metabolic activation of N-nitrosamines (NAs). Key cytochrome P450 (CYP) enzymes implicated in this process include CYP2E1, CYP2A6, and CYP3A4. In rats, the CYP2A6 orthologues are CYP2A1 (female-dominant) and CYP2A2 (male-dominant), while CYP3A4 is orthologous to CYP3A1 and CYP3A2 (Cross & Ponting, 2021; Martignoni et al., 2006; Yang et al., 1990). The functional differences between human CYP2E1 and its murine and rat orthologues are relatively minor, with significant substrate overlap. However, CYP2E1 expression levels are markedly higher in hamsters compared to rats. Due to its similarity to the human enzyme, the rat serves as an appropriate model species for investigating CYP2E1-mediated metabolism (Martignoni et al., 2006). Regarding

CYP3A, four isoforms are expressed in humans (CYP3A4, CYP3A5, CYP3A7, and CYP3A43), with CYP3A4 being the most abundant in hepatic tissue. In contrast, the rat genome encodes six CYP3A isoforms: CYP3A1, CYP3A2, CYP3A9, CYP3A18, CYP3A23, and CYP3A62 (Martignoni et al., 2006). Among these, CYP3A1 and CYP3A2 serve as the primary orthologues of human CYP3A4 and are exclusively expressed in the liver (Martignoni et al., 2006). For this study, particularly in the enhanced Ames test, induced hamster S9 liver homogenates are utilized. The CYP450 enzyme composition in hamsters differs from that of both humans and rats in terms of isoform expression, activity, and substrate specificity. These variations can influence the metabolic activation and detoxification of NAs, highlighting the importance of species selection in toxicological studies. A comprehensive summary of interspecies CYP450 differences, as reviewed by Evans et al. 2025, is presented in **Figure 1**.

Comparative P450 Activity Based on Literature Review.

	Human CYP-like Activity	
Human CYP	**Rat**	**Hamster**
2A6	2A1 2A2	2A8
2C8	2C13	?
2C9	2C11	2C25
2C19	2C24	2C28
2D6	2D1	2D27
2E1	2E1	2E1
3A4	2 C	3A10

Figure 1: Comparison of orthologue CYP450 enzymes by Evans et al. (2025).

As previously mentioned, N-nitrosamines (NAs) undergo metabolic activation and bioactivation primarily through cytochrome P450 (CYP450) enzymes, which are localized in the endoplasmic reticulum of hepatocytes and other cell types. To assess the general susceptibility of selected nitrosamine drug substance-related impurities (NDSRIs) to CYP450-mediated metabolism, in vitro metabolism assays will be conducted using induced hamster liver S9 fractions. The metabolic reaction rate for each NDSRI will be determined as a function of substrate concentration. To further elucidate the specific CYP450 isoforms involved in NDSRI metabolism, experiments will be conducted using recombinant CYP450 enzymes. For this purpose, EasyCYP® bactosomes (XenoTech), which contain microsomal CYP450 enzyme systems, will be utilized in the presence and absence of calf thymus DNA (ctDNA). These commercially available formulations provide a biologically relevant system for studying membrane-bound CYP450 subfamilies. This approach offers two key advantages: 1.) it facilitates the identification of specific CYP450 isoforms responsible for the metabolic degradation of individual NAs, and 2.) it provides a cell-free, proof-of-principle assay that can be applied to future studies. A panel of CYP450 enzymes essential for the metabolism of various active pharmaceutical ingredients (APIs), NAs, and NDSRIs has been defined by Bellec et al. (1996) and the ICH M12 guideline. This panel includes CYP1A1, CYP1A2, CYP2A6, CYP2B6, CYP2C8, CYP2C9, CYP2C19, CYP2D6, CYP2E1, and CYP3A4-enzymes known to play a critical role in xenobiotic metabolism. In this study, we will adhere to these selected enzymes and follow the established protocols from MUTAMIND 1, which are outlined in the subsequent section.

Incubation with metabolic competent systems used for toxicological assessments

Our experimental workflow involves a systematic screening approach designed to detect specific NDSRI metabolism pathways. We will leverage the wealth of analytical methods and insights gained from the MUTAMIND study. It is planned to use the following screening protocol including five steps of investigation (**Figure 2**).

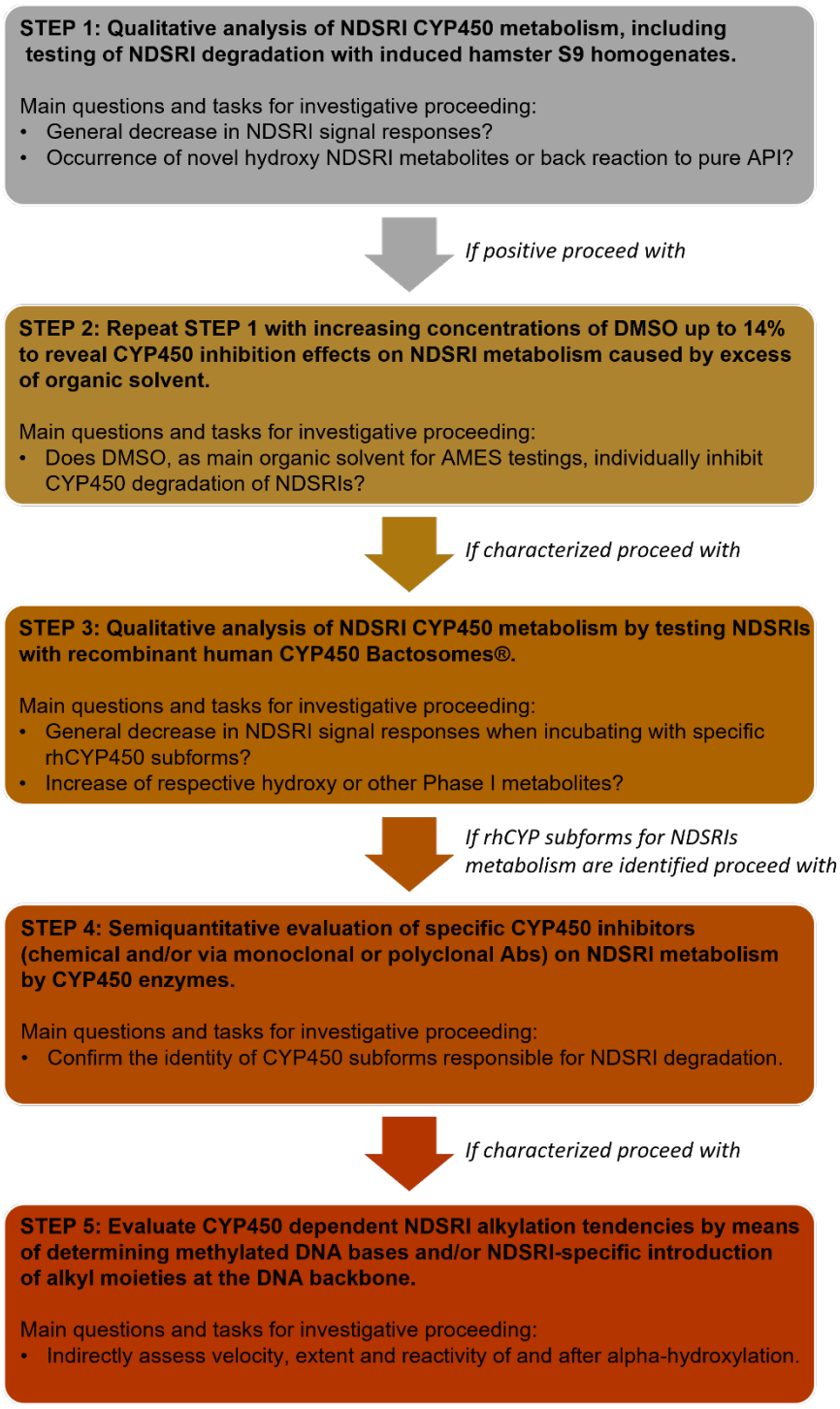


Figure 2: Five step investigation plan for the determination of NDSRI metabolites and specific DNA adducts.

Incubations with S9 homogenates

Test principle:

To guarantee stable metabolic conversion by the S9-mix, in particular by the CYP450 system, it is of utmost importance to specify the present enzymatic activity. Therefore, the most relevant CYP450 isoforms will be initially examined in the S9-mix using CYP450 isoform-specific substrates, which are degraded to respective metabolites in the presence of active enzymes. An overview of the S9 quality test and the used substrates/metabolites are listed in **Table 5**.

Table 5: Overview of the substrates/metabolites used for the S9-quality and metabolic competence check of the liver cell models.

CYP450 isoforms (human)	**Orthologue(s) (rat)***	**Substrate**	**Metabolite**	**Co-Factor**
CYP3A4	CYP3A9	Midazolam	Hydroxymidazolam	NADPH/H+
CYP2D6	CYP2D3	Dextromethorphan	Dextrorphan	NADPH/H+
CYP1A2	CYP1A2	Phenacetine	4-Acetaminophen (Paracetamol)	NADPH/H+
CYP2E1	CYP2E1	Chlorzoxazone	Hydroxychlorzoxazone	NADPH/H+
CYP2C8	CYP2C22**	Amodiaquine	N-deethyl-Amodiaquine	NADPH/H+
CYP2C9	CYP2C12	Tolbutamide	Hydroxytolbutamide	NADPH/H+
CYP2B6	CYP2B1/2B2	Bupropion	Hydroxybupropion	NADPH/H+
CYP2C19	CYP2C13/2C55	Mephenytoin	Hydroxymephenytoin	NADPH/H+
CYP2A6	CYP2A1/2A2	Nicotine, Pilocarpine	Cotinine/Norcotinine, Hydroxypilocarpine	NADPH/H+

*Except CYP2A6 according to Hammer, Schmidt, Marx-Stoelting, Potz, and Braeuning (2021). **Bhatt *et al.* (2022); Qian *et al.* (2010)

Experimental procedure:

All substrates are mixed individually with respective amounts of the S9-mix or liver cell lysates, phosphate buffered saline (pH 7.4), and the NADPH/H+ co-factor. The well-established assay is performed at 37°C and constant mixing for 45 min. In the same way, co-incubations in the absence of substrates and S9-mix excludes spontaneous/non-enzymatic conversion of the substrates or already present amounts of the respective metabolite in the substrate stock solutions. It is planned to implement pilocarpine as novel CYP2A6 substrate into our testing regime.

The final incubation set-up will consist of:

- 183 µL 100 mM PBS, containing 5 mM MgCl2 (pH7.4)
- 5 µL rat/hamster S9-mix (20 mg protein/mL)
- 10 µL 20 mM NADPH tetrasodium salt in PBS
- 2 µL substrate solution

The substrate concentration will be as follows:

CYP450 isoforms (human)	Substrate	Substrate concentration
CYP3A4	Midazolam	[25 µM] (Wandel et al., 1994)
CYP2D6	Dextromethorphan	[5 µM] (Kerry, Somogyi et al. 1994)
CYP1A2	Phenacetine	[50 µM] (Polasek, Miners, 2006)
CYP2E1	Chlorzoxazone	[120 µM] in PBS (Bachmann and Sarver 1996)
CYP2C8	Amodiaquine	[10µM] in PBS (Li, Björkman et al. 2002)
CYP2C9	Tolbutamide	[80 µM] in PBS (Zhang, Peng et al. 2022)
CYP2B6	Bupropion	[20 µM] in PBS (Turpeinen, Nieminen et al. 2004)
CYP2C19	Mephenytoin	[100 µM] in PBS (Lasker, Wester et al. 1998)
CYP2A6	Nicotine, Pilocarpine	Nicotine [60 µM] (Yamazaki, Inoue et al. 1999) Pilocarpine [10 µM] in PBS (Endo et al., 2007)

After the incubation procedure, all samples are treated with -20°C cold acetonitrile in threefold sample volume for protein precipitation. Subsequently, the protein aggregates are separated from the supernatant via centrifugation. All supernatants are evaporated at 60°C to obtain a dried residue. The residues are constituted with equal volumes of 0.1% formic acid and further subjected to LC-MS measurements. Here, a highly sensitive MRM experiment is performed to detect the metabolites, by considering the results from the negative controls (without the addition of substrate or S9-mix). Afterwards metabolic ratios are calculated by peak area integration and the following equation:

$$metabolic\ ratio\ [\%] = \frac{peak\ area_{metabolite}}{(peak\ area_{metabolite} + peak\ area_{substrate})} * 100$$

Peak areas are reported by the mass spectrometry (MS) detector in arbitrary units (counts per second, CPS). Metabolic activity will be assessed semi-quantitatively by comparing peak areas across all samples. However, direct quantification of these peak areas and conversion into molar concentrations—requiring calibration with authentic metabolite standards—is not within the scope of this study. Quantitative data, such as those needed for structure-activity relationship (SAR) analyses or calculations of pharmacological intrinsic activity, are not the primary focus. For each experimental condition, three technical replicates will be performed to ensure analytical reliability. Additionally, in the later phases of the study, biological replicates may be reanalyzed after several months to confirm metabolic capacity and data reproducibility. Following a defined incubation period, the metabolic ratio will be determined and correlated with both the percentage of substrate conversion and the protein concentration used in the assay. This allows for the calculation of metabolic activity according to the following equation:

$$metabolic\ activity\ = \frac{metabolic\ ratio\ [\%]}{t[min]/protein\ amount[mg]}$$

A very good conversion by S9 enzymes is expected for the reactions of the individual substrates, since this is a highly enriched CYP450 enzyme preparation that can recognise and convert a large number of substrates or molecular structures. Since it is currently unknown to what extent and to which type NDSRIs are metabolised, this must be clarified by experiments. It is planned to start with a concentration of 50 µM NDSRI in the presence/absence of S9 mix and to observe the decrease of the area under the curve (AUC) over time, which is an indication of metabolic conversion. In parallel, an MS/MS experiment will be used to determine whether new hydroxymetabolites or specific cleavage products arise that may already be described in the literature for the related APIs. If the concentration of the NDSRIs decreases due to S9 enzymes but no hydroxymetabolites or cleavage products arise, this could be an indication of unknown or reactive unstable metabolites. If the AUC of individual NDSRIs remains constant during incubation with S9 mix, this can be interpreted as resistance to CYP450 metabolism. Metabolism via phase II enzymes or no metabolism is conceivable. Additionally, in case of significant NDSRI degradations, increasing concentrations of DMSO will be added to investigate the inhibition effect of organic solvents on CYP450 activity. Planned concentrations will be 0.1, 0.5, 1, 5 and 14%.

Incubations with EasyCYP® Bactosomes

For our purposes we will use human recombinant EasyCYP® Bactosomes, which are characterized by already adjusted protein concentrations and have high CYP450 oxidoreductase activity, which guarantees good metabolic degradation rates under standardized conditions. Following the main CYP450 enzymes responsible for drug degradation in accordance to the ICH M12 guideline, EasyCYP® will be ordered for the following enzymes.

- CYP1A2
- CYP3A4
- CYP2D6
- CYP2C19
- CYP2E1
- CYP2B6
- CYP2A6
- CYP2C9
- CYP2C8

After the preincubation of the EasyCYP® preparations with NADPH in PBS for 5 minutes, the positive controls as well as NDSRIs were separately added to each aliquot and further incubated for 4h at 37°C. The general protocol and composition for the incubation of positive controls as well as the NDSRIs will be as follows (**Table 6 and Table 7**).

Table 6: General protocol for rhCYP incubations listing the composition in microliters. In case that ctDNA is added for adduct analysis the respective listed volume must be fortified into the incubation approach. Otherwise, the volume can be substituted by 17 µL of PBS.

EasyCYP® (10 mg/mL Protein) [µL]	PBS, pH 7.4 [µL]	NDSRI (1 M working solution) [µL]	ctDNA (1763,4 ng/µl) [µL]	NADPH (20mM in PBS) [µL]
5	72	1	17	5

Table 7: Utilized positive controls for bactosomal incubations.

	EasyCYP® (10 mg/mL Protein)	PBS, pH 7.4	CYP Substrate	NADPH (20mM in PBS)
CYP1A2R	5 µl	89 µl	Phenacetine (50µM) 1 µl	5 µl
CYP3A4R	5 µl	89 µl	Midazolam (25µM) 1 µl	5 µl
CYP2D6R	5 µl	89 µl	Dextromethorphan (1µM) 1 µl	5 µl
CYP2C19R	5 µl	89 µl	S-Mephenytoin (50 µM) 1 µl	5 µl
CYP2E1R	5 µl	89 µl	Chlorzoxazone (120µM) 1 µl	5 µl
CYP2B6R	5 µl	89 µl	Bupropion (50 µM) 1 µl	5 µl
CYP2A6R	5 µl	89 µl	Nicotine (30 µM) 1 µl	5 µl
CYP2C9RH	5 µl	89 µl	Tolbutamide (20 µM) 1 µl	5 µl
CYP2C8R	5 µl	89 µl	Amodiaquine (10 µM) 1 µl	5 µl

Similar to the incubations with S9 homogenate, the reaction is stopped by the addition of ACN (-20°C) and a protein precipitation is induced. After centrifugation at 14,000 rcf for 5 min, 1 mL of distilled water is added to the supernatant and the sample is transferred to Chromabond® HLB 96-well plates for solid phase extraction. This purification is carried out according to the manufacturer's instructions. The samples are then analysed by LC-MS/MS. In case a CYP450 enzyme is identified that causes the degradation of NDSRI, an optional counter-test can be carried out. For this purpose, CYP450 inhibitors are added to the approach, which should prevent the conversion of the NDSRI to the metabolite. Similarly, NDSRIs can be incubated again with S9 mix with the addition of a specific inhibitor. It is intended to use the recommendations of the ICH M12 guideline and the following inhibitors in excess (**Table 8**)

Table 8: Recommended CYP inhibitors by the ICH M12 guideline. For CYP2E1 it is intended to use chlormethiazole as inhibitor. For CYP2A6 tranylcypromine will serve as reported inhibitor.

CYP Enzyme	Inhibitor
CYP1A2	α-Naphthoflavone, Furafylline*
CYP2B6	Clopidogrel*, Ticlopidine*, Thiotepa*
CYP2C8	Gemfibrozil glucuronide*, Montelukast, Phenelzine*
CYP2C9	Sulfaphenazole, Tienilic acid*
CYP2C19	Loratadine, Ticlopidine*
CYP2D6	Paroxetine*, Quinidine
CYP3A	Azamulin*, Itraconazole, Ketoconazole, Troleandomycin*

* Designated as time dependent inhibitor. When used, those inhibitors should be pre-incubated with the experimental system.

Liquid chromatography – tandem mass spectrometry analysis

General settings

During all studies liquid chromatography – mass spectrometry (LC-MS) measurements will be performed with a Sciex QTRAP® 6500 triple quadrupole mass spectrometers equipped with a Shimadzu Nexera® ultra-performance liquid chromatograph. This system is capable to work under high pressure up to 400 bar allowing the use of small particle sizes in chromatographic columns to increase peak sharpness and the methods' selectivity.

NDSRI precursor and metabolite identification after S9 and bactosomal incubations

The depletion of nitrosamine drug substance-related impurities (NDSRIs) and the concurrent formation of their metabolites over time will be monitored using a systematic LC-MS/MS approach. This methodology integrates incubations with induced hamster S9 fractions and recombinant human bactosomes to assess metabolic transformation. For LC-MS analysis, the previously described analytical setup will be employed to separate parent NDSRIs and their respective metabolites based on their physicochemical properties. A mass spectrometer will be operated in both positive and negative ionization modes, depending on the analyte's chemical characteristics, to ensure sensitive and selective detection. To quantitatively assess NDSRI degradation, calibration curves will be generated, allowing for time-dependent concentration monitoring. The formation of NDSRI metabolites, if sufficiently stable, will be evaluated qualitatively and semi-quantitatively, given the current lack of reference standards for these metabolites. The analytical workflow will begin with a well-established LC-MS setup, utilizing the Agilent Poroshell® C18 column (100 × 3 mm, 2.7 µm). The primary screening focus will be on hydroxylated metabolites of NDSRIs, using targeted enhanced product ion (EPI) screening and precalculated mass transitions. Additionally, potential API-derived metabolites—which may also be relevant to NDSRI metabolism—will be examined. These include metabolic transformations such as ester hydrolysis and demethylation. A detailed overview of the data representation and analysis strategy is provided in **Figures 3 and 4**.

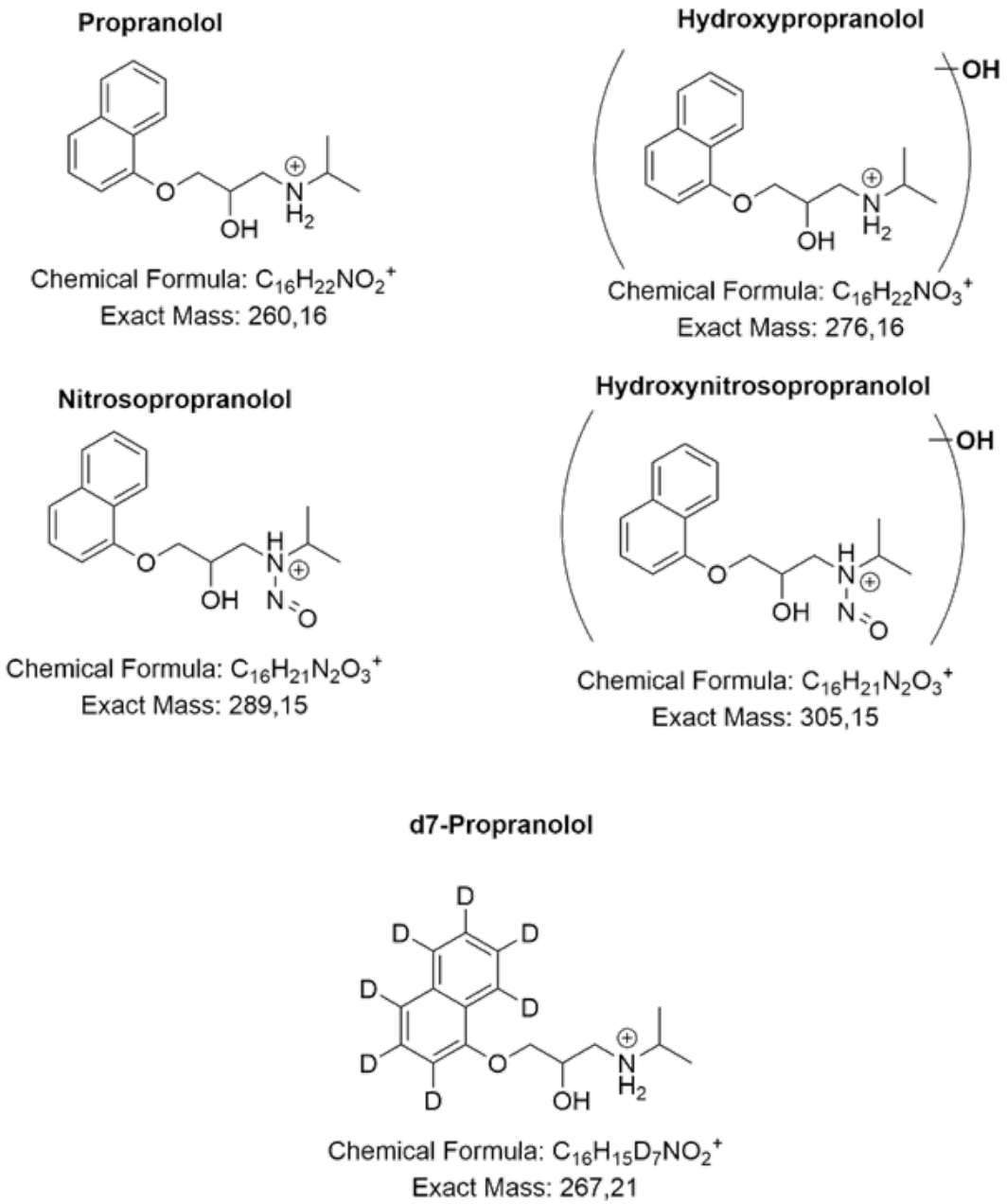


Figure 3: Utilized m/z values for standard untargeted hydroxylation products of propranolol as well as nitrosopropranolol. d7-propranolol was used as internal standard.

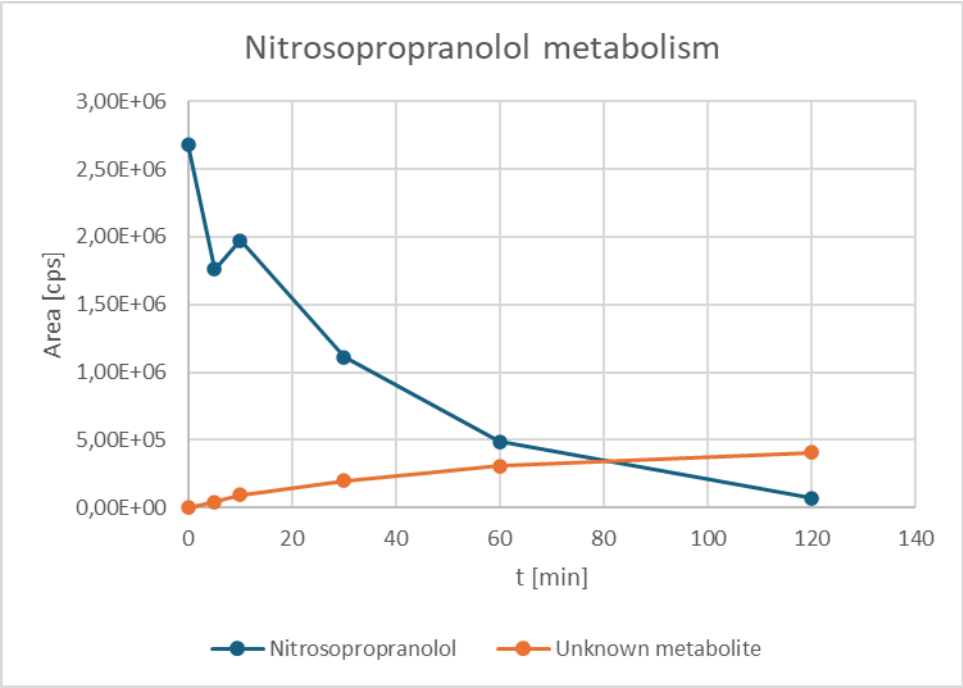


Figure 4: Mean signal responses of N-Prop and an unknown metabolite after PHH incubation for 120 min. Here, no hydroxynitrosopropranolol was determined after incubation. Same ion transitions (m/z 289 -> m/z 259, loss of nitroso group) as nitrosopropranolol but more polar (elutes earlier than NA-Propra). n=3.

This methodology provides valuable insights into the metabolic pathways and potential risks associated with NDSRIs. The incubation protocol involves preparing mixtures containing the NDSRI compound, appropriate cofactors such as NADPH, and either hamster S9 fractions or human bactosomes. These mixtures are maintained under optimal conditions, including specific temperature and pH, to ensure enzymatic activity. Aliquots are taken at predetermined time points at 0, 15, 30, 60, and 120 minutes, to capture the kinetic profile of NDSRI metabolism. To terminate the reactions, enzymatic activity in the aliquoted samples is quenched using cold organic solvents in form of acetonitrile. The quenched samples are then centrifuged to remove precipitated proteins, and the supernatant is collected for analysis. For LC-MS analysis, the above-mentioned analytical setup is employed to separate the parent NDSRI and its metabolites based on their physicochemical properties. A mass spectrometer, operating in appropriate ionization modes (positive or negative), is used to detect and quantify the analytes. Quantification and data analysis involve generating calibration curves using authentic standards of the NDSRI and its metabolites to ensure accurate quantification. The concentrations of the parent NDSRI and metabolites at each time point are determined, and concentration-time profiles are plotted to visualize the depletion of the NDSRI and the formation of metabolites. Kinetic parameters, such as the NDSRI depletion, as well as the formation rate of metabolites, are calculated. Comparative analysis entails contrasting the metabolic profiles obtained from hamster S9 fractions with those from human bactosomes. This assessment of species-specific differences in metabolism can inform the selection of appropriate animal models for in vivo studies and predict human metabolic pathways. This systematic approach enables the elucidation of metabolic pathways and the assessment of interspecies differences, providing critical insights during the drug development process.

Adduct analysis

So far, the BfArM has the highly sensitive method for detecting DNA methylation, particularly at deoxyguanosine, which was also used in MUTAMIND 1. It is planned to investigate further DNA alkylations or reactions with NDSRIs for one or two substances. The determination of DNA adducts will be performed with predefined targeted as well as untargeted experiments. In case of targeted experiments, *m/z* values of pre-calculated nucleoside adducts will be integrated in an enhanced product ion experiment. Here, the linear ion trap of the third quadrupole is engaged to significantly increase the sensitivity and limit of detection. Structure elucidation is mandatory to confirm DNA alkylation. In case of twofold, or even threefold, alkylated nucleosides or to detect alkylated nucleosides with unpredicted residues, untargeted experiments will be performed with neutral loss experiments. Here, the loss of deoxy ribose is monitored to detect uncommon residues or respective mass shifts. A large number of publications contain references to the analysis of DNA nucleosides by LC-MS/MS, so that extensive research and review of these methods was carried out in advance (He et al., 2019). Initially, standard chromatographic conditions were tested to get first insights with regards to the retention time and ionization tendency under ESI conditions. The following parameters and equipment were initially utilized for these purposes:

Column: Waters XSelect® HSS T3 XP (100 x 3 mm. 2.5 µm)
Eluents: Channel A: 0,2% formic acid
Channel B: acetonitrile, LC-MS grade

AMES test

General

The bacterial reverse mutation assay, or Ames test, is a mutagenicity test, which is in regulatory use, since the first versions of the OECD TG471 (*Salmonella typhimurium*) and TG 472 (*Escherichia coli*) guidelines, which were issued in 1983. Only minor changes were implemented in the document when revised in 1997 (merge of TG472 with TG471) and 2020. Based on its good performance in detecting rodent carcinogens with e.g., sensitivity of 49.4%, specificity of 80.3%, concordance of 62.9%, positive predictivity of 73.7% and negative predictivity of 55.1%, as calculated from 988 substances by Matthews, Kruhlak, Cimino, Benz, and Contrera (2006) [see also Kirkland, Aardema, Henderson, and Muller (2005) for 717 compounds], the Ames test represents a key test and the entry assay in different test batteries for genotoxicity testing. Over the four decades of use, thousands of test substances were investigated for their mutagenic potential. The bacterial tester strains, the test procedures and the test conditions used are described in detail in OECD TG471. The guideline takes into account that some chemical classes need a specific test design for reliable prediction of mutagenicity (e.g. azo-dyes and diazo compounds, glycosides etc.). These include the plate incorporation and pre-incubation methods, the addition of metabolic enzyme systems (S9 liver homogenate) from different species (e.g., rat, hamster), and variation in liver S9 protein content (10 up to 30%). Based on prior discussion with EMA, deviations from the standard procedure are acceptable if scientifically justified.

Ames data sets obtained in MUTAMIND 1

The selected NDSRIs which should be investigated for their suitability to be used as positive controls were partly already tested in MUTAMIND1 with Salmonella strain TA 1535, TA 100 and WP2 *uvrA*. As starting point the revealed data sets were summarized as follows:

Table 9: AMES data of selected NAs (data base MUTAMIND 1).

CAS	N-nitrosamine name	Lowest mutagenic concentration (in µg/plate)		
		TA 1535	TA 100	WP2 uvrA
2470278-90-9	N-nitroso-rasigelene	333	100	1000
2680527-91-5	N-nitroso-duloxetine	33	100	2500
2755871-02-2	N-nitroso-varenicline	1000	1000	negative
92432-50-3	N-nitroso-trimetazidine	33	100	negative
84418-35-9	N-nitroso-propranolol	5000	5000	negative
55855-43-1	N-nitroso-desmethyl-diphenhydramine	n.t.	n.t.	n.t.
**32635-81-7*	*N-nitroso-betahistine*	*3*	*3*	*10*

*Nitrosamine will not be tested in first instance, but is listed for sake of completeness
n.t.: not tested in MUTAMIND1 project

The most sensitive strain was defined as the strain with the highest response (ratio of treated group versus solvent control). All strains were tested up to 5000 µg/plate as requested by OECD 471. The lowest test concentration was 3 µg/plate. The data sets in Table 3 shows outcomes of Ames tests using DMSO as solvent and 30% (v/v) non-induced hamster S9 mix as metabolization system.

Types of S9 homogenates

During the present project one batch of non-induced hamster S9 homogenate, which is produced at ICCR-Rossdorf GmbH, and two commercially available batches of induced hamster S9 homogenate, obtained from MOLTOX, USA, will be used.

The hamster S9 liver microsomal fraction prepared inhouse will be obtained from non-induced livers of Syrian golden hamsters. The protein concentration in the S9 preparation is usually between 20 and 45 mg/ml. Each batch of S9 homogenate is routinely tested with 2-aminoanthracene as well as Congo red before use in the Ames test to ensure metabolic activity. Furthermore, the sensitivity of the batch to metabolize N-nitrosamines will be proven using 1-Cyclopentyl-4-nitrosopiperazine (1-CPNA).

An appropriate quantity of S9 supernatant is thawed and mixed with S9-cofactor solution. The amount of S9 supernatant is normally 30% (v/v). The concentrated cofactor solution yields the following concentrations in the S9 mix: 80 mM $MgCl_2$, 33 mM KCl, 20 mM Glucose-6-phosphate, 2.8 units/ml glucose-6-phosphate-dehydrogenase, 4 mM NADP, 2.0 mM NADH, 2.0 mM FMN in 100 mM sodium-ortho-Phosphate-buffer, pH 7.4. During setup of the experiment the S9-mix is stored on ice. Preparation of the S9-mix is performed according to Ames et al. (1977) and Michael J Prival and Mitchell (1982).

The induced hamster S9 will be stored under identical conditions as the inhouse produced S9 homogenate, maintaining constant quality at -70°C or below. The protein content ranges from 25to 40 mg/mL. The S9 mix will be prepared using the same cofactor solution as for the non-induced S9 and will be used as 30%(v/v) in the mix.

Impact of solvent – DMSO

The influence of organic solvents on the mutagenic response of NAs was also addressed in the literature. When DMSO was used as the solvent in Ames tests, no mutagenicity was detected for NDMA and a decreased response for NDEA (Y. Mori, Yamazaki, & Konishi, 1987; Yahagi et al., 1977). This result had been subsequently linked to the inhibition of CYP450, in particular, CYP2E1 by DMSO (Jia & Liu, 2007). Recently, the effect of DMSO, DMF and acetonitrile on induced rat and hamster liver S9 was investigated. Again, significant inhibition of CYP2E1 activity was present (Lynch & Harvey, 2022).

CYP2E1 is the predominant P450 isoform mediating metabolism of small or terminal NAs, and, if inhibited by DMSO as a solvent, it would lead to a reduction of metabolic activation, as in the case of NDMA, and could, in principle, lead to negative test results. For larger NAs, such as the symmetrical compounds N-nitrosodi-n-propylamine, N-nitrosodi-n-butylamine and N-nitrosodi-n-isobutylamine, positive results were instead reported (Andrews & Lijinsky, 1980). This in line with the observation that different CYP isoforms are able to metabolize NAs (Trejo-Martin et al., 2022), including, for example, CYP2A6 for small to medium NAs, and CYP3A4 for large compounds (Cross & Ponting, 2021).

The effects of various Ames test conditions were investigated by Bringezu and Simon (2022) that analyzed the mutagenic potential of NDMA, NDEA, N-nitroso-dipropylamine, and N-nitrosodi-butylamine. These results led to the conclusion that the differences between using low volumes of DMSO (1.6% v/v) and water on the Ames test results were minimal except for certain simple nitroso alkylamines metabolized by CYP2E1 (e.g., reduced mutagenic activity with DMSO for NDMA, NDEA) (Trejo-Martin et al., 2022). Reviewing the data set compiled in the Mutamind project, solvents had no or only a minor effect on the mutagenic potential of the NAs. In rare cases a lowered response was observed. Based upon this knowledge it is recommended using the lowest concentration of solvent required, while avoiding precipitation and cytotoxicity. This issue will be addressed using different concentrations of DMSO in the final mixture in objective 2 using the plate incorporation and pre-incubation assay.

Test procedures

Frozen bacterial stocks are thawed and pre-cultured for up to 8 h (required bacterial density: 10^8-10^9 cells/mL). Then bacteria are mixed with the test item preparation (formulated in an adequate vehicle) without or with a metabolic activating system (e.g., induced hamster liver S9 homogenate, non-induced hamster liver S9 homogenate) and plated on selective agar (without histidine or tryptophan, depending on the tester strain). Negative, solvent and positive controls are tested in parallel for acceptability reasons. After a suitable period of incubation (48 - 72 h), revertant colonies are counted by means of a computerized analysis system and recorded. In the case of test item precipitation on the plates, manual scoring may be necessary. All incubations are performed at 37°C. Three plates per test group are tested in parallel.

The arithmetic mean revertant rate and the standard deviation per test group as well as the relative revertant factor, compared to the corresponding vehicle control, are calculated and reported. A test item is considered as a mutagen, if a biologically relevant increase in the number of revertants of twofold or above (strains TA 98, TA 100, and EC WP2 *uvr*A) or threefold or above (strains TA 1535 and TA 1537) the spontaneous mutation rate of the corresponding solvent control is observed. A dose dependent increase in the number of revertant colonies below the threshold is regarded as an indication of a mutagenic potential if reproduced in an independent second experiment. However,

whenever the colony counts remain within the historical data range of negative and solvent controls, such an increase is not considered biologically relevant.

Bacterial toxicity will be taken into account while assessing the mutagenicity data. Toxicity of the test item results in a reduction in the number of spontaneous revertants (below a factor of 0.5) or a clearing of the bacterial background lawn.

The *Salmonella typhimurium* and *E. coli* reverse mutation assay is considered acceptable, if it meets the following criteria of OECD TG471:

1 Regular background growth in the negative and solvent controls;
2 The spontaneous reversion rates in the negative and solvent controls are in the range of the current historical control data of the testing laboratory;
3 The positive control substances should produce an increase above the threshold of twofold (strains TA 98, TA 100, and EC WP2 *uvr*A) or threefold (strains TA 1535 and TA 1537) of the colony count of the corresponding solvent control;
4 A minimum of five analyzable dose levels should be present with at least three dose levels showing no signs of toxicity, evident as a reduction in the number of revertant colonies below the indication factor of 0.5.

Plate-incorporation assay

In the standard Ames test (plate incorporation) all ingredients are mixed and poured onto the selective agar plates (without histidine or tryptophan, depending on the tester strain) and incubated for 48 to 72 h in an incubator at 37°C:

- 100 µL* of test item solution at each dose level (solvent or reference mutagen solution = positive control),
- 500 µL of S9-mix for experiments with metabolic activation or S9-mix substitution buffer for experiments without metabolic activation (7 parts of 100 mM sodium-ortho-phosphate-buffer pH 7.4 and 3 parts of a 0.15 M KCl solution),
- 100 µL of bacterial suspension (from pre-culture of the respective strains),
- 2000 µL overlay agar (sterile; TA strains: 7 g agar-agar, 6 g NaCl, 10.5 mg L-histidine x HCl x H_2O, 12.2 mg biotin add 1 L in deionised water; EC strain: 7 g agar-agar, 6 g NaCl, 10.2 mg tryptophan add 1 L in deionised water)

For the pre-incubation method, with direct incubation of the test item with the bacteria in solution, the following materials are mixed in a test tube, and subsequently incubated at 30°C for 30 min:

- 100 µL* test item solution at each dose level (solvent or reference mutagen solution = positive control),
- 500 µL of S9-mix for experiments with metabolic activation or S9-mix substitution buffer for experiments without metabolic activation (7 parts of 100 mM sodium-ortho-phosphate-buffer pH 7.4 and 3 parts of a 0.15 M KCl solution),
- 100 µL of bacterial suspension (from pre-culture of the strains)

After pre-incubation, 2.0 mL overlay agar (approx. 45°C; contents see above) will be added to each tube. The mixture is poured on selective agar plates (without histidine or tryptophan depending on the

tester strain used). After solidification, the plates are incubated upside down for 48 to 72 h in an incubator (de Serres & Shelby, 1979).

*Depending on the solubility of the tested nitrosamine with regard to the goal of reducing the concentration of the solvent in the final mixture the applied volumes of the test solution and solvent control may differ from the above-mentioned volume.

Bacterial strains

A certain specificity of NAs to revert the missense Ames tester strains with excision-repair deficiency i.e., TA 1535 and TA 100 was evident in former studies. The base-pair substitution strain TA 1535 was, therefore, preferentially selected to perform several *in vitro* bacterial mutagenicity studies for this compound class (see e.g., (S. Y. Lee & Guttenplan, 1981; Nagao, Suzuki, Yasuo, Yahagi, & Seino, 1977; Rao, Cox, Allen, Epler, & Lijinsky, 1981; Rao, Hardigree, Young, Lijinsky, & Epler, 1977; Rao et al., 1979). The *Salmonella* Typhimurium strain YG7108, a derivative of *Salmonella* Typhimurium TA 1535 is highly sensitive to the mutagenic properties of methylating agents. It lacks two genes (*ada* and *ogt*) coding for the DNA repair enzyme O^6-methylguanine DNA methyltransferase (Yamada, Sedgwick, Sofuni, & Nohmi, 1995). YG7108 appears to show a high sensitivity to N-alkylnitrosamines, may be, due to the lack of such DNA repair enzymes (Fujita & Kamataki, 2001).

It is clearly demonstrated that the strains outlined in the respective OECD TG471 guideline are adequate to detect the mutagenic response of NAs. More specifically, TA 100 and TA 1535 are able to identify mutagenicity of this subclass of NAs studied. In addition, it was also shown that E. coli WP2 *uvrA* is highly sensitive to detect the mutagenic activity of the tested NAs and was, therefore, combined with TA 1535 and TA 100 in the MUTAMIND 1 project.

In MUTAMIND 2 testing of NAs will include all strains recommended in OECD test guideline TG471 "*because initial and emerging molecular analyses indicated that, collectively, they permitted the recovery of all six classes of base-substitution mutations, three classes of frameshift mutations, and some small deletions*" (R. V. Williams et al., 2019). These bacterial strains have multiple/different modes of reversion, and each strain has a particular mutation target (hotspot). The recorded response of the Ames test may thereby depend on sensitivity of the target site to the DNA-reactive moiety and the susceptibility of the specific DNA adduct-base to result in mutations (Levy et al., 2019; G. M. Williams, 2018; R. V. Williams et al., 2019).

The histidine dependent strains are derived from *Salmonella typhimurium* strain LT2 through mutations in the histidine locus. Additionally due to the "deep rough" (rfa^-) mutation they possess a faulty lipopolysaccharide envelope which enables substances to penetrate the cell wall more easily. A further mutation causes a reduction in the activity of an excision repair system. The latter alteration includes mutational processes in the nitrate reductase and biotin genes produced in a UV-sensitive area of the gene named *uvrB*$^-$. In the strains TA 98 and TA 100 the R-factor plasmid pKM 101 carries the ampicillin resistance marker.

Strain WP2 and its derivatives carry the same defect in one of the genes for tryptophan biosynthesis. Tryptophan-independent (Trp^+) mutants (revertants) can arise either by a base change at the site of the original alteration or by a base change elsewhere in the chromosome so that the original defect is suppressed. This second possibility can occur in several different ways so that the system seems capable of detecting all types of mutagen which substitute one base for another. Additionally, the *uvrA*

derivative is deficient in the DNA repair process (excisable repair damage). Such a repair-deficient strain may be more readily mutated by agents.

When summarized, the mutations of the *Salmonella typhimurium* strains and the *Escherichia coli* strain used in this study can be described as follows:

Table 10: characteristics of the proposed bacterial strains.

Strains	**Genotype**	**Type of mutations indicated**
Salmonella typhimurium		
TA 1537	*his* C 3076; *rfa*$^-$*; uvrB*$^-$	frame shift mutations
TA 98	*his* D 3052; *rfa*$^-$*; uvrB*$^-$; R-factor	" "
TA 1535	*his* G 46; *rfa*$^-$; uvrB$^-$	base-pair substitutions
TA 100	*his* G 46; *rfa*$^-$*; uvrB*$^-$; R-factor	" "
Escherichia coli		
WP2 *uvrA*	*trp*$^-$; *uvrA*$^-$	base-pair substitutions and others

Regular checking of the properties of the *Salmonella typhimurium* and *Escherichia coli* strains regarding the membrane permeability, ampicillin resistance; UV sensitivity, and amino acid requirement as well as normal spontaneous mutation rates is performed according to Ames *et al.* (1977) and Maron and Ames (1983). In this way it is ensured that the experimental conditions set down by Ames are fulfilled.

The bacterial strains TA 1535, TA 1537, TA 98, TA 100, and WP2 *uvrA* were obtained from Trinova Biochem GmbH (35394 Gießen, Germany).

Test procedure of objective 1: Positive control selection

Based on solubility data N-nitroso-rasigelene, N-nitroso-duloxetine and N-nitroso-propanolol can be tested with a minimum DMSO concentration of 1.15% in the final mixture, achieving the maximum test concentration of 5000 µg/plate. N-nitroso-trimetazidine was dissolved in DMSO at 230 mg/mL resulting in a DMSO concentration of 3.5% in the final mixture, achieving 5000 µg/plate. Regarding N-nitroso-varenicline and N-nitroso-desmethyl-dephenyhydramine solubility trials will be performed. It is aimed to keep the concentration of DMSO for this NAs as low as possible as well.

The assay method will follow the pre-incubation as described above. For each NDSRI six concentrations will be tested using two batches of induced hamster S9 mix and one batch of non-induced hamster S9 mix, including appropriate negative control (solvent control) and strains specific positive controls (2-AA). The top dose will be 5000 µg/plate, based on OECD 471. The tester strains will be TA 98, TA 1537, TA 1535, TA 100 and WP2 *uvrA.*

Test procedure of objective 2a & 2b: Evaluation of solvent concentration on positive control activity

Based on the outcome of objective 1, one of the S9 batches used in the previous step will be used for investigation of possible solvent effects of DMSO.

Therefore, preincubation and plate incorporation method will be applied. The choice of N-nitrosamines to be implemented in this objective needs to be discussed based on the results of objective 1. Only sensitive strains showing robust responses will be included in this approach. That means a minimum increase in the number of revertants of twofold or above (strains TA 98, TA 100, and EC WP2 *uvr*A) or threefold or above (strains TA 1535 and TA 1537). Strains showing no positive effects or only borderline effects will be excluded, in general. Furthermore, the increase should be at intermediate concentrations within the range of 3 to 5000 µg/plate. This criterium in combination with a high solubility in DMSO is needed to apply different quite low DMSO concentrations in the reaction mixture.

It is planned to test for the selected N-nitrosamines at least four appropriate concentrations in the selected strains in the plate incorporation assay and in the pre-incubation assay. The aimed DMSO concentration are numerically different in the plate incorporation and the pre-incubation assay. This is due to the different total volume of the reaction mix (with or without overlay agar, cf. Test procedures) but apply to the same volume of DMSO in the test substance preparation itself.

The aimed DMSO concentrations are as follows:

Plate incorporation assay: 0.3%, 1% and 4% (v/v)

Pre-incubation assay: 1%, 3% and 14% (v/v)

References

ATSDR. (2017). Toxicological Profile for N-Nitrosodiphenylamine. 113.

ATSDR. (2022). Toxicological Profile for N-Nitrosodimethylamine (NDMA) Draft for Public Comment. 216.

Akutsu T, Kobayashi K, Sakurada K, Ikegaya H, Furihata T, Chiba K. Identification of human cytochrome p450 isozymes involved in diphenhydramine N-demethylation. Drug Metab Dispos. 2007 Jan;35(1):72-8. doi: 10.1124/dmd.106.012088. Epub 2006 Oct 4. PMID: 17020955.

Ames, B. N., McCann, J., & Yamasaki, E. (1977). Methods for detecting carcinogens and mutagens with the Salmonella/mammalian microsome mutagenicity test. In B. J. K. e. al. (Ed.), Handbook of Mutagenicity Test Procedures. Amsterdam: Elsevier.

Andrews, A. W., & Lijinsky, W. (1980). The mutagenicity of 45 nitrosamines in the Salmonella typhimurium. Teratog Carcinog Mutagen, 1(3), 295-303.

Appel, K. E., Ruf, H. H., Mahr, B., Schwarz, M., Rickart, R., & Kunz, W. (1979). Binding of nitrosamines to cytochrome P-450 of liver microsomes. Chemico-Biological Interactions, 28(1), 17-33.

Appel, K. E., Rühl, C. S., & Hildebrandt, A. G. (1985). Oxidative dealkylation and reductive denitrosation of nitrosomethylaniline in vitro. Chemico-Biological Interactions, 53(1-2), 69-76.

Appel, K. E., Ruhl, C. S., & Spiegelhalder, B. (1984). Dinitrosation of diphenylnitrosamine in vivo. Toxicology Letters, 23, 353-358.

Bachmann K, Sarver JG. Chlorzoxazone as a single sample probe of hepatic CYP2E1 activity in humans. Pharmacology. 1996 Mar;52(3):169-77. doi: 10.1159/000139381.

Bellec, G., Dreano, Y., Lozach, P., Menez, J. F., & Berthou, F. (1996). Cytochrome P450 metabolic dealkylation of nine N-nitrosodialkylamines by human liver microsomes. *Carcinogenesis, 17*(9), 2029-2034.

Bhatt S, Manhas D, Kumar V, Gour A, Sharma K, Dogra A, Ojha PK, Nandi U. Effect of Myricetin on CYP2C8 Inhibition to Assess the Likelihood of Drug Interaction Using In Silico, In Vitro, and In Vivo Approaches. ACS Omega. 2022 Apr 4;7(15):13260-13269. doi: 10.1021/acsomega.2c00726. PMID: 35474783; PMCID: PMC9026026.

Bringezu, F., & Simon, S. (2022). Salmonella typhimurium TA 100 and TA 1535 and E. coli WP2 uvrA are highly sensitive to detect the mutagenicity of short Alkyl-N-Nitrosamines in the Bacterial Reverse Mutation Test. Toxicol Rep, 9, 250-255.

Carlson, E. S. (2019). METABOLIC ACTIVATION AND DNA-DAMAGING PROPERTIES OF CARCINOGENIC N-NITROSAMINES. University of Minnesota.

Cusack KP, Koolman HF, Lange UE, Peltier HM, Piel I, Vasudevan A. Emerging technologies for metabolite generation and structural diversification. Bioorg Med Chem Lett. 2013 Oct 15;23(20):5471-83. doi: 10.1016/j.bmcl.2013.08.003.

Cross, K. P., & Ponting, D. J. (2021). Developing Structure-Activity Relationships for N-Nitrosamine Activity. Comput Toxicol, 20.

de Serres, F. J., & Shelby, M. D. (1979). Recommendations on data production and analysis using the Salmonella/microsome mutagenicity assay. Mutation Research/Environmental Mutagenesis and Related Subjects, 64(3), 159-165.

Fujita, K., & Kamataki, T. (2001). Role of human cytochrome P450 (CYP) in the metabolic activation of N-alkylnitrosamines: application of genetically engineered Salmonella typhimurium YG7108 expressing each form of CYP together with human NADPH-cytochrome P450 reductase. Mutat Res, 483(1-2), 35-41.

Evans, K., Boitnotte, S., Zeiger, E., Cheung, J., Lynch, A., A comparative analysis of select P450 enzymes in uninduced and PB/BNF-induced hamster and rat liver S9, Mutation Research - Genetic Toxicology and Environmental Mutagenesis, Volume 902, 2025, 503855, ISSN 1383-5718, https://doi.org/10.1016/j.mrgentox.2025.503855.

Endo, T., Ban, M., Hirata, K., Yamamoto, A., Hara, Y., Momose, Y. Involvement of CYP2A6 in the Formation of a Novel Metabolite, 3-Hydroxypilocarpine, from Pilocarpine in Human Liver Microsomes,

Drug Metabolism and Disposition, Volume 35, Issue 3, 2007, Pages 476-483, ISSN 0090-9556, https://doi.org/10.1124/dmd.106.013425.

George, J., Tsuchishima, M., & Tsutsumi, M. (2019). Molecular mechanisms in the pathogenesis of N-nitrosodimethylamine induced hepatic fibrosis. Cell Death & Disease, 10(1), 1-9.

Guengerich, F. P., & Avadhani, N. G. (2018). Roles of Cytochrome P450 in Metabolism of Ethanol and Carcinogens. Paper presented at the Alcohol and Cancer. Retrieved from http://dx.doi.org/10.1007/978-3-319-98788-0_2

Gustafson A, King C, Rey JA. Lorcaserin (Belviq): A Selective Serotonin 5-HT2C Agonist In the Treatment of Obesity. P T. 2013 Sep;38(9):525-34. PMID: 24273398;

Hammer, H., Schmidt, F., Marx-Stoelting, P., Potz, O., & Braeuning, A. (2021). Cross-species analysis of hepatic cytochrome P450 and transport protein expression. Arch Toxicol, 95(1), 117-133.

He X, Xia Q, Wu Q, Tolleson WH, Lin G, Fu PP. Primary and secondary pyrrolic metabolites of pyrrolizidine alkaloids form DNA adducts in human A549 cells. Toxicol In Vitro. 2019 Feb;54:286-294. doi: 10.1016/j.tiv.2018.10.009.

Hecht, S. S. (1998). Biochemistry, biology, and carcinogenicity of tobacco-specific N-nitrosamines. Chem Res Toxicol, 11(6), 559-603.

ICH M12 guideline https://www.ema.europa.eu/en/ich-m12-drug-interaction-studies-scientific-guideline

Ihler, Friedrich & Kloos, B & Spiegel, Jennifer & Weiss, Bernhard & Canis, Martin & Bertlich, Mattis. (2021). A possible synergistic effect of Betahistine and Selegiline on the improvement of cochlear micorcirculation. Laryngo-Rhino-Otologie. 100. 10.1055/s-0041-1728479.

Jackson PJ, Brownsill RD, Taylor AR, Resplandy G, Walther B, Schwietert HR. Identification of trimetazidine metabolites in human urine and plasma. Xenobiotica. 1996 Feb;26(2):221-8. doi: 10.3109/00498259609046702. PMID: 8868005.

Jia, L., & Liu, X. (2007). The Conduct of Drug Metabolism Studies Considered Good Practice (II): In Vitro Experiments. Current Drug Metabolism, 8(8), 822-829.

Kassem MG, Al Hossaini AM. Varenicline. Profiles Drug Subst Excip Relat Methodol. 2012;37:389-411. doi: 10.1016/B978-0-12-397220-0.00010-6. Epub 2012 Mar 19. PMID: 22469324.

Kirkland, D., Aardema, M., Henderson, L., & Muller, L. (2005). Evaluation of the ability of a battery of three in vitro genotoxicity tests to discriminate rodent carcinogens and non-carcinogens I. Sensitivity, specificity and relative predictivity. Mutat Res, 584(1-2), 1-256.

NL Kerry, AA Somogyi, F Bochner, G Mikus. The role of CYP2D6 in primary and secondary oxidative metabolism of dextromethorphan: in vitro studies using human liver microsomes. British Journal of Clinical Pharmacology, Volume38, Issue3, 1994, Pages 243-248, https://doi.org/10.1111/j.1365-2125.1994.tb04348.x

Lantz RJ, Gillespie TA, Rash TJ, Kuo F, Skinner M, Kuan HY, Knadler MP. Metabolism, excretion, and pharmacokinetics of duloxetine in healthy human subjects. Drug Metab Dispos. 2003 Sep;31(9):1142-50. doi: 10.1124/dmd.31.9.1142. PMID: 12920170.

Iarc. (2000). Some Industrial Chemicals - IARC monographs on the evaluation of carcinogenic risks to humans. Some industrial chemicals Some Industrial Chemicals – IARC monographs on the evaluation of carcinogenic risks to humans. Some industrial chemicals (Vol. 77, pp. 403-438). Lyon: International Agency for Research on Cancer.

Lasker JM, Wester MR, Aramsombatdee E, Raucy JL. Characterization of CYP2C19 and CYP2C9 from human liver: respective roles in microsomal tolbutamide, S-mephenytoin, and omeprazole hydroxylations. Arch Biochem Biophys. 1998 May 1;353(1):16-28. doi: 10.1006/abbi.1998.0615.

Lecht S, Haroutiunian S, Hoffman A, Lazarovici P. Rasagiline - a novel MAO B inhibitor in Parkinson's disease therapy. Ther Clin Risk Manag. 2007 Jun;3(3):467-74. PMID: 18488080;

Lee, S. Y., & Guttenplan, J. B. (1981). A correlation between mutagenic and carcinogenic potencies in a diverse group of N-nitrosamines: determination of mutagenic activities of weakly mutagenic N-nitrosamines. Carcinogenesis, 2(12), 1339-1344.

Levy, D. D., Zeiger, E., Escobar, P. A., Hakura, A., van der Leede, B. M., Kato, M., et al. (2019). Recommended criteria for the evaluation of bacterial mutagenicity data (Ames test). Mutat Res Genet Toxicol Environ Mutagen, 848, 403074.

Li XQ, Björkman A, Andersson TB, Ridderström M, Masimirembwa CM. Amodiaquine clearance and its metabolism to N-desethylamodiaquine is mediated by CYP2C8: a new high affinity and turnover enzyme-specific probe substrate. J Pharmacol Exp Ther. 2002 Feb;300(2):399-407. doi: 10.1124/jpet.300.2.399. PMID: 11805197.

Li, Y., & Hecht, S. S. (2022). Metabolic Activation and DNA Interactions of Carcinogenic N-Nitrosamines to Which Humans Are Commonly Exposed. Int J Mol Sci, 23(9), 4559.

Li, X., Le, Y., Guo, X., King, S. T., Dorsam, R. T., Atrakchi, A. H., McGovern, T. J., Davis-Bruno K. L., Keire, D. A., Heflich, R., Mei, N., Mutagenicity and genotoxicity evaluation of 15 nitrosamine drug substance-related impurities in human TK6 cells, Regulatory Toxicology and Pharmacology, Volume 154, 2024, 105730, ISSN 0273-2300, https://doi.org/10.1016/j.yrtph.2024.105730.

Liu, Y., & Glatt, H. (2008). Mutagenicity of N-nitrosodiethanolamine in a V79-derived cell line expressing two human biotransformation enzymes. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 643(1-2), 64-69.

Loeppky, R. (2007). Mechanistic studies on the formation and metabolism of nitrosamines. Cancer Research, 67(9_Supplement), SS05-03.

Lynch, A., & Harvey, J. N. (2022). N-Nitrosamine Genotoxicity Investigations @ GSK.

Maron, D.M. and B.N. Ames (1983) Revised methods for the Salmonella mutagenicity test; Mutation Research 113, 173-215

Martignoni, M., Groothuis, G. M. M., & de Kanter, R. (2006). Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opinion on Drug Metabolism & Toxicology*, *2*(6), 875–894. https://doi.org/10.1517/17425255.2.6.875

Matthews, E. J., Kruhlak, N. L., Cimino, M. C., Benz, R. D., & Contrera, J. F. (2006). An analysis of genetic toxicity, reproductive and developmental toxicity, and carcinogenicity data: II. Identification of genotoxicants, reprotoxicants, and carcinogens using in silico methods. Regul Toxicol Pharmacol, 44(2), 97-110.

Mori, Y., Yamazaki, H., & Konishi, Y. (1987). Mutagenicity of N-nitrosodiethanolamine in the Salmonella/microsome test. Mutation Research Letters, 192(2), 91-94.

Nagao, M., Suzuki, E., Yasuo, K., Yahagi, T., & Seino, Y. (1977). Mutagenicity of N-butyl-N-(4-hydroxybutyl)nitrosamine, a bladder carcinogen, and related compounds. Cancer Research, 37(2), 399-407.

OECD. (2020). Ninth Addendum to OECD Guidelines for Testing of Chemicals, Section 4, No. 471, Bacterial Reverse Mutation Test: Organisation for Economic Co-operation and Development.

PAterson, J.W., Conolly, M.E., Dollery, C.T. *et al.* The pharmacodynamics and metabolism of propranolol in man. *Pharmacol. Clin.* **2**, 127–133 (1970). https://doi.org/10.1007/BF00420338

Polasek TM, Elliot DJ, Miners JO. Measurement of human cytochrome P4501A2 (CYP1A2) activity in vitro. Curr Protoc Toxicol. 2006;Chapter 4:Unit4.19. doi: 10.1002/0471140856.tx0419s27.

Prival, M. J., King, V. D., & Sheldon, A. T., Jr. (1979). The mutagenicity of dialkyl nitrosamines in the Salmonella plate assay. Environ Mutagen, 1(2), 95-104.

Prival, M. J., & Mitchell, V. D. (1982). Analysis of a method for testing azo dyes for mutagenic activity in Salmonella typhimurium in the presence of flavin mononucleotide and hamster liver S9. Mutation Research/Environmental Mutagenesis and Related Subjects, 97(2), 103-116.

Qian L, Zolfaghari R, Ross AC. Liver-specific cytochrome P450 CYP2C22 is a direct target of retinoic acid and a retinoic acid-metabolizing enzyme in rat liver. J Lipid Res. 2010 Jul;51(7):1781-92. doi: 10.1194/jlr.M002840. Epub 2010 Feb 10. PMID: 20147703; PMCID: PMC2882718.

Rao, T. K., Cox, J. T., Allen, B. E., Epler, J. L., & Lijinsky, W. (1981). Mutagenicity of N-nitrosopyrrolidine derivatives in Salmonella (Ames) and Escherichia coli K-12 (343/113) assays. Mutation Research, 89(1), 35-43.

Rao, T. K., Hardigree, A. A., Young, J. A., Lijinsky, W., & Epler, J. L. (1977). Mutagenicity of N-nitrosopiperidines with Salmonella typhimurium/microsomal activation system. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 56(2), 131-145.

Rao, T. K., Young, J. A., Lijinsky, W., & Epler, J. L. (1979). Mutagenicity of aliphatic nitrosamines in Salmonella typhimurium. Mutation Research, 66(1), 1-7.

RDKit: Open-source cheminformatics. https://www.rdkit.org

Routledge PA, Shand DG. Clinical pharmacokinetics of propranolol. Clin Pharmacokinet. 1979 Mar-Apr;4(2):73-90. doi: 10.2165/00003088-197904020-00001. PMID: 378502.

Python: Van Rossum, G., & Drake, F. L. (2009). *Python 3 Reference Manual*. Scotts Valley, CA: CreateSpace.

Schrenk, D., Schwarz, M., & Tennekes, H. A. (1982). A novel pathway of nitrosamine metabolism in liver microsomes: Denitrosation of nitrosamines by cytochrome P 450. In S. Snyder (Ed.), Advances in experimental medicine and biology: Biological reactive intermediates. II: B chemical mechanisms and biological effects. (pp. 136:1157-1164). New York, NY: Plenum Press.

Turpeinen M, Nieminen R, Juntunen T, Taavitsainen P, Raunio H, Pelkonen O. Selective inhibition of CYP2B6-catalyzed bupropion hydroxylation in human liver microsomes in vitro. Drug Metab Dispos. 2004 Jun;32(6):626-31. doi: 10.1124/dmd.32.6.626.

Trejo-Martin, A., Bercu, J. P., Thresher, A., Tennant, R. E., Thomas, R. F., Cross, K., et al. (2022). Use of the bacterial reverse mutation assay to predict carcinogenicity of N-nitrosamines. Regul Toxicol Pharmacol, 105247.

Val L, Chen LS, Mendes GD, De Nucci G. Comparative bioavailability of betahistine tablet formulations administered in healthy subjects. Arzneimittelforschung. 2010;60(7):440-4. doi: 10.1055/s-0031-1296309. PMID: 20712134.

Wakabayashi, K., Nagao, M., Kawachi, T., & Sugimura, T. (1982). Mechanism of appearance of mutagenicity of N-nitrosodiphenylamine with norharman. IARC Scientific Publications(41), 695-707.

WANDEL, C., BÖCKER, R., BÖHRER, H., BROWNE, A., RÜGHEIMER, E., MARTIN, E. Midazolam is metabolized by at least three different cytochrome P450 enzymes, British Journal of Anaesthesia, Volume 73, Issue 5, 1994, Pages 658-661, ISSN 0007-0912, https://doi.org/10.1093/bja/73.5.658.

Wang T, Yang L, Hua J, Xie H, Jiang X, Wang L. Simultaneous bioanalysis of rasagiline and its major metabolites in human plasma by LC-MS/MS: Application to a clinical pharmacokinetic study. J Pharm Biomed Anal. 2016 Jun 5;125:280-5. doi: 10.1016/j.jpba.2016.04.003.

Williams, G. M. (2018). Are all Ames Strains in the OECD Mutagenicity Test Guideline 471 useful and necessary? An analysis of large mutagenicity data sets for the IWGT.

Williams, R. V., DeMarini, D. M., Stankowski, L. F., Jr., Escobar, P. A., Zeiger, E., Howe, J., Cross K.P. (2019). Are all bacterial strains required by OECD mutagenicity test guideline TG471 needed? Mutat Res Genet Toxicol Environ Mutagen, 848, 503081.

Yahagi, T., Nagao, M., Seino, Y., Matsushima, T., Sugimura, T., & Okada, M. (1977). Mutagenicities of N-nitrosamines on salmonella. Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 48(2), 121-129.

Yamazaki H, Inoue K, Hashimoto M, Shimada T. Roles of CYP2A6 and CYP2B6 in nicotine C-oxidation by human liver microsomes. Arch Toxicol. 1999 Mar;73(2):65-70. doi: 10.1007/s002040050588.

Yamada, M., Sedgwick, B., Sofuni, T., & Nohmi, T. (1995). Construction and characterization of mutants of Salmonella typhimurium deficient in DNA repair of O6-methylguanine. J Bacteriol, 177(6), 1511-1519.

Yang, C. S., Yoo, J.-S. H., Ishizaki, H., & Hong, J. (1990). Cytochrome P450IIe1: Roles in Nitrosamine Metabolism and Mechanisms of Regulation. *Drug Metabolism Reviews, 22*(2-3), 147-159.

Zhang T, Peng T, Rao J, Wang K, Qiu F. Quantitation of Diclofenac, Tolbutamide, and Warfarin as Typical CYP2C9 Substrates in Rat Plasma by UPLC-MS/MS and Its Application to Evaluate Linderane-Mediated Herb-Drug Interactions. J Anal Methods Chem. 2022 Mar 10;2022:1900037. doi: 10.1155/2022/1900037.

PharmGKB

M. Whirl-Carrillo[1], R. Huddart[1], L. Gong, K. Sangkuhl, C.F. Thorn, R. Whaley and T.E. Klein. "An evidence-based framework for evaluating pharmacogenomics knowledge for personalized medicine" *Clinical Pharmacology & Therapeutics* (2021) Sep;110(3):563-572. doi: 10.1002/cpt.2350. Epub 2021 Jul 22.

M. Whirl-Carrillo, E.M. McDonagh, J.M. Hebert, L. Gong, K. Sangkuhl, C.F. Thorn, R.B. Altman and T.E. Klein. "Pharmacogenomics knowledge for personalized medicine" Clinical Pharmacology & Therapeutics (2012) Oct;92(4):414-7.

(A) An Evidence-Based Framework for Evaluating Pharmacogenomics Knowledge for Personalized Medicine. *Clinical pharmacology and therapeutics.* 2021. Whirl-Carrillo Michelle, Huddart Rachel, Gong Li, Sangkuhl Katrin, Thorn Caroline F, Whaley Ryan and Klein Teri E.PMID:34216021 DOI:10.1002/cpt.2350

Drugbank

Knox C, Wilson M, Klinger CM, Franklin M, Oler E, Wilson A, Pon A, Cox J, Chin NEL, Strawbridge SA, Garcia-Patino M, Kruger R, Sivakumaran A, Sanford S, Doshi R, Khetarpal N, Fatokun O, Doucet D, Zubkowski A, Rayat DY, Jackson H, Harford K, Anjum A, Zakir M, Wang F, Tian S, Lee B, Liigand J, Peters H, Wang RQR, Nguyen T, So D, Sharp M, da Silva R, Gabriel C, Scantlebury J, Jasinski M, Ackerman D, Jewison T, Sajed T, Gautam V, Wishart DS. DrugBank 6.0: the DrugBank Knowledgebase for 2024. Nucleic Acids Res. 2024 Jan 5;52(D1):D1265-D1275. doi: 10.1093/nar/gkad976.