SPECIFIC CONTRACT

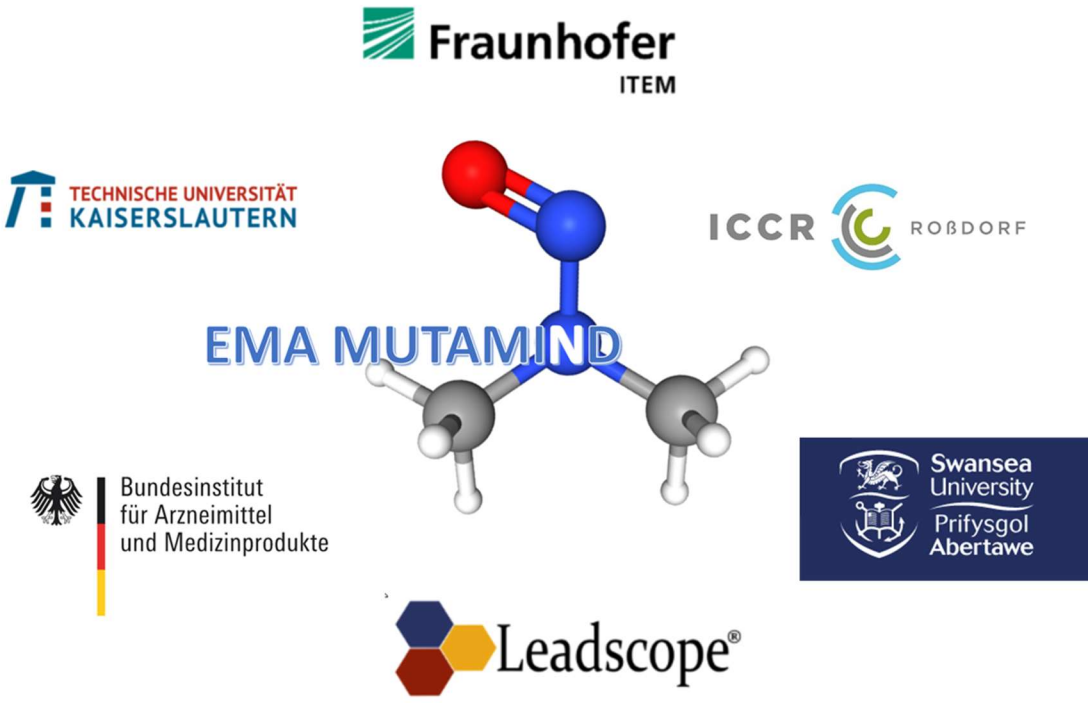
No. 02

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

In vitro mutagenicity methodology for nitrosamines

Deliverable 2:

Basic study protocols for the bacterial mutagenicity assay and the *in vitro* Comet assay (liver cell models) with nitrosamines, including results of methodological literature searches and protocol optimization strategies



The EMA MUTAMIND Logo was designed based on: https://pubchem.ncbi.nlm.nih.gov/compound/6124#section=3D-Conformer

Contractual Submission Date: 17/06/2022, update 07.09.2022

Actual Submission Date: 07/09/2022

Responsible partner: Fraunhofer ITEM

Content

1 Introduction and aims

The project "*In vitro* mutagenicity methodology for nitrosamines" aims 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 nitrosamines (NAs). A carefully selected set of reference NAs, active pharmaceutical ingredient (API)-derived NAs and supporting reference compounds is needed to demonstrate reproducibility, sensitivity (the proportion of genotoxic carcinogens that generate positive results), and specificity (the proportion of non-genotoxic compounds that generate a negative result) of each test model and provide data to estimate and compare the genotoxic potency of test compounds in the two different *in vitro* assays. One part of the project focuses on evaluation and optimization of the Ames test to improve its sensitivity in detecting the mutagenic potential of NAs. The other part is dedicated to evaluation and optimization of the *in vitro* Comet assay with metabolically competent liver cell models as a complementary or alternative assay for detection of potentially mutagenic/carcinogenic NAs and for respective risk assessment.

The present document, describes the basic study protocols for performing the bacterial mutagenicity assay and the *in vitro* Comet assay (liver cell models) on NAs, including results of methodological literature searches and protocol optimization strategies. Additionally, the document provides information on critical study parameters such as provision/purchase of NAs, API-related NAs and human hepatocytes, metabolic competence of both metabolic activation systems (rat and hamster S9-mix) and *in vitro* cell models (HepG2 cells, primary human and rat hepatocytes), solubility issues with potential solvent-mediated inhibition of CYP450 isoforms and stability and purity. This study will also provide information describing how to assess and modify critical study parameters. The basic protocols for the Ames and *in vitro* Comet assay testing, as given in the present document, should be regarded as starting point for evaluation of critical parameters and final optimization. They are based on the well-established methods of the different partners and benefit from the respective historical control data and specific experience of the laboratories. Methodological details, given in this study protocol should be regarded as preliminary, and are subject to changes during the ongoing study.

2 Test compounds

2.1 NA compounds selected for Ames test and *in vitro* Comet assay

Overlapping NA compounds for the Ames test and the *in vitro* Comet assay with liver cell models were selected based upon specific physicochemical properties, available data on mutagenicity and carcinogenicity and complex selection criteria. The recent selected compound panel aimed at covering amongst others different structural classes with different data background regarding their mutagenic (Ames test) and carcinogenic potential, different mechanisms of metabolic activation and includes API-derived NAs. The NAs and API-derived NAs depicted in ***Table 1*** (CAS No., if available, in parentheses) were chosen as priority one and two for the Ames test (42 compounds, originally 25 NAs were planned), including the overlapping priority 1 compounds for the *in vitro* Comet assay with liver cell models (10 compounds, marked in grey). A complete overlap in compounds is needed at the end of the project to enable comparison of the Ames test with the *in vitro* Comet assay, primarily, regarding sensitivity. The calculation of specificity, concordance as well as positive and negative predictivity will be limited, due to the low number of carcinogenicity- and/or Ames test-negative NAs in the compound panel. Therefore, the respective calculations might not be statistically meaningful enough to establish broad conclusions.

In total 18 API-related compounds were included in the Ames test panel, with three of them i.e., N-nitrosofolic acid (parent compound widely used as dietary supplement), N-nitrosodesloratadine (parent compound widely used as antihistamine), and N-nitrososalbutamol (parent compound widely used as asthma medication), also planned to be tested in the *in vitro* Comet assay with three different liver cell models. In case of negative Ames test data for these three API-derived compounds at least one API-derived compound will be replaced by an Ames test-positive one for *in vitro* Comet assay testing. NDMA and NNN, marked in red color in ***Table 1***, will be used as starting compounds for establishment and optimization purposes. In total 13 compounds with no Ames test and cancer data were included, with 12 of them being bulky, API-derived compounds. Bulky cyclic and aryl NAs contain rings of five or more members, while bulky substituted NAs contain substituted rings, acyclic substituents, branched acyclic substituents or carbon chain substituents larger than 5 atoms. Additionally, 10 carcinogenicity and Ames test positive NAs, 4 carcinogenicity positive and Ames test negative compounds, 1 carcinogenicity and Ames test negative NA, 1 carcinogenicity negative without Ames test, 1 carcinogenicity negative, but Ames test positive NA, 3 carcinogenicity positive NAs without Ames test data, 6 with positive Ames tests, 1 carcinogenicity weak positive without Ames test data, 1 carcinogenicity positive NA with unclear Ames data and 1 NA with unclear data were included. Additional NAs, investigated in other recent studies, (e.g. 4-N-nitroso-hydrochlorothiazide), might also be tested to help to establish reproducibility of the test results. The depicted compound panel should be regarded as preliminary, as compounds might be withdrawn, due to unexpected non-availability, too high prices or technical issues, and might be replaced by others with high priority ranking.

Table 1*:* Nitrosamines and API-derived nitrosamines chosen for the present project.

Chosen nitrosamines with structural classes and CAS No.			
Simple	**Bulky, aryl**	**Bulky, cyclic**	**Bulky, substituted/ substituted dialkyl**
N-Nitrosodimethylamine (NDMA; 62-75-9; positive control)	N-Nitrosodiphenylamine (86-30-6)	Dinitrosopentamethylene-tetramine (101-25-7)	N-Nitrosodiethanolamine (1116-54-7)
Diethylamine,1,1'-dimethyl-N-nitroso- (601-77-4)	Nitrosomethylaniline (614-00-6);	N-Nitrosoproline (7519-36-0)	Nitroso-n-methyl-n-(2-phenyl)ethylamine (13256-11-6)
Methyl-t-butylnitrosamine (2504-18-9)	p-Fluoro-N-methyl-N-nitrosoaniline (937-25-7)	**N-Nitrosonornicotine (NNN; 16543-55-8; positive control)**	2-Methoxy-N-(2-methoxyethyl)-N-nitrosoethanamine (67856-65-9)
Dipentylnitrosamine (13256-06-9)	N-Nitroso-N-methyl-4-aminopyridine (16219-99-1)	Nitroso-heptamethyleneimine (20917-49-1)	Methyl(acetoxymethyl) nitrosamine (56856-83-8)
2-Propanamine, N-ethyl-N-nitroso- (16339-04-1)	Benzenamine, N-methyl-N-nitroso-4-[2-(4-quinolinyl) ethenyl]- (16699-10-8)	Dinitrosohomopiperazine (55557-00-1)	4-(Methylnitrosamino)-1-(3-pyridyl)-1-(butanone) NNK (64091-91-4)
2-Propanamine, N-methyl-N-nitroso- (30533-08-5)	N-Nitrosochlorodiazepoxide (51715-17-4)	N-Nitroso-4-benzoyl-3,5-dimethylpiperazine (61034-40-0)	4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol NNAL (76014-81-8)
Bulky, API-derived			
N-Nitrosoephedrine (17608-59-2)	N-Nitrosofolic acid (29291-35-8)	N-Nitroso-Nortriptyline (55855-42-0)	4-N-Nitroso-hydrochlorothiazide (63779-86-2)
N-Nitrosopropanolol (84418-35-9)	N-Nitrosotrimetazidine (92432-50-3)	N-Nitrosometoprolol (138768-62-4)	N-Nitrosofluoxetine (150494-06-7)
N-Nitrosodesloratadine (1246819-22-6)	N-Nitrosorasagiline (2470278-90-9)	N-Nitrosobumetanide (2490432-02-3)	N-Nitrosoduloxetine (2680527-91-5)
N-Nitrosofurosemide* (2708280-93-5)	N-Nitrosolorcaserin (2724616-80-0)	N-Nitrosovarenicline (2755871-02-2)	N-Nitrosochloroquine* (nocas-3)
N-Nitrosoenalapril (nocas-5)	N-Nitrososalbutamol (nocas-9)		

*No provider identified up to now. Candidate for replacement.

2.2 Sourcing and distribution of NA compounds

Sourcing of the NA and API-related NAs is proving to be more difficult than expected. On the one hand, for some API-related compounds, no commercial provider could be identified, using different search strategies and 2 originally chosen compounds had, therefore, already to be replaced by others. In other cases, collaboration with industry (which was already started), or custom synthesis or synthesis at BfArM as a project partner is needed to be able to test the compounds. On the other hand, some of the selected compounds with commercial providers are extremely expensive, when large amounts of high purity are required (e.g. for the Ames test gram amounts are essential). Reducing the requirement for purity to about 95%, makes some compounds more affordable, but false-positive test outcomes related to specific impurities then cannot be excluded, and will have to be considered during data interpretation. Further efforts will be needed to resolve these issues.

The supply of the chosen NAs and development of solubilizing SOPs (see 2.3 for pre-test on solubility) will be done in close collaboration of all testing facilities i.e., BfArM, Fraunhofer ITEM, TU Kaiserslautern and ICCR. Known susceptibility of the different cell models and Ames tester strains to specific solvents as well as safety of employees will be considered. As the preferred method, compounds will be ordered by one specific partner. The compounds will then be provided to the other partners in the form of adequate aliquots (either stock solutions or powder/liquid samples). As an alternative, the compounds will be ordered from the respective suppliers with aliquots directly sent to the different partners. This process will also be used for compounds provided by industry. BfArM will then perform the pre-tests on solubility (see 2.3), stability and purity (see 2.4).

2.3 Pre-tests on solubility

The solubility of NAs, to be reconstituted/diluted by non-critical solvents for bacterial/cell survival and/or enzyme stability, is crucial for all experiments. Highly concentrated stock solutions with completely dissolved compounds will be generated and diluted with cell culture medium 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. Respective experiments are described in section 3.3. In general, to solely test for solubility, initial NA stock solutions of 50 mg/ml will be prepared, starting with the preferred solvents for the biological tests i.e., water and dimethyl sulfoxide (DMSO). In the case of solubility problems or potential inhibition of metabolic enzymes by DMSO (refer to section 3.3), other solvents such as ethanol (EtOH), methanol (MeOH), acetonitrile or N,N-dimethylformamide (DMF) will be considered, depending on the NA in question. 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 re-tested in the presence of S9-mix, which is known to improve solubility of certain compounds due to protein binding. All samples will be evaluated by considering opacity, re-precipitation of the solution, and color change. As background information for solubility testing, concentrations needed to induce DNA damage were extracted from the methodological *in vitro* comet assay literature search with liver cell models, which demonstrated that millimolar concentration are usually needed to induce DNA-strand-breaks. For details see ***Table 2***. Notably, the concentrations described in Table 2 are from experiments/studies performed with liver cell models under similar methodological conditions as proposed for this project (refer to section 5). No references are given in the table, as it only serves as a guide and starting point for concentration selection. If not

otherwise stated, cells were incubated for 24 h, but experiments with 4 h incubation periods were also identified.

Table 2: Concentrations found in literature for positive outcomes of *in vitro* Comet assay experiments.

Compound	NDMA	NDMA	NDMA	NDMA	NDEA	NDBA	NPIP	NPYR
Cell type	HepG2	HepG2	HepG2	PHH	HepG2	HepG2	HepG2	HepG2
S9-mix used	no	yes	no	no	no	no	no	no
Concentration positive_min	10 mM (4 h); 90 mM	5 mM (4 h)	10 mM	10 mM	3 mM	0.3 mM (+fpg)	9 mM (+fpg)	3 mM
Concentration positive_max	10 mM (4 h); 200 mM	5 mM (4 h)	135 mM	10 mM	3 mM	44 mM	44 mM (+fpg)	50 mM
Solvents	Medium/PBS	Medium	DMSO	Medium	DMSO	DMSO	DMSO	DMSO

2.4 Estimation of stability and purity

Prior to the laboratory experiments, it is of utmost importance to avoid non-specific or adverse effects on cell viability, metabolism or NA reactivity due to impurities to obtain valid results. Therefore, all NAs will be screened for impurities by means of LC-MS full scan analysis, in particular for the compounds with lower purity (≤ 95%). A determination range of 50 – 1000 Da is applied on standard stock solutions of NAs with 1 µg/ml to reveal and identify undesired signals. One limitation, however, will be the inability to screen for inorganic compounds, such as heavy metals or other salts. Additionally, stability experiments will be performed by incubating the NAs [1 µg/ml] with assay–specific matrices, including the used cell culture media for the *in vitro* Comet assay experiments, and the relevant organic solvents, which were determined by solubility testing (refer to section 2.3). Solvents will be chosen individually for the different NAs not only based on solubility, but also on information provided in the literature with regard to relevant metabolic enzymes (see ***Table 3*** and ***Table 4***) and their potential inhibition by certain solvents (***Table 6***). At respective time points (t[h] = 0, 1, 2, 4, 8, 16, 24, and 48) aliquots of each stability test sample will be subjected to LC-MS analysis to evaluate the time-dependent decrease of signal intensity, which directly correlates with non-specific degradation and/or non-specific binding. Instability of NAs might result in lower than planned concentrations during cell incubation, and finally in lower relative DNA alkylation rates. Stability should, therefore, be adequate.

The tests for impurities and stability used here are primarily for internal study documentation and for the validity of future results. Instability of the compounds or a high degree of impurities could lead to a falsification of results. Therefore, we have specified that all compounds to be tested should be stable and free of impurities in amounts that could cause toxic side reactions. This will be evaluated case-by-case and no general statement can be given. If instability is observed, the degree must be determined, and novel degradation products identified. Another crucial point is the speed of degradation. These parameters will have also to be discussed individually, and implications subsequently weighed for the Ames test, *in vitro* Comet assay or other approaches. It is, therefore, difficult to set a general limit. Considering the high prices for NAs with a purity level > 95% a compromise between purity levels and price must be made within this study. Whether an impurity will have an impact on the test results will have to be discussed on a case-by-case basis. As far as feasible, we will try to identify critical impurities, which occur at high levels via LC-MS. The manufacturer's certificate of analysis will also be carefully assessed, and, in the event of a high level of contamination or critical individual compounds, the distributor will be consulted to clarify the nature of impurities.

3 Metabolism

3.1 Identification of enzymes, relevant for the metabolism of the selected NAs/NA classes

To obtain background information on enzymes relevant for both metabolic activation of NAs and metabolism of related APIs the literature was searched. The results are given in ***Table 3*** and ***Table 4***. With regard to metabolic activation of NAs, CYP2E1 is known to be important for metabolism of smaller NAs i.e., N-nitrosodimethylamine (NDMA) and N-nitrosodiethylamine (NDEA), whereas for the bulky compound 4-(Methylnitrosamino)-1-(3-pyridyl)-1-(butanone) (NNK), several CYP subfamilies were shown to take part in metabolism such as 1A2, 2A6, 3A4, 2D6, 2F1, 3A5, 2B6, 2B7, 2E1 and 2C8 for humans and 1A, 1A2, 2A4, 2A13, 2B1 and 3A for rats. For 4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanol (NNAL) 2A6 and 2A13 are described to be the relevant enzymes.

Table 3: CYP450 enzymes relevant for metabolic activation of some chosen NAs. Predominant CYP450 enzymes are shown 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)

Notably, many drug-metabolizing enzymes can vary between species (*e.g.,* human versus rodent) in their isoform composition, expression, and activity levels. Of particular importance for the metabolic activation of NAs are amongst others CYP2E1, CYP2A6 (rat orthologues expressed in liver tissue: CYP2A1–female dominant/CYP2A2-male dominant) and CYP3A4 (rat orthologue: CYP3A1/CYP3A2) (Cross & Ponting, 2021; M. Martignoni, G. M. M. Groothuis, & R. de Kanter, 2006; C. S. Yang, Yoo, Ishizaki, & Hong, 1990). For CYP2E1 the differences between humans and mouse/rat orthologues are not large and widely overlap in the respective substrates (though CYP2E1 expression is significantly higher in hamster versus rat). Therefore, rats are a good model species for investigations on CYP2E1-dependent metabolism (M. Martignoni, G. M. Groothuis, & R. de Kanter, 2006). Regarding CYP3A, four CYP3A isoforms are expressed in humans i.e., CYP3A4, -3A5, -3A7 and 3-A43, with highest abundance

of CYP3A4 in the liver. In rats, six CYP3A forms were identified, including CYP3A1, -3A2, -3A9, -3A18, -3A23 and -3A62 (Marcella Martignoni et al., 2006). Rat CYP3A1 and CYP3A2 are the orthologues of human CYP3A4 and are expressed only in the liver (Marcella Martignoni et al., 2006). For more information see 5.3.

Table 4: CYP450 isoforms responsible for the degradation of some APIs with nitrosated counterparts.

Nitrosated API	CAS No.	CYP450 involved in metabolism of the non-nitrosated API	References
N-Nitrosochloroquine	nocas-3	1A2, 2C8, 2C19, 2D6, 3A4/5	(Rendic & Guengerich, 2020)
N-Nitrosoduloxetine	2680527-91-5	1A2, 2D6, other	(Drugbank, 2022)
N-Nitrosolorcaserin	2724616-80-0	1A1, 1A2, 2A6, 2B6, 2C19, 2D6, 2E1, 3A4, FMO1	(Gustafson, King, & Rey, 2013)
N-Nitrosonortriptyline	55855-42-0	2D6, 1A2, 2C19, 3A4	(Drugbank, 2022)
N-Nitrosofluoxetine	150494-06-7	1A2, 2B6, 2C9, 2C19, 2D6, 3A4, 3A5	(Drugbank, 2022)
N-Nitrosorasagiline	2470278-90-9	1A2, others	(Drugbank, 2022)
N-Nitrosofurosemide	2708280-93-5	2C11, 2E1, 3A1, 3A2	(K. H. Yang, Choi, Lee, Lee, & Lee, 2009)
N-Nitrosodesloratadine	1246819-22-6	2C8	(Kazmi, Yerino, Barbara, & Parkinson, 2015)
N-Nitrosobumetanide	2490432-02-3	CYP450	(Brater, 1991)

As already mentioned, NAs are metabolically activated by CYPs located in the endoplasmic reticulum of e.g., liver cells. To identify the key contributors to metabolism of the chosen NA subclasses, metabolism assays with liver microsomes will be used. Reaction rate (DNA alkylation) will be determined as a function of concentration for each NA. For the identified key CYPs, recombinant enzymes will serve to identify their contribution to the respective metabolism rate. Currently, no protocols exist for the direct determination of DNA alkylation by NAs solely using recombinant CYP450 enzymes and calf thymus DNA (ctDNA). In the present project, it is planned to implement a respective protocol by using CYP-specific Corning® Supersomes™ in the presence of ctDNA. These commercially available formulations consist of a CYP450 microsomal system, capable to present membrane-bound subgroups of metabolic enzymes. On the one hand, this approach simplifies the identification of specific CYP450 subgroups, which are responsible for individual metabolic degradation of NAs, and on the other hand, this assay is not cell-dependent and can serve as proof-of-principle assay for future studies. Bellec, Dreano, Lozach, Menez, and Berthou (1996) defined a panel of CYP450 enzymes, which play a key role in the metabolism of different NAs, including CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (Bellec et al., 1996). It is planned to use both, the selected enzymes and assay conditions, as described in this publication. All enzymes are available as standardized supersomal preparation by Corning®.

DNA adducts on ctDNA will finally be determined by LC-MS using 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, thus, allowing the use of small particle sizes in chromatographic columns to increase peak sharpness and method selectivity. DNA adducts will be detected with pre-defined 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 production 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.

3.2 Metabolic activity of S9-fractions and liver cell models

Results from Task 3.1 on the most relevant CYPs for activation of each NA class will be used to evaluate metabolic competence of the different *in vitro* model systems (primary human and rat hepatocytes as well as HepG2 cells) and S9-fractions (rat and hamster) needed for Ames testing and the *in vitro* Comet assay with HepG2 cells, respectively. A large batch of rat S9-fraction has already been prepared by ICCR and by default characterized concerning protein content and with regard to metabolic activity by using both 2-aminoanthracene and benzo[a]pyrene. This S9-fraction will be distributed to all partners to ensure standard conditions at all laboratories (ICCR, ITEM and BfArM). The same S9-fraction will also be used in the round-robin study, if HepG2 cells will turn out to be not metabolically competent enough. The S9-fractions and the liver cell models will be characterized in more depth for metabolic competence by BfArM. CYP450-specific marker substrates for different CYP450 enzyme subclasses will, therefore, be incubated with respective aliquots of rat and hamster S9-mix or with the liver cell models. Subsequently, the metabolic degradation and the conversion rate are determined by LC-MS. **Fehler! Verweisquelle konnte nicht gefunden werden.** provides an overview of the substrates with concentrations planned for use.

Table 5: Overview of the substrates/metabolites used for the S9-quality and metabolic competence check of the liver cell models. All CYPs listed in this table have the co-factor NADPH/H+ (human).

CYP450 isoforms (human)	Orthologue(s) (rat)*	Substrates with concentrations and references	Metabolite
CYP3A4	CYP3A9	Terfenadine (100 µM) (Jurima-Romet, Wright et al. 1998)	Hydroxyterfenadine
CYP2D6	CYP2D3	Dextromethorphan (5 µM) (Kerry, Somogyi et al. 1994)	Dextrorphan
CYP1A2	CYP1A2	Caffeine (200 µM) (Notarianni, Oliver et al. 1995)	Paraxanthine
CYP2E1	CYP2E1	Chlorzoxazone (120 µM) (Bachmann and Sarver 1996) & Zopiclone** (50 µM)	6-Hydroxychlorzoxazone & Zopiclone-N-oxide
CYP2C9	CYP2C12	Tolbutamide (80 µM) (Zhang, Peng et al. 2022)	4-Hydroxytolbutamide
CYP2B6	CYP2B1/2B2	Bupropion (20 µM) (Turpeinen, Nieminen et al. 2004)	Hydroxybupropion
CYP2C19	CYP2C13/2C55	S-Mephenytoin (100 µM) (Lasker, Wester et al. 1998)	4-Hydroxymephenytoin
CYP2A6	CYP2A1/2A2	Nicotine (60 µM) (Yamazaki, Inoue et al. 1999)	Cotinine/Norcotinine

*Except CYP2A6 according to Hammer, Schmidt, Marx-Stoelting, Potz, and Braeuning (2021). ** Zopiclone is more sensitive than chlorzoxazone, but less specific. Therefore, both compounds will be tested (Yamazaki, Inoue et al. 1999).

It is expected that orthologous CYP450 subclasses from rat, as depicted in **Fehler! Verweisquelle konnte nicht gefunden werden.** will convert these substrates in the same way as the related human isoforms, since there is homology of the amino acid sequence of about 75%. A possible influence by phase II metabolism, especially glucuronidation, is expected only for tolbutamide, mephenytoin, dextromethorphan, and nicotine. However, many substrates have to first undergo phase I metabolism to be further metabolized via phase II to generate glucuronides (nicotine is an exception). In all metabolic competence assays in the present project, the experimental conditions and co-factors have been selected so that only CYP450 enzymes can be appropriately investigated. Essential co-factors for the investigation of e.g. phase II enzymes such as uridine-5-diphosphoglucuronic acid (UDPGA) or 3'-phosphoadenosine-5'-phosphosulfate (PAPS) will not be added. The focus will, thus, be on phase I metabolism only. The detailed protocols given below will also be used to assess the impact of organic solvents on metabolic activity.

Due to the importance of activity detection for CYP2E1 and its involvement in the toxicity of NDMA, NDEA and smaller nitrosamines, two substrates i.e., chlorzoxazone and zopiclone will be tested for this enzyme. Notably, substrates are specific for the detection of human CYP450 enzymes. However, in this *in vitro* assay, both rat and hamster liver enzymes are expected to metabolize these substrates, and corresponding CYP-specific NAs, by orthologous enzymes.

Characterization of rat and hamster S9-fractions:

To guarantee stable metabolic conversion of NAs by the used S9-fractions, their enzymatic activities will be determined using a protocol established at the BfArM. Since the quality of the preparations can be expected to be consistent due to a standardized manufacturing procedure and an Ames test quality check using both 2-aminoanthracene and benzo[a]pyrene (see also 4.3.6), the enzyme preparations will only be examined once before start of the AMES tests and *in vitro* Comet assays. If a repeat assay will be required, the conversion rate of substrates will be re-tested. The final incubation set-up will consist of:

- 183 µL 100 mM PBS, containing 5 mM $MgCl_2$ (pH7.4)
- 5 µL rat/hamster S9-mix (20 mg protein/mL)
- 10 µL 20 mM NADPH tetra sodium salt in PBS
- 2 µL test substrate

Final substrate concentrations are given in **Fehler! Verweisquelle konnte nicht gefunden werden.**. After a pre-incubation time of 5 min, NADPH, as essential co-factor, will be added to start the incubation. All substrates are incubated with S9-mix on an individual basis to avoid inhibition or side reactions. The incubation time will be 60 min at 37°C and at constant mixing at 550 rpm using a thermomixer. Parallel incubations in the absence of S9-mix will be used to exclude spontaneous/non-enzymatic conversion of the substrates or already present amounts of the respective metabolite in the substrate stock solutions (negative control). It is planned to also use this approach to investigate the impact of certain solvents on CYP450 enzyme activities. For details see 3.3.

Characterization of HepG2 cells and primary rat and human hepatocytes:

The characterization of the metabolic competence of the used liver cell models i.e., HepG2 cells and rat and human hepatocytes should be performed under optimal conditions, avoiding cell stress, such as unnecessary freeze-thaw cycles. It is, therefore, planned to perform the incubations directly in the respective cell incubators, using the same incubation conditions, as described in section 5 for the NAs.

The incubation time, incubation buffer and conditions should correspond as closely as possible to the final *in vitro* Comet assay conditions. After incubation, both the cell supernatant and the cells are sent to BfArM and processed by solid phase extraction and analyzed for conversion of the substrates. In case of cell incompatibility, the substrate quantities are adjusted or exchanged for an alternative substrate for a corresponding enzyme. Parallel incubations without cells will be used to exclude spontaneous/non-enzymatic conversion of the substrates or already present amounts of the respective metabolite in the substrate stock solutions (negative control). All incubations will be performed in triplicate.

Sample handling and LC-MS measurements:

After incubation, all samples will be treated with -20°C cold acetonitrile in a threefold sample volume for protein precipitation. The resulting protein aggregates will be separated from the supernatant via centrifugation and supernatants evaporated at 60°C to obtain a dried residue. The residues will be constituted with equal volumes of 0.1% formic acid and further subjected to LC-MS measurements. Here, a highly sensitive MRM experiment will be performed to detect the metabolites, by considering the results from the negative controls. As already given under 3.1, LC-MS/MS measurements will be performed with a Sciex QTRAP® 6500 triple quadrupole mass spectrometers, equipped with a Shimadzu Nexera® ultra-performance liquid chromatograph.

By using the metabolic ratio, it will be semi-quantitatively determined, which S9-fraction or liver cell model shows the highest activity for a certain CYP450 enzyme, when equimolar amounts of substrate are used. Metabolic ratios will be calculated by peak area integration using the following equation:

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

Peak areas are reported by the MS detector as arbitrary units in counts-per-second. The metabolic activity can be compared semi-quantitatively relative to all samples. A direct quantification of the peak areas and conversion into molar quantities, which would require a calibration of all metabolites, is not intended for the present project, since quantitative data, e.g. for structure-activity relationships or calculations of pharmacological intrinsic activity, are not in the foreground. For each approach, of course, 3 technical replicates will be performed. In the further course of the study, biological replicates might be again measured after a few months to confirm metabolic capacity and quality.

After a certain incubation time, the metabolic ratio can finally be related to the percentage of conversion and the amount of protein used, enabling to calculate the metabolic activity. Calculation follows the given equation:

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

qRT-PCR analyses for evaluation of metabolic competence of the liver cell models:

For evaluation of metabolic competence of the *in vitro* liver cell models, CYP450 gene expression will, additionally, be screened by real time quantitative polymerase chain reaction (qRT-PCR). Data will

subsequently be compared with the results of the metabolic activity check, the data given by the provider of the primary human hepatocytes and with relevant literature data. The DNA purification and digestion procedure will be done according to the following protocol:

Liver cell models to be investigated:

- Primary rat hepatocytes
- Primary human hepatocytes
- HepG2 cells
- Human liver slices (optional only)

Kits and reagents:

- RNAprotect (Qiagen)
- RNeasy mini Kit (Qiagen)
- QiaShredder columns (Qiagen)
- QuantiTect® Reverse Transcription Kit (Qiagen)
- QuantiTect® SYBR® Green PCR Kit (Qiagen)
- QuantiTect Primer Assays (human and rat CYP450 and reference genes 18S rRNA or GAPDH)

Devices:

- Nanodrop™ (ThermoFisher)
- Bioanalyzer™ (Agilent)
- Thermocycler
- LightCycler 480™ (Roche)

Protocol:

- **RNA isolation:**

  Starting material: at maximum 1 x 10^7 cells, or 30 mg of fresh or frozen tissue, or 15–20 mg RNAprotect stabilized tissue.

RNA Isolation with RNeasy mini kit, according to manufacturer's protocol:

1. *Add 1/100 volume of ß-mercaptoethanol to the required volume of RLT Buffer (e.g.: 1ml RLT + 10 µl ß-mercaptoethanol).*
2. *For < 5 x 10^6 cells: 350 µl RLT buffer (+ 3.5 µl ß-mercaptoethanol).*
3. *For < 1 x 10^7 cells: 600 µl RLT buffer (+ 6 µl ß-mercaptoethanol).*
4. *Mix cells with RLT buffer and pipette complete lysate onto a QiaShredder column. (Centrifuge for 2 min at 13,000 g).*
5. *Pipette 1 volume of 70% ethanol onto the lysate in the Collection Tube and mix. (e.g.: 350 µl lysate + 350 µl ETOH).*
6. *Pipette 700 µl sample onto a RNeasy column with Collection Tube and centrifuge for 30 sec. Centrifuge at 8000 g. (RNA is bound on the membrane)*
7. *Discard Collection Tube, previously dumping the liquid into a sealable waste container*
8. *Set column on new Collection Tube*
9. *Pipette 700 µl RW1 onto the column and centrifuge for 30 sec. Centrifuge at 8000 g*
10. *Discard Collection Tube, previously dumping the liquid into a sealable waste container*
11. *Set column on new Collection Tube*
12. *Pipette 500 µl RPE onto the column and centrifuge for 30 sec. Centrifuge at 8000 g*
13. *Discard Collection Tube, previously dumping the liquid into a sealable waste container*
14. *Set column on new Collection Tube*
15. *Pipette 500 µl RPE onto the column and centrifuge at 8000 g for 2 min.*
16. *Place the column on a new collection tube and centrifuge at 13,000 g for 1 min.*
17. *Place the column on a labeled 1.5 ml Eppendorf tube and pipette 30-50 µl RNase-free water onto the membrane and elute with 1 min at 8000 g*

18. *Freeze RNA at -80 °C*

- **RNA quantification and quality determination:**
  RNA quality and quantity will be determined by absorbance measurement using a Nanodrop™ device. RNA ratios A_{260}/A_{280}: 1.8–2.2, and A_{260}/A_{230}: >1.7 are accepted for further analysis. Externally extracted RNA is additionally evaluated using Bioanalyzer™ (Agilent).

- **cDNA synthesis via reverse transcriptase PCR:**
  The cDNA will be synthesized using the "QuantiTect® Reverse Transcription Kit", according to the manufacturer's protocol:

1. *Thaw all reagents and centrifuge briefly, then place on ice*
2. *In a sterile, nuclease-free Eppendorf tube (0.2 ml) prepare template RNA mix on ice: gDNA Wipeout Buffer (7x) 2 µl, RNA 0.5 µg, nuclease-free water up to 14 µl*
3. *Incubate for 2 min at 42°C, then store on ice.*
4. *Prepare reverse transcription master mix: Quantiscript Reverse Transcriptase 1 µl, Quantiscript RT Buffer (5x) 4 µl ; RT Primer Mix 1 µl ,Template RNA 14 µl*
5. *Mix carefully (do not vortex!) and, if necessary, centrifuge briefly.*
6. *In a thermal cycler, call up RT program: 15 min. 42°C, 3 min. 95°C , then stop the reaction on ice!*

- **Quantitative real-time PCR:**
  qRT-PCR will be performed using the "QuantiTect® SYBR® Green PCR Kit" on a LightCycler 480™, according to the manufacturer's protocol. Analysis will be performed in technical triplicates; using "QuantiTect Primer Assays":

1. *Thaw QuantiTect SYBR Green Mastermix (2x), PCR-grade Water, cDNA and QuantiTect Primerassay (10x).*
2. *Dilute the cDNA with PCR-grade Water 1:10.*
3. *For one sample, pipette and mix the following reagents together; for duplicates or triplicates, use 2 or 3 times the amount, respectively: QuantiTect Primer Assay (10x) 2 µl, QuantiTect SYBR Green Mastermix (2x) 10 µl, Nuclease-free water 6 µl, cDNA 2 µl*
4. *Pipet mix into a white 96 well PCR plate and seal with the appropriate foil.*
5. *Centrifuge the PCR plate at 3000 g for 2-3 min and store it in the dark at 4°C until measurement*
6. *Measure on Lightcycler 480™, define plate allocation under subsets and samples, assign file name, place plate in Lightcycler and start QuantiTect SYBR Green Run Protocol:*

   ***PCR Initial Heat Inactivation*: 95°C for 15 min**
   ***Denaturation*: 94°C for 15 s**
   ***Annealing*: 55 °C 30 s**
   ***Cycles:* 40**
   ***Extension*: 72 °C 30 s**

- **Data-Analyses:**

  CYP450 gene expression will be quantified from measured Ct values and normalized to the reference genes 18S rRNA or GAPDH. In addition to the use of internal reference genes, external normalization will be performed by means of adjusted amounts of RNA to compare real-time PCR data generated from different experiments and cell types. Here, reference RNA will be provided by Takara®, Japan.

3.3 Impact of solvents on metabolic activity

The impact of solvents on metabolic activity is an important topic to consider, and a key parameter for test protocol optimization. Biological assays such as the Ames test and the *in vitro* Comet assay are generally performed under physiological conditions in aqueous media and at neutral pH. Therefore, it is important to dissolve the test compounds in appropriate solvents, which do not interfere with the

respective assays. Compounds are preferentially dissolved in aqueous vehicles but many compounds require organic solvents due to poor water solubility.

One of the most frequently used organic solvents for *in vitro* experiments is DMSO, which is superior to other organic solvents, due to its cell compatibility and solving characteristics. DMSO is frequently used, when compounds are hardly soluble in water. In standard Ames tests, DMSO concentrations of up to 4 - 14% are used, depending on the type of assay. For *in vitro* Comet assays with e.g. liver cells much lower solvent concentrations can usually be applied, as DMSO can already inhibit cell growth at concentrations > 0.5% (v/v). While cell lines like HepG2 are more sensitive to DMSO, primary rat hepatocytes can sustain DMSO concentrations of up to 2%. Solvent sensitivity is, thus, highly cell type-specific and will need case by case decisions based on the pre-experiment on solubility and the experience with the different cell models. Alternative non aqueous solvents include EtOH, MeOH acetonitrile, and DMF. Notably, DMSO has been shown to inhibit CYP450 enzymes particularly at concentrations used in *in vitro* mutagenicity assays. For instance, DMSO has been shown to decrease mutagenicity of short alkyl chain NAs like NDMA and NDEA as a result of reduced metabolic activation (Yukio Mori et al., 1985; Yahagi et al., 1977). The main NA-relevant human drug metabolizing enzymes are CYP3A4, CYP2D6, CYP2C9 and CYP2C19, while small NAs are usually metabolized by CYP2E1, CYP2A6 and CYP1A2. For NDMA and NDEA metabolism, CYP2E1 is the predominant CYP450 isoform. CYP2E1 has a very small active site, enabling metabolic conversion of only small or terminal NAs. Nevertheless, a variety of enzymes can participate in metabolism and also metabolic activation of NAs. For example, CYP2A6 is also important for small to medium NAs, and CYP3A4 is associated with metabolic activation of larger compounds (Cross & Ponting, 2021). In a publication from Bringezu and Simon (2022) the impact of DMSO on NA metabolism was briefly summarized and the authors concluded that DMSO has the biggest impact on the mutagenicity of NDMA. Larger chain aliphatic NAs remained positive with DMSO, using induced rat liver S9-mix (phenobarbital/beta-naphthoflavone) (Bringezu & Simon, 2022). However, only relatively small amounts of DMSO and highly concentrated stock solutions were used leaving the question if concentrations higher than 1.6% (v/v) are problematic.

To evade negative impact on metabolic capacity, DMSO is planned to be used, in the present project, at a maximum concentration of 0.5% in the *in vitro* Comet assay. To substantiate this decision and to aid in data interpretation and optimization of the Ames test, the influence of DMSO and, may be, other solvents like acetonitrile, methanol or DMF on metabolic capacity will be re-investigated by incubation of CYP450 substrates with S9-mix in the presence of different solvent concentrations and controlled by parallel samples without addition of organic solvents. At least four DMSO/solvent concentrations, i.e., 0.5, 1, 5, and 14% (v/v; total volume including S9-mix and CYP450-specific substrates), will be investigated, using, in principle, the protocol listed under section 3.2 for determination of metabolic competence of the used rat and hamster S9-fractions. A concentration of 14% was previously determined as clearly inhibiting DMSO concentration by (Yahagi et al., 1977). Inhibiting effects will result in lower substrate conversion rates, and may be, false negative results.

Table 6: Inhibition of CYP450 enzymes in different models systems by certain solvents, maximum NOEC concentrations in percent (v/v).

Test System	Solvent	Conc. [%]	Incubation [min.] substrate	NOEC [% solvent] of NA-relevant CYP450 enzymes								References
				3A4	2D6	2C9	2C19	2E1	2A6	1A1	1A2	
Human												
Human hepatocytes	DMSO	0.1, 1, 2	60	< 0.1	>2	0.1	0.1	<0.1	>2	-	>2	(Easterbrook, Lu, Sakai, & Li, 2000)
	Methanol			>2	>2	0.1	>2	0.1	>2	-	>2	
	Acetonitrile			>2	>2	>2	>2	>2	>2	-	>2	
Pooled human liver microsomes	Acetonitrile	0.1, 0.5, 1	10 Testosterone	>1	-	-	-	-	-	-	-	(Iwase et al., 2006)
	Methanol			>1	-	-	-	-	-	-	-	
	Ethanol			>1	-	-	-	-	-	-	-	
	1-Propanol			0.5	-	-	-	-	-	-	-	
	DMF			>1	-	-	-	-	-	-	-	
	PG			>1	-	-	-	-	-	-	-	
	PEG			>1	-	-	-	-	-	-	-	
	DMSO			0.1	-	-	-	-	-	-	-	
	Acetonitril	0.1, 0.5, 1	10 Midazolam	>1	-	-	-	-	-	-	-	
	Methanol			>1	-	-	-	-	-	-	-	
	Ethanol			>1	-	-	-	-	-	-	-	
	1-Propanol			>1	-	-	-	-	-	-	-	
	DMF			>1	-	-	-	-	-	-	-	
	PG			>1	-	-	-	-	-	-	-	
	PEG			>1	-	-	-	-	-	-	-	
	DMSO			>1	-	-	-	-	-	-	-	
Human CYP expressing lymphoblastoid cell microsomes	Methanol	0.1, 0.3, 1, 3, 5	20	1	0.3	3	1	-	3	0.3	3	(Busby, Ackermann, & Crespi, 1999)
	Ethanol			1	0.3	3	<0.1	-	1	0.1	1	
	DMSO			0.1	0.1	3	0.1	-	3	1	3	
	Acetonitrile			1	1	>5	1	-	1	<0.3	1	
Human liver microsomes	Methanol	0.2, 0.5, 1, 5	2	1	1	0.2	1	0.5	>5	-	>5	(Chauret, Gauthier, & Nicoll-Griffith, 1998)
	DMSO			<0.2	1	0.2	0.2	<0.2	>5	-	>5	
	Acetonitrile			1	1	5	1	1	1	-	>5	
Human liver microsomes	Methanol	1	5 - 60 (substrate-specific)	>1	>1	<1	>1	<1	>1	-	<1	(Hickman, Wang, Wang, & Unadkat, 1998)
	DMSO			>1	>1	<1	>1	<1	>1	-	<1	
	Acetonitrile			>1	>1	>1	>1	>1	<1	-	>1	
	Acetone			>1	>1	>1	<1	<1	<1	-	>1	
	DMF			>1	<1	<1	<1	<1	<1	-	>1	
	Isopropanol			<1	>1	<1	<1	<1	>1	-	>1	

Table 6: Continued.

Test System	Solvent	Conc. [%]	Incubation [min.] substrate	NOEC [% solvent] of NA-relevant CYP450 enzymes								References
				3A4	2D6	2C9	2C19	2E1	2A6	1A1	1A2	
Human												
Human liver microsomes	DMSO	0.25, 0.5, 1, 2	18	-	-	-	-	-	-	-	<0.2	(Nirogi et al., 2011)
	Acetonitrile			-	-	-	-	-	-	-	2	
	Methanol			-	-	-	-	-	-	-	2	
Recombinant human CYP450s expressed in *E. coli*	Ethanol	0.5, 1, 2.5, 5, 10	5	1	1	0.5	<0.5	-	-	-	2.5	(Baird, Begg, Pritchard, & Voice, 2005)
	Acetonitrile			1	0.5	2.5	1	-	-	-	-	
	Methanol			2.5	1	0.5	1	-	-	-	5	
	PEG			<0.5	1	2.5	0.5	-	-	-	1	
	DMSO			<0.5	1	2.5	<0.5	-	-	-	5	
				3A	2D	2C		2E		1A		
Rat												
Rat liver microsomes	Acetonitrile	0.1, 0.25, 0.5, 0.75, 1	No information	-	-	-		>1		-		(Patil et al., 2015)
	PEG400			-	-	-		>1		-		
	Methanol			-	-	-		0.5		-		
	Acetone			-	-	-		0.25		-		
	Ethanol			-	-	-		<0.1		-		
	n-Propanol			-	-	-		<0.1		-		
	Isopropanol			-	-	-		<0.1		-		
	DMF			-	-	-		<0.1		-		
	DMSO			-	-	-		<0.1		-		
	Dioxane			-	-	-		<0.1		-		
Rat liver microsomes	Methanol	0.1, 0.25, 0.5, 2.5, 5, 10	30 - 60 (substrate dependent)	5	1	0.5		0.1		0.5		(D. Li et al., 2010)
	Acetonitrile			5	1	1		2.5		1		
	DMSO			2.5	2.5	1		<0.1		0.5		
	Acetone			2.5	0.5	1		<0.1		1		
	Ethanol			2.5	0.5	0.1		5		0.5		

Conc.: Concentration; **DMF**: N,N-dimethylformamide; **DMSO**: Dimethylsulfoxide; **NOEC**: No observed effect concentration, no effect is defined as CYP inhibition < 25%; **PG**: propylene glycol; **PEG**: polyethylene glycol; **-**: not tested at all; **<**: No lower concentration tested; **>**: No higher concentration tested; **green**: not critical; **orange**: partly critical; **red:** critical.

In this context, **Table *6*** summarizes the results of a literature search regarding inhibition of CYP450 enzyme activities by certain solvents (i.e. human CYP3A4, CYP2D6, CYP2C9, CYP2C19, CYP2E1, CYP2A6, CYP1A1, CYP1A2 and their rat orthologues). To aid in choosing the right solvents and solvent concentrations for different NAs and the different *in vitro* models the highest no observed effect concentrations (NOECs) are given, with <25% inhibition defined as no effect level. The table clearly shows that inhibition of CYPs by solvents is solvent type-, CYP450 isozyme-, model system-, test type-, and test substrate-dependent. The study by Easterbrook et al. (2001) conducted with primary human hepatocytes (PHH) is considered of high relevance for the present project, as PHH will also be used here as a model system. The study showed that DMSO strongly inhibited CYP3A4, CYP2C9, CYP2C19 and CYP2E1 already at concentrations of 0.1% (v/v) and lower, whereas, for example, acetonitrile mediated no inhibition at 2%. For PHH also methanol seems not to be a good choice for small NAs as CYP2E1 was also inhibited at a concentration of 0.1%. Regarding rat liver microsomes CYP2E was found to be most prone to inhibition by different solvents such as MeOH, acetone, EtOH, DMSO, and others (D. Li et al., 2010; Patil, Kamble, Shah, & Iyer, 2015), with acetonitrile being less critical. Due to its volatility acetonitrile will not be the solvent of first choice for safety reasons, irrespective of the fact that pre-experiments with HepG2 cells indicated no cytotoxicity after 24 h of incubation (data not shown).

4 Ames testing

4.1 General introduction

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 a scientific justification, deviations from the standard procedure are acceptable and are in part required for meaningful results.

4.2 Results of methodologically focused literature search for Ames testing of NAs

N-Nitrosamines generally appear to act as mutagenic carcinogens, and they require metabolic activation to generate reactive intermediates, which subsequently can lead to carcinogenicity. Literature has shown that the standard Ames test is highly sensitive for prediction of NA mutagenicity (Cross, 2022; Trejo-Martin et al., 2022). However, sensitivity can be further improved by varying the specific experimental protocol, making Ames-negative compounds challenging to classify as to their

potential carcinogenic risk (Heflich, 2022). Specifically, mutagenic potency of NAs in the Ames test can be affected by the following test parameters currently under investigation (Heflich, 2022):

1. Choice of species for used S9 fraction i.e., rat, hamster, and mouse
2. Concentration of S9 fraction
3. Pre-incubation vs. plate incorporation assay
4. Length of pre-incubation
5. Slightly acidic pH during pre-incubation
6. Choice of vehicle/solvent
7. Tester strains employed
8. Growth stage and concentration of the tester strains

Bringezu and Simon (2022) recently investigated the impact of various Ames test conditions on the test outcome in mutagenicity testing of NAs. The authors analyzed the mutagenic potential of NDMA, NDEA, N-nitrosodipropylamine, and N-nitrosodibutylamine. Their results finally suggested that testing for bacterial mutagenicity using the OECD TG471 protocol and considering pre-incubation for short chain substitutions, in general, leads to reliable results [test parameters: tester strains TA98, TA100, TA1535, TA1537, EC WP2 uvrA; 10% (v/v) ß-naphthoflavone/phenobarbital-induced rat liver S9 homogenate; solvent DMSO; 60 min of pre-incubation at 37°C]. More specifically, this study showed that the bacterial reverse mutation assay, applying the established plate incorporation or pre-incubation protocols, and using the *Salmonella* Typhimurium strains TA100 and TA1535 and E. coli WP2 uvrA was suitable to predict the mutagenicity of NAs in the presence of phenobarbital/β-naphthoflavone-induced rat liver S9.

Furthermore, an ongoing project (Heflich, 2022), is focusing on the strategy for testing NA impurities and Drug Substance-related NA Impurities (NDSRIs), and is investigating the following variety of conditions (where over 1,000 plates are being tested per compound):

- Tester strains: TA1535, TA100, TA98, TA1537, WP2 uvrA (pKM101)
- 10 and 30% Aroclor-induced rat and hamster liver S9
- Pre-incubation times of 30 and 60 min (plate incorporation used as backup)
- Limited solvent concentration (<3.6%); priority: H_2O, acetone, DMSO

Preliminary findings from this study generally suggested the largest dose responses using the following conditions:

- Assay strains TA1535, TA100, and WP2 uvrA (pKM101)
- Using hamster liver S9 fraction rather than rat liver S9 fraction for metabolic activation
- Activation using 30% S9 rather than 10% S9
- Assays using preincubation time periods of 60 min rather than 30 min

A preliminary outcome of the study is that no unique positive test results were obtained using only one experimental condition (i.e., all positives were obtained under more than one condition, but more than one bad choice can result in a negative response).

Regarding the issues related to the Ames protocol, EMA discussed the use of *in vitro* mutagenicity data for carcinogenicity potency ranking of NAs (EMA, 2020). It is noted that "*available Ames assay data are highly predictive for a qualitative prediction of carcinogenicity in rodent studies, but problematic for estimation of carcinogenic potency*". The issues that have been identified in this context are:

- *"N-nitrosamines need to be activated metabolically and the artificial rat liver S9-mix used for simulation of metabolism in in vitro assays only provides limited metabolic competence;*
- *Available Ames assays use different doses, and also the strains used are often not the same. It is known that the quantitative results in Ames assays vary from laboratory to laboratory and also intra-laboratory variations are not negligible;*
- *Published Ames data are highly variable in quality;*
- *The four to five Salmonella Typhimurium strains and one E. coli strain used in standard GLP Ames assays have different sensitivities and specificities for mutagenicity.*

All standard Ames tester strains are alkyl transferase proficient and effectively repair alkylated guanine caused by small alkyl-N-nitrosamines."

4.2.1 Type of S9 homogenates

As reviewed by Bringezu and Simon (2022), there are different studies indicating that metabolic activation of NAs using S9-mix was affected by species differences regarding the S9 fraction used, and suggesting the use of hamster S9 for the class of NAs (Lijinsky & Andrews, 1983; M. J. Prival, King, & Sheldon, 1979; Raineri, Poiley, Andrews, Pienta, & Lijinsky, 1981). Use of hamster S9 was reported to better metabolically activate some NAs than rat S9 under certain conditions (Lijinsky & Andrews, 1983). In 1979, Rao, Young, Lijinsky, and Epler (1979) concluded that the Ames test was not sensitive enough for carcinogenicity hazard prediction of aliphatic NAs. However, such studies were not conducted according to current OECD TG471 guideline. Current testing results, using historical and re-tested disparate results, no longer support this conclusion (Cross, 2022; Trejo-Martin et al., 2022), provided that appropriate conditions are employed. This requires an assessment with knowledge of the mechanism for the subclasses of NAs being studied based upon their chemical structures (Cross & Ponting, 2021; Dobo et al., 2022).

While Bringezu and Simon (2022) noted that CYP2E1 levels might not be adequately present in the tests with rat liver S9 under certain conditions, their work showed that rat S9-mix was capable of detecting mutagenicity of the NAs, when the tests were performed using the OECD TG471 guideline recommendations (*Salmonella* and *E. coli* strains). Whereas in earlier investigations, the induction of rat liver enzymes was performed using phenobarbital 1% (v/v) in drinking water, the tests by Bringezu and Simon (2022) used a combination of phenobarbital/β-naphthoflavone (PB/NF) as inducing agents. In addition, the inducers were administered via oral gavage, and might, therefore, lead to higher exposure of the rats, as compared to administration via drinking water. Such procedural differences in the enzyme induction might be responsible for the S9 species differences in the observed mutagenic responses between the two studies. The data by Bringezu and Simon (2022) indicated that PB/NF-induced rat liver S9 seems adequate for metabolic activation of NAs, and that the procedure used is suitable for the detection of mutagenic activity of NAs *in vitro*. In the respective S9-mix, 10% (v/v) S9-fraction was used.

In relation to S9 concentration, the literature, notably, reported that the mutagenic response of NAs can be larger, when using high percentages of S9-fraction in the S9-mix, as in the case of N-nitrosodibenzylamine (Schmezer, Brendler, Tompa, & Pool, 1990). Others have also shown that incubation time and higher amounts of S9-fraction can increase the mutagenic response of NAs (Bartsch, Malaveille, & Montesano, 1975; Michael J Prival & Mitchell, 1982; Rao et al., 1979). These observations, related to S9-mix (10% versus 30%), have been referred to as anecdotal evidence by (Lynch & Harvey, 2022).

A recent literature analysis confirmed that, historically, tests using rat S9-fraction had similar sensitivities than tests with hamster S9-fraction, (though, test data available using hamster S9 were limited), and that tests using hamster S9 may result in decreased specificity. It was observed that hamster S9 had a stronger dose-response (revertants/dose) for some compounds, although testing with rat S9 still produced positive study call results amongst those compounds (Trejo-Martin et al., 2022). In early studies with NAs, such as NDMA and NDEA, some experimental conditions had to be adjusted to see positive test results, when using rat S9-fraction (e.g. solvent, pre-incubation). In such instances, both negative and positive studies may be reported in the literature, with the more recent follow-up studies using the OECD TG471 guideline where appropriate conditions, based on the knowledge of activation mechanisms for individual nitrosamines, resulted in positive outcomes.

4.2.2 Pre-incubation versus plate incorporation method

As reviewed by Bringezu and Simon (2022), the plate incorporation and the pre-incubation methods have historically led to some conflicting bacterial mutagenicity study results, as reported in the literature. For example, in early testing, the plate incorporation method failed to detect the mutagenic effect of NAs with short-alkyl chains (e.g., NDMA), whereas the pre-incubation method led to positive results for the same compounds (Bartsch et al., 1975; Yahagi et al., 1977). Araki et al. reported that the protocol can affect the mutagenic response/potency, although mutagenicity was detected at all conditions used (Araki, Muramatsu, & Matsushima, 1984). The tests performed by (Bringezu & Simon, 2022) followed the OECD TG471 guideline recommendations and indicated the suitability of both the plate incorporation and pre-incubation protocols for detecting NAs, but that pre-incubation should be considered for NAs with short-alkyl chains. Indeed, according to OECD TG471 short chain aliphatic NAs like NDMA and NDEA may be detected more efficiently using the pre-incubation method. Specifically, Bringezu and Simon (2022) applied the following conditions for the pre-incubation experiments: the same volumes of bacterial suspension, test or control materials and S9-mix or phosphate buffer were mixed and incubated for 60 min at 37° C. After this pre-incubation period, 2 mL of molten top agar were added, and the mixture poured onto the selective agar plates. The plates were then incubated for 48 h at 37° C before final revertant analysis.

A recent literature analysis confirmed that, historically, the plate incorporation and pre-incubation test showed comparable performance and that the sensitivity and accuracy of the two methods were very similar (Trejo-Martin et al., 2022).

4.2.3 Impact of solvents

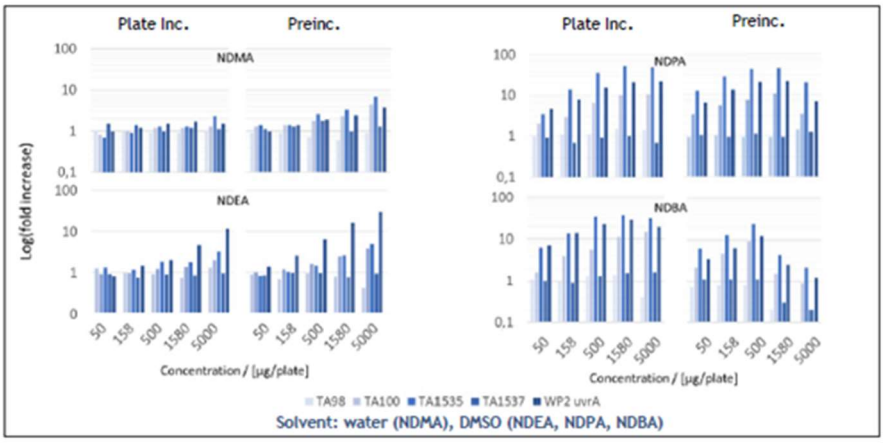
DMSO is known to inhibit several drug-metabolizing enzymes such as P450 enzymes that are involved in metabolic activation of chemicals, and this has even been observed at concentrations as low as 1 % or less (Busby et al., 1999; Chauret et al., 1998; Easterbrook et al., 2000; F. Peter Guengerich, 2000; Hickman et al., 1998; Kawalek & Andrews, 1980). The effect of regular organic solvents (DMSO, EtOH, MeOH, acetonitrile, and acetone) at different concentrations (0.1, 0.25, 0.5, 1, 2.5, 5, and 10% v/v) on P450-mediated metabolism using rat liver microsomes have been investigated by co-incubation of each solvent and the corresponding substrate of different CYP450 isoforms i.e., CYP1A, CYP2C, CYP2D, CYP2E, and CYP3A. It was observed that the sensitivity of the various P450 isoforms to particular organic solvents using rat liver microsomes was not identical, but, all of the tested solvents exhibited a significant inhibition on the activities of P450s at 10% (v/v). In particular, the rat CYP2D and CYP2E enzymes were much more sensitive to all of the solvents than the other tested enzymes, with the exception of acetonitrile (D. Li et al., 2010).

Identification of the metabolic pathways, responsible for drug metabolism, is a regulatory requirement. For nitroso-APIs the identity of the enzymes responsible for the metabolism of the parent API is, therefore, known in most cases. However, although it seems likely, it cannot be assumed that the same enzyme would be involved in activation of the nitroso-API. Additionally, it cannot be assumed that for relatively few APIs, which are not or barely metabolized, no metabolic activation of the nitroso-derivative (e.g. alpha-carbon hydroxylation) will occur. As has been described, several CYPs (>10) can participate and compete in human drug metabolism. Some drugs are preferentially metabolized by single CYPs, while for others several CYPs may be involved in various capacities. However, although phase I metabolism primarily involves oxidation mechanisms, the same metabolites are not necessarily formed at the same site of the API. As the identity of CYPs, responsible for metabolic activation of nitroso-APIs, cannot be confirmed without experimentation, it may not be possible to select the most appropriate solvent, based only on the knowledge of metabolic pathways. As metabolic pathways could potentially involve one or many CYP450 enzymes, no single solvent might be ideal, and a careful case-by-case reflection will be needed to decide on the most appropriate solvent for *in vitro* testing, considering amongst others information on the most sensitive enzyme(s), solubility of the NA compound and concentrations to be tested.

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. The results of this study are summarized in the figure below by Trejo-Martin et al. (2022), adapting the original figure of the corresponding publication (Bringezu & Simon, 2022). 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). Based upon this knowledge, this study recommends using the lowest concentration of solvent required, while avoiding precipitation and cytotoxicity.



DMSO has the most impact on inhibiting mutagenicity for NDMA. Larger chain aliphatic Nitrosamines are positive with DMSO induced rat liver S9 (phenobarbital/ß-napthoflavone)

For more general, non- Ames-focused information on potential solvent effects on NA-relevant CYP450 enzyme activities, see also paragraph 3.3 and ***Table 6***.

4.2.4 Bacterial strains used for Ames testing of NAs

A certain specificity of NAs to revert the missense Ames tester strains with excision-repair deficiency i.e., TA1535 and TA100 was evident in older studies. The base-pair substitution strain TA1535 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 TA1535 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).

The recent work by Bringezu and Simon (2022) clearly demonstrated that the strains outlined in the respective OECD TG471 guideline are adequate to detect the mutagenic response of NAs. More specifically, TA100 and TA1535 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 should, therefore, be combined with TA1535 and TA100 for appropriate and meaningful bacterial mutagenicity testing. The genotype of all the bacterial strains and their types of mutations are summarized in ***Table 7***.

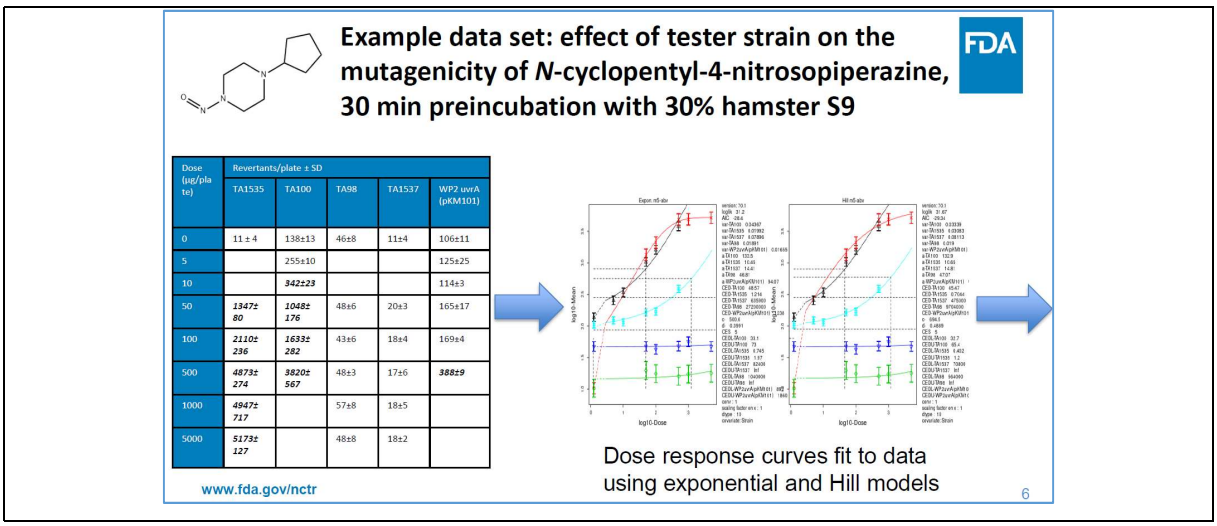
It is useful to remember in the context of bacterial mutagenicity testing of NAs that these strains are recommended in OECD test guideline TG471 "*because initial and emerging molecular analyses indicated that, collectively, they permitted the recovery of all six classes of base-substitution mutations, 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

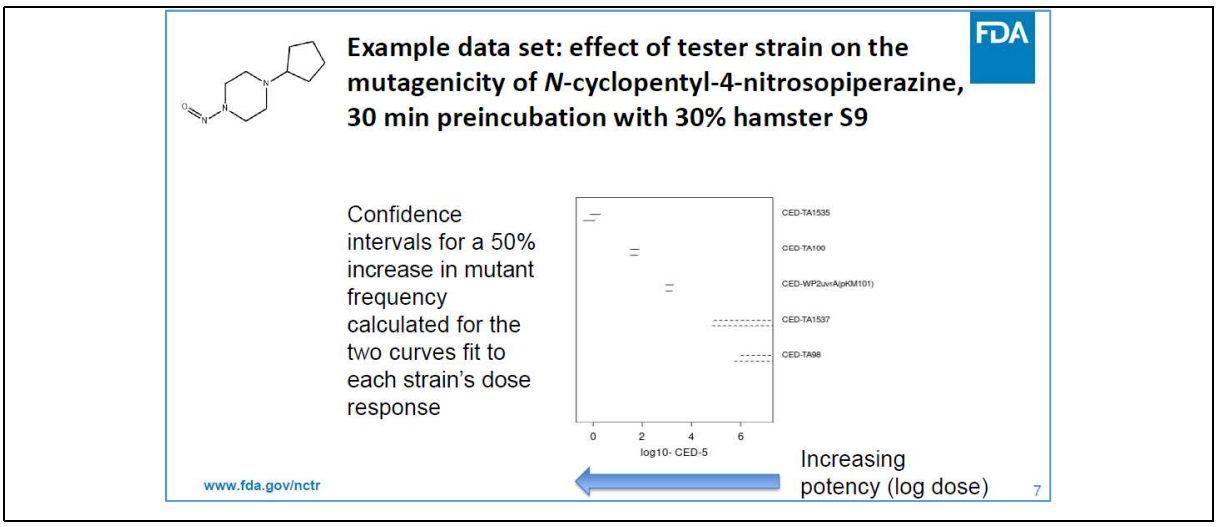
target site to the DNA-reactive moiety and the susceptibility of the specific DNA adduct-base to result in mutations (Levy et al., 2019; G. M. Williams, 2018; R. V. Williams et al., 2019).

Table 7*:* Characteristics of the proposed *Salmonella typhimurium* and *E. coli* 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	" "
E. coli		
EC WP2 *uvrA*	trp$^-$; uvrA$^-$	base-pair substitutions and others

Relative benchmark dose (BMD) mutagenicity potency values (and corresponding confidence intervals) are currently being considered to help in assessment of the optimum methodological conditions for the Ames test, as shown in the picture below from the work currently done by U.S Food and Drug Administration (FDA), National Center for Toxicological Research, Division of Genetic and Molecular Toxicology (Heflich, 2022). In this work, dose response curves were fit to data (slide 6 of the picture below) using the Proast BDM software (Wills, Johnson, et al., 2016; Wills, Long, et al., 2016) to derive BMD confidence intervals (slide 7), where: 1) the mutagenicity potency is on the x-axis; 2) the BMD confidence interval is the length of each line and a longer line means less confidence; and 3) dotted lines are results of not meeting the 2-fold rule (where a positive response is considered as twice the background mutation rate). The Benchmark response (BMR) (or CEST) level of 50% increase in mutants over background was selected as the point of initial increase in dose response (resulting in a BMD_{50} calculation). This analysis can illustrate the relative sensitivity of comparative approaches as well as indicating whether a more sensitive approach would change the Ames study outcome.





4.3 Basic methodology used as a starting point for assay optimization

Bacterial reverse mutation assays give reliable data on the ability of a chemical to interact with DNA and to induce gene mutations. The test system determines the frequency at which a chemical abolishes the effect of the forward mutation. The genetic target presented to a chemical is small, specific and selective. Therefore, several bacterial strains or a single bacterial strain with multiple markers are necessary to overcome mutagen specificity. The reversion of bacteria from growth-dependence on a particular amino acid (histidine or tryptophan) to the ability to grow in the absence of that amino acid (reversion from auxotrophy to prototrophy) is the most widely used marker. The *Salmonella typhimurium* histidine (*his*) and the *E. coli* tryptophan (*trp*) reversion systems measures $his^- \rightarrow his^+$ and $trp^- \rightarrow trp^+$ reversions, respectively. The *Salmonella typhimurium* (TA) and *E. coli* (EC) strains, which are recommended for OECD TG471 compliant testing were originally constructed to differentiate two types of point mutations, namely base pair substitutions (e.g., TA1535, TA100, and EC WP2 *uvr*A) and frameshift (e.g., TA1537, TA98) mutations (see ***Table 7***). All bacterial strains are regularly checked regarding membrane permeability, ampicillin resistance, UV sensitivity, and amino acid requirement as well as spontaneous mutation rates and responsiveness to selected reference mutagens to ensure identity of the strains and appropriate test performance.

4.3.1 Test procedure

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 rat 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:

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.

4.3.2 Solubility / Vehicle selection

A homogeneous test item preparation is required for testing. Thus, prior to testing, selected solvents are used for solubility testing. The solvent is chosen based on its solubility properties and its relative non-toxicity to the bacteria (Maron, Katzenellenbogen, & Ames, 1981). The possible inhibitory effect of the solvent on the enzymatic activity of the metabolizing enzymes has to be taken into account, when selecting the most appropriate solvent (see also paragraphs 3.3 and 4.2.3 for more detailed information on potential solvent effects with regard to metabolic activation). The following solvents are routinely used as vehicles in the Ames test: deionized water (most preferred), DMSO, acetone, EtOH, MeOH, tetrahydrofuran (THF), and 1% carboxymethylcellulose (CMC). Since some solvents (i.e., EtOH, acetone, and THF) are toxic to bacteria, a lower top concentration than the normal 5000 µg/plate of the test item might be applied, if one of these solvents has to be used, due to specific requirements of certain NAs. This may result in disregarding the recommendations on top dose given in the current OECD TG471 guideline.

4.3.3 Dose selection / Top dose

As required by the current OECD TG471 guideline in its effective version (2020) the maximum concentration to be tested is 5000 µg/plate, unless limited by toxicity or solubility issues of the test item. The concentration range covers at least two logarithmic decades. At least six adequately spaced concentrations are tested (e.g. 5000, 2500, 1000, 333, 100, 33, and 10 µg/plate). In general, dose selection of follow-up experiments is based on the results of the previous experiments.

4.3.4 Standard plate incorporation test procedure

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)

4.3.5 Pre-incubation test procedure

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 37°C for 60 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 compatibility of the solvent with bacterial growth and the test procedure (plate incorporation or pre-incubation method) the applied volumes of the test solution and solvent control can differ from the above-mentioned volumes.

4.3.6 Rat liver S9 homogenate

Due to the limited capacity for metabolic activation of potential mutagens in *in vitro* methods a mammalian-derived exogenous metabolic activation system is necessary. For test items, which have to be metabolized by hepatic enzyme systems, S9 liver homogenate will be added to the incubation mix in the form of S9-mix. Phenobarbital/β-naphthoflavone-induced rat liver S9 will be used as the metabolic activation system. The protein concentration in the S9 preparation is usually between 20 and 45 mg/mL. Each batch of S9-homogenate is routinely tested before use for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the bacterial reverse mutation assay.

An appropriate quantity of S9-fraction is thawed and mixed with an S9-cofactor solution to result in a final concentration of approx. 10% (v/v) homogenate in the S9-mix. Cofactors are added to the S9-mix to reach the following concentrations in the S9-mix: 8 mM $MgCl_2$, 33 mM KCl, 5 mM glucose-6-phosphate, 4 mM NADP in 100 mM sodium-ortho-phosphate-buffer, pH 7.4. During the experimental setup, the S9-mix is stored on ice until use. Preparation of the S9-mix is performed according to Ames,

McCann, and Yamasaki (1977). To boost metabolic activation of certain test items the final concentration of S9-fraction can be increased to approx. 30% (v/v) in the S9-mix for Ames testing.

4.3.7 Hamster liver S9 homogenate

The hamster S9 liver microsomal fraction 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.

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 in an ice bath. Preparation of the S9-mix is performed according to Ames et al. (1977) and Michael J Prival and Mitchell (1982).

4.3.8 Positive controls

Table 8: List of specific positive controls for the different tester strains.

Tester strain	Positive control	Concentration	Solvent
TA 1535, TA 100 without S9 mix	Sodium azide (NaN_3)	10 µg/plate	deionised water
TA 1537, TA 98 without S9 mix	4-Nitro-o-phenylene-diamine (4-NOPD)	10 µg/plate (TA 98) 50 µg/plate (TA 1537)	DMSO
EC WP2 *uvr*A without S9 mix	Methyl methane sulfonate (MMS)	2.0 µL/plate	deionised water
TA 1535, TA 1537, TA 98, TA 100, EC WP2 *uvr*A with rat S9 mix	2-Aminoanthracene (2-AA)	2.5 µg/plate (all TA strains) 10.0 µg/plate (EC WP2 *uvr*A)	DMSO
TA 1535, TA 1537, TA 100, EC WP2 *uvr*A with hamster S9 mix	2-Aminoanthracene (2-AA)	2.5 µg/plate (all TA strains) 10.0 µg/plate (EC WP2 *uvr*A)	DMSO
TA 98 with hamster S9 mix	Congo red	500 µg/plate	deionised water

The stability of the positive control substances in solution is unknown, but a mutagenic response in the expected range will be sufficient evidence of biological stability.

4.4 Strategy for optimization of the Ames test protocol for mutagenicity testing of NAs

In total, 42 compounds were carefully selected (see 2.1.) with high priority for testing in the bacterial mutagenicity assay (see ***Table 1***). The selected compounds are representative for the large variety of NA classes (e.g., small and large NAs, API-related ones, NA of special interest). The metabolic activity of the rat S9 liver homogenate batches, used in the experimental part of this project, was pre-tested after production of the S9-fraction, and will be determined in more depth, as described under 3.2.

Based on a broad literature search on mutagenicity and metabolic activation of NAs and using the Leadscope database of 281 compounds with Ames study results, 158 positive NAs with a total of 1511 individual strain tests were examined (each tested in standard OECD strains, and without and with metabolic activation, more specifically rodent liver S9). There was only one compound with a negative

Ames strain-specific test with S9-mix, which was positive only without S9-mix in the same strain. This compound was judged by the author to be a direct mutagen due to a non-NA functional group. Based on this result, it was decided to perform the Ames tests, in the present study, only in the presence and not in absence of S9-mix. Additionally, it has been clearly demonstrated in the literature (Bringezu & Simon, 2022) that some tester strains are more susceptible to NA-induced mutagenicity than others. Thus, we decided to use the three most sensitive bacterial standard Ames tester strains only (see below). This reduced set-up, as compared to standard OECD TG471 guideline studies, will enable us to test at least the originally planned 25 compounds for mutagenicity in the Ames test. However, with the overall expectation that NAs should be mutagenic in the Ames test and to avoid missing a positive compound, those compounds that test negative with S9-mix, will be re-tested without metabolic activation.

Initially, we will focus on defining the optimal test conditions for the bacterial reverse mutation assay with NAs regarding the exposure scenario, the metabolic activation system, solvents and the tester strains. This will be done by using NDMA, NDEA and eight additional model compounds. These will include priority 1 compounds (see 2.1), which will also be tested in the *in vitro* Comet assay with liver cell models. The tests will be performed with the selected set of three bacterial Ames assay tester strains i.e., TA 1535, TA 100 and EC WP2 *uvr*A. The pre-incubation method will be applied, due to its more sensitive outcome in previous investigations. In general, induced rat S9 liver homogenate [10% (v/v) S9-fraction] will be applied in the first run, but if tested negative a repeat experiment with 30% (v/v) induced rat S9 liver homogenate and/or hamster S9 liver homogenate [30% (v/v) S9-fraction] will be performed, in accordance with OECD TG471 (OECD 2020). At this phase we will strongly focus on the influence of the applied solvent on the mutagenicity of the NAs (see ***Table 9***).

Table 9: For each nitrosamine the following conditions will be tested

Condition	Solvent (µl)			
	Water*	DMSO*	Methanol*	Acetone*
Rat S9	100	7, 30, 100	7, 30	7, 30
Hamster S9**	100	7, 30, 100	7, 30	7, 30

*Number of concentrations tested may be limited by solubility, but 100 µl is too toxic for bacteria
** In case of additional experiments

Stock preparations of the compounds in deionized water, DMSO, acetone and methanol will be prepared. A top dose of 5 mg/plate, as specified in OECD TG471, will be tested if feasible. With regard to the applied volume of solvent used in the experiments the highest concentration in the stock preparation should be 50 mg/mL in water (application volume 100 µL), 714, 167 or 50 mg/mL in DMSO (application volume 7 µL, 30 µl or 100 µL, respectively), 714 or 167 mg/mL in acetone or MeOH (application volume 7 µL or 30 µl, respectively) (see ***Table 9***). In case of lower compound solubility in the respective solvent the highest feasible concentration (e.g. homogeneous suspension) will be used as top concentration.

The pre-incubation method will be performed in the presence of 10% (v/v) phenobarbital/ß-naphthoflavone-induced rat S9-mix. The pre-incubation procedure will last for 60 min at 37°C.

Compounds, which show no clear mutagenic effect using induced rat liver S9 homogenate with 10% (v/v) S9-fraction, will be re-tested with an increased concentration of 30% (v/v) S9-fraction. Case by case, the use of 30% (v/v) non-induced hamster S9-mix can be more appropriate. In this case, a similar approach, as described above for rat S9-mix, will be applied (see ***Table 9***).

Appropriate negative and positive controls, based on the laboratory's broad historical control data base and considering safety at work, will be run in parallel to check for suitability of the test system. The reliability of the obtained test results in this experimental phase will be checked against existing literature results, wherever possible. Finally, based on the outcome of testing of the initial set of ten NA compounds the most sensitive experimental setup will be defined, and a preliminary optimized test protocol will be issued, including parameter like the most suitable solvent(s)/solvent concentrations, rat S9 homogenate versus hamster S9 homogenate (if data are available), 10% (v/v) versus 30% (v/v) S9-fraction (if data are available) and tester strain selection.

An additional 15 compounds (remaining priority 1 compounds) will be tested to check the preliminary optimized protocol from the previous phase. Based on the data of the first phase of the project the most suitable solvents and most suitable solvent concentrations will be used. If the compounds test negative under the optimized protocol using two different solvents they will be tested under other conditions (e.g. pre-incubation or plate incorporation, hamster or rat S9, different solvent, if not water soluble). The data will assist in defining appropriate positive and negative control substances (including simple and complex NAs). The hypothesis of high sensitivity of the selected setup - preliminary test protocol – will be again reviewed and potentially updated. Shortcomings in the preliminary test protocol will thereby be addressed.

In the final phase (budget has to be extended), if no further modifications are needed, an additional set of at least 13 compounds (selected priority 2 compounds) will be investigated, according to the final optimized testing protocol. If the compounds test negative, they will be tested under two other conditions, if appropriate. Predictivity of the obtained mutagenicity data for rodent carcinogenicity (existing database) will finally be analyzed. The analysis will elucidate reproducibility (comparison to existing data in the literature), sensitivity (correct identification of genotoxic carcinogens), and specificity (correct identification of non-genotoxic compounds). Positive predictivity (reflecting the false positive rate) and negative predictivity (reflecting the false negative rate) are further benchmarks for a final judgement on the proposed optimized protocol. The outcome of the complete experimental testing in the bacterial reverse mutation assay (Ames test) will be summarized in a final report. This document will describe the optimal conditions for testing and will contain the final optimized test protocol for the Ames test with NAs.

4.5 Ames fluctuation test

The criteria for study design, as defined in the optimized test protocol based on the requirements of OECD TG471 (OECD, 2020) will finally be transferred to a miniaturized version of the bacterial mutagenicity assay, i.e., the Ames fluctuation test. This Ames test variant is already mentioned in the updated OECD TG471 guideline in paragraph 8 (Principles of the test method) as "fluctuation method". At least five representative and meaningful compounds, which were already tested using the optimized standard test protocol, will be tested. In contrast to the OECD TG471 plate incorporation or pre-incubation test method, the Ames fluctuation test is performed in a 24-well format for test article exposure and subsequently in a 384-well format for revertant selection. In this assay, revertant analysis is not based on colony counting, but on a color change of the medium from purple to yellow indicating bacterial growth and subsequent growth-dependent pH changes (bromocresol purple is a pH indicator dye). The Ames fluctuation method, therefore, needs less test article, is less time consuming (simple colorimetric analysis) and is more appropriate for higher throughput screening than the classical Ames test. The miniaturized Ames test protocol, which will be used in the present project, was already evaluated in an international round-robin study (see Reifferscheid et al. (2012) for methodological

details) and the methodology is also available as ISO norm for genotoxicity screening of water and waste water (ISO, 2012). Before final mutagenicity testing of NAs with the Ames fluctuation test, the standard method will be adapted based on the optimized Ames testing protocol for NAs. A final check on transferability will be performed by comparing the outcome of the different Ames test methodologies with issuance of the final performance assessment.

For the Ames fluctuation assay, overnight cultures of the different types of bacterial strains, also used in the standard Ames experiments will be prepared. Therefore, 20 µl of thawed bacterial suspensions are added to 20 ml of Nutrient Broth and are grown overnight (12 ± 2 h) at 37 °C using a shaking incubator. For TA98 and TA100 20 µl of an ampicillin stock (50 mg/ml in NaOH) is added. At the end of overnight growth OD600 of the 1:10 diluted cultures are measured using a bio-photometer and FAU (formazine attenuation unit) values are calculated to estimate bacterial numbers. For cytotoxicity and mutagenicity testing overnight cultures are diluted with exposure medium to strain-specific FAU values. Cytotoxicity testing is performed, if needed, with TA98 to be able to get sufficiently high OD600 values, with OD600 measurement and, thus, turbidity representing the endpoint. The exposure medium consists of magnesium sulfate, citric acid monohydrate, di-potassium-hydrogen phosphate, sodium ammonium phosphate D (+) glucose monohydrate, biotin, histidine or tryptophan (E. coli). Histidine or tryptophan are added in such amounts to enable approximately 2 cell divisions. In total at least 50 ml of diluted bacterial suspensions are needed.

As testing was decided to be performed with S9-mix only, the following materials are mixed in wells of a 24-well plate (A plate) and subsequently incubated at 37°C for 100 min using a shaking incubator:

- 10 µL test item solution at each dose level (solvent = negative control or reference mutagen solution = positive control; see standard Ames testing for types of positive controls)
- 490 µL of bacterial suspension (from diluted overnight cultures of the strains)
- 17 µL of S9-mix
- Total incubation volume 517 µL

Every 24-well plate harbors both a negative/solvent control, a positive control and 6 concentrations of the compound both in triplicate. For cytotoxicity testing with TA 98 the incubation volume is doubled. As highest concentration the first concentration with clear cytotoxicity or first concentration with compound precipitation is used for mutagenicity testing. This standard protocol might change depending on the optimized conditions, as defined in the standard Ames test experiments, and the compatibility of the respective solvent with the bacteria.

For cytotoxicity testing OD600 values of the TA98 wells of the A plate are measured and FAU values are calculated. Before OD600 measurement wells of a new 24-well plate (B plate) are filled with 2.5 ml of pH indicator medium (exposure medium without histidine or tryptophan, but with bromocresol purple) and after OD600 measurement, 500 µl of the incubation suspension per well of the A plate are transferred to wells of the B plate. For all other bacterial strains, 2.5 ml of indicator medium are directly added per well to the wells of the respective A plate. From every 24-well plate three 384-well plates (one per replicate) are then prepared with 48 wells (volume 50 µl) generated from one well of the 24-well plate. In total 144 wells per treatment are finally analyzed for bacterial growth after incubation for 48 h at 37 °C using an incubator. For incubation the 384-well plates are placed in lockable plastic bags to avoid exsiccation of the plates.

Finally, wells are inspected by the naked eye regarding a color change from purple to yellow (bacterial growth). Even a slight change is judged as positive. The number of revertant wells (yellow) of the

compound-treated wells are compared with the respective negative/solvent control. When an increase in revertant wells of a factor of more than three occurs, mutagenicity is claimed. The results of the Ames fluctuation test will ultimately be compared with the standard assay procedure, particularly regarding sensitivity.

5 *In vitro* Comet assay with liver cell models

5.1 General introduction

The *in vitro* single-cell gel (SCG)/Comet assay represents a test principle for identifying genotoxic agents in non-dividing cultured mammalian cells and for further characterizing the types of DNA damage. Additionally, the Comet assay can be used to monitor DNA repair in time course studies. The assay is based on the electrophoretic mobility of DNA fragments embedded in agarose on slides, enabling the detection of DNA damage on the single cell level (***Figure 1***). Based on separation of the two DNA strands upon alkaline treatment, the alkaline version (pH > 13) of the Comet assay can detect DNA single-strand breaks (SSB) and DNA double-strand breaks (DSB), DNA-DNA and DNA-protein cross-links, alkali-labile sites (ALS) and SSB arising as DNA repair intermediates. DNA damage is detected as DNA migrating out of the cell nucleus during single-cell gel electrophoresis, resembling a comet tail. In particular, the measured tail intensity (TI; % DNA in tail) is proportional to the number of DNA strand breaks induced.

There exist various modifications of the methodology in order to specify positive data, including 1) the use of different pH conditions (alkaline vs. neutral) to discriminate between SSB, DSB and ALS, 2) detection of DNA-DNA and DNA-protein cross-links by simultaneous exposure of the cells to an inducer of DNA strand breaks (e.g. gamma irradiation) and subsequent quantification of the reduction in DNA migration, and 3) the use of DNA-glycosylases to investigate specific DNA-base modifications, like the oxidative DNA lesion 8-Oxoguanine.

The *in vitro* Comet assay, which will be evaluated in the present project for its potential to predict NA mutagenicity and carcinogenicity, may employ cell lines or primary cell cultures. Here, we will use primary rat and human hepatocytes (without S9-mix) and the liver cell line HepG2 (most likely without and with S9 mix, depending on the pre-experiments on metabolic competence performed under 3.2), which are considered to be metabolically competent and NA-relevant.

Notably, the alkaline Comet assay represents an indicator test, which doesn't detect irreversible DNA damage such as mutations, but DNA damage that is still repairable. It can, thus, not be directly concluded from a positive Comet assay that a substance is mutagenic and/or carcinogenic *in vivo*. Due to the transient nature of DNA damage, and in part rapid DNA repair, incubation times and cell/tissue sampling are crucial parameters.

There is currently no international adopted OECD Technical Guidance Document available for the *in vitro* Comet assay, while there is adoption of the *in vivo* version of the assay. The *in vivo* alkaline Comet assay, which may employ different kinds of tissues like blood and liver as "systemic tissues" or lung, stomach, and gut as "first site of contact tissues", is covered by OECD TG489. Besides OECD TG489, which provides also methodological hints for *in vitro* testing, there are also numerous publications available on the mostly used protocols for the *in vitro* Comet assay (Azqueta & Collins, 2013; Moller et al., 2020; OECD, 2016; Tice et al., 2000; Vasquez & Frotschl, 2016), which will be evaluated and used for optimizing the *in vitro* Comet assay protocols with liver cell models.

The *in vitro* alkaline Comet assay harbors both advantages and disadvantages. Advantages include generation of data on a single cell level, usage of diverse cells and tissues possible, independent of cell division, low cell number needed, relatively rapid and easy to perform methodology with economic advantages, use of digital imaging avoids evaluator bias, high sensitivity and specificity and coverage of mechanistic aspects, when adapting the protocol.

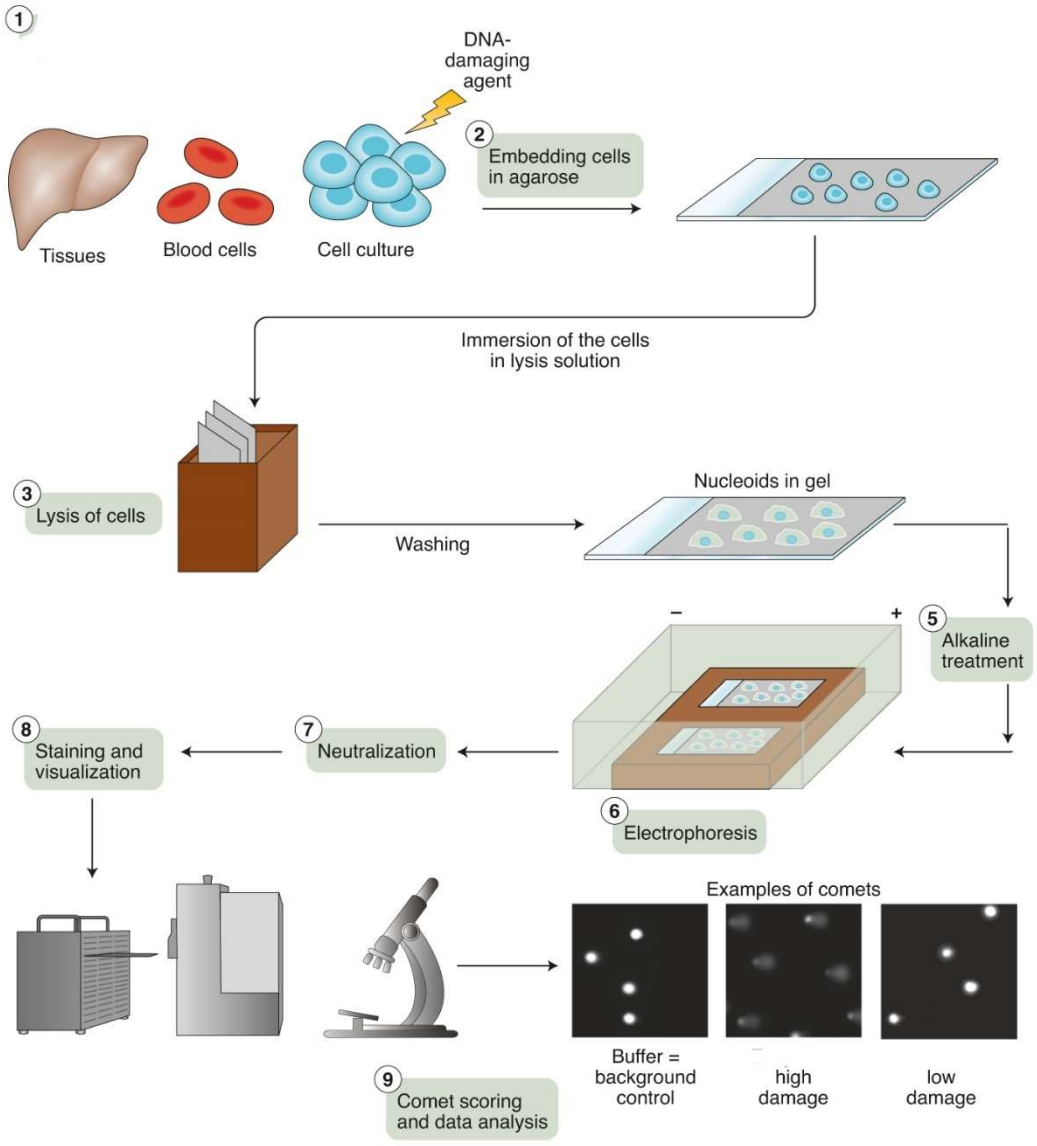


Figure 1: Scheme depicting the alkaline Comet assay procedure adapted from (Moller et al., 2020)

Disadvantages of the assay are that differentiation of cell types is hardly possible, evaluation can be time-consuming, only a small cell sample is evaluated (statistical issue), the assay detects only pre-mutagenic lesions, but no fixed DNA damage, personal scoring might be evaluator biased, kinetics is highly important due to ongoing DNA repair, and data interpretation is sometimes difficult, particularly for highly cytotoxic or pro-apoptotic agents. The latter aspect requires parallel cytotoxicity testing, e.g., by assessing cell counts, lactate dehydrogenase (LDH) leakage or metabolic capacity of the cells (MTT or resazurin reagents). In the present study, cytotoxicity testing will also be used for concentration-

range finding to avoid unspecific effects due to excessive cytotoxicity. Only concentrations will be used for the main experiment which mediate less than 50 % cytotoxicity according to the ICH S2 (R1) guidance (EMA, 2012) document for *in vitro* genotoxicity testing.

5.2 Methodological literature search on *in vitro* Comet assay with NAs and liver cell models

To evaluate the existing data pool on *in vitro* and *in vivo* alkaline Comet assays with NAs and to aid in compound selection, a deep literature research on *in vitro* and *in vivo* alkaline Comet assays with NAs was performed (see **Appendix 1**). In total, this search yielded 107 publications on this topic, the majority (80) of which addressed *in vitro* studies with different primary and tumor cell types, whereas 37 publications covered *in vivo* data. The literature search confirmed that the assay seems, in principle, be able to mirror both positive Ames test and carcinogenicity outcomes for different NA species. In total 23 NAs with *in vitro* and 15 NAs with *in vivo* Comet assay data were identified (see **Table *10***).

Table 10: Compounds with Comet assay data, as identified by deep literature search (Appendix 2)**.**

Compound	**CAS-No.**	***In vitro***	***In vivo***
N-Nitrosodiethylamine (NDEA)	55-18-5	X	X
N-Nitrosomorpholine (NMOR)	58-89-2	X	X
N-Nitrosodimethylamine (NDMA)	62-75-9	X	X
N-methyl-N -nitro-N-nitrosoguanidine (MNNG)	70-25-7	X	X
N-nitrosodiphenylamine (NDPhA)	86-30-6	X	-
N-Nitrosopiperidine (NPIP)	100-75-4	X	X
N-Nitrosodi-N-propylamine (NDPA)	621-64-7	X	X
N-Methyl-N-nitrosourea (MNU)	684-93-5	X	X
N-Ethyl-N-nitrosourea (ENU)	684-93-5	X	X
N-Nitrosodi-N-butylamine (NDBA)	924-16-3	X	X
N-Nitrosopyrrolidine (NPYR)	930-55-2	X	X
N-Nitrosodicyclohexylamine	947-92-2	X	-
N-Nitrosodiethanolamine (NDELA)	1116-54-7	X	X
N-ethyl-N-nitro-N-nitrosoguanidine (ENNG)	4245-77-6	X	-
N-Nitrosoethylmethylamine (NEMA)	10595-95-6	X	X
1-nitroso-4-methylpiperazine (NMPz)	16339-07-4	X	-
3-N-nitroso-oxazolidin-2-one	38347-74-9	X	-
N-Nitroso(acetoxymethyl)methylamine (NDMAOAc)	56856-83-8	X	-
S-Nitrosoglutathione	57564-91-7	X	-
4-(Methylnitrosamino)-1-(3-pyridyl)-1-butanone (NNK)	64091-91-4	X	X
N'-Nitrosonornicotine	80508-23-2	X	
4-(acetoxymethylnitrosamino)-1-(3-pyridyl)-1-butanone (NNKOAc)	127686-49-1	X	
1-Ethyl-1-nitrosourea (NEU)	759–73–9	X	
N-Butyl-N-(4-hydroxybutyl)nitrosamine (BBN)	3817-11-6		X
N-Nitrosoproline (NPro)	7519-36-0		X

In the next step, the evaluation of the results of the literature search was narrowed down to publications focusing on the liver. This search resulted in 24 (*in vivo*) and 27 *(in vitro*) publications on the corresponding subject. The fact that about 65% of the *in vivo* Comet studies are based on findings in the liver further highlighted the importance of this organ in the context of genotoxicity testing of NAs. The sheer number of publications in itself, also illustrates the still limited data situation regarding NAs and Comet assay investigations. For *in vitro* liver models only 12 studies with NAs could be identified, which were checked in detail with regard to methodologically important parameters (see **Appendix 3**). Most of these liver-focused *in vitro* studies were performed in HepG2 cells, followed by studies in HepaRG and PHH. The fact that S9-mix as exogenous metabolic activation system was only used in one HepG2 study by Hong et al. (2018) indicated that the metabolic competence of the *in vitro* models was not or only partially considered in the evaluated studies, and seems to represent an important parameter for the study outcome, particularly, when using HepG2 cells. When evaluating the positive *in vitro* Comet assay findings with liver cell models (22 publications), notably millimolar concentrations of NAs were needed to induce DNA strand breaks, and, thus, a positive outcome (see also ***Table 2***). In the case of the negative outcomes (7 publications) it is noteworthy that the NA concentrations were in part lower. At the same time, negative *in vitro* Comet assay results occurred especially in rather unusual liver models such as HiHeps, Hep3B and JHH6. But, HepG2 cells, as established and well-known liver cell model, also showed some negative or inconclusive results, which seemed to be more related to cell origin, cell exposure, concentrations and solvents used than to methodological shortcomings regarding the Comet assay itself.

When compiling the methodological approach of these studies, it is generally noticeable that the essential information regarding the performance of the *in vitro* Comet assay (Moller et al., 2020) is partially incomplete with more or less information gaps (see **Appendix 3**), limiting evaluation of the test outcomes. Notably, there were no marked differences between the published protocols pointing to the Comet assay methodology not to be of major influence, if appropriately applied. This result of the literature search supported the decision not to start with harmonized *in vitro* Comet assay protocols in the present project, but to use the well-established protocols of the three partners and to set more value on appropriately characterized cell models and clearly defined cell culture and cell exposure conditions. In the present project, HepG2 cells were, for example, directly obtained from a cell repository and were deeply characterized (e.g., population doubling, morphology, chromosome number, response to known genotoxins, performance in the *in vitro* alkaline Comet assay etc.), prior to experiments with NAs. A common working batch was prepared for the whole project and all partners will use the same batch, the same culture conditions and a limited number of passages (2 – 12; see also 5.6). Additionally, potential solvent effects will be considered.

5.3 Liver cell models for mutagenicity testing of NAs

Metabolic competence of cells to provide human relevant NA testing is considered a key feature of a useful test system. Most carcinogenic NAs need to be metabolically activated to become carcinogenic. The complexity and species specificity of metabolic competence is, therefore, highly important for both NAs and the relevance of the test system. Appropriately cultured primary cells, maintaining the specific *in vivo* metabolism of a certain tissue, are considered the gold standard. Established cell lines always need to be validated against primary cells or also the respective tissue *in vivo.* In the case of missing relevant, enzyme activities, these have to be complemented for meaningful results. This will be done by using broadly accepted exogenous metabolic activation systems, like S9-mix, or by stably transfecting and expressing relevant genes in cell lines.

Liver is considered the most sensitive tissue for most NAs and liver also represents the tissue with highest metabolic competence. Therefore, liver models were chosen for the *in vitro* Comet assay experiments in this project. Different *in vivo* Comet assay studies have already shown positive results for NAs in the liver (see 5.2). Primary human and rat liver cells as well as HepG2 cells, as an established human liver cell line, will be employed and evaluated. They will finally be compared regarding competence to predict both Ames test results and the carcinogenic potential of NAs. HepG2 cells are ubiquitously available for every laboratory from the most important international cell repositories, whereas primary cells are more difficult to obtain and need experience for isolation and/or appropriate handling. The advantage of primary cells, however, is the relevance for the species they are derived considering metabolic competence. Metabolic competence of the used primary cells and HepG2 cells will be evaluated and validated in the present project. HepG2 cells will be complemented with S9 mix, if necessary.

Primary human hepatocytes

Metabolic activation of NAs is catalyzed by a variety of CYP450 isoforms, namely CYP2E1, CYP2A6, CYP2B1, CYP2C9 and CYP3A4 among others (Bellec et al., 1996; Y. Li & Hecht, 2022). The ideal hepatic *in vitro* model would thus express all relevant CYPs in physiological composition. Primary human hepatocytes (PHH) are regarded as gold standard for *in vitro* studies involving metabolism of xenobiotics (Gomez-Lechon, Tolosa, Conde, & Donato, 2014). However, the maintenance of specific metabolic functions in culture is challenging and depends amongst others on the three-dimensional environment and extracellular matrix composition (Xu, 2021). For instance, culture in a collagen sandwich (Fahrig, Rupp, Steinkamp-Zucht, & Bader, 1998) or 3D hydrogels (H. J. Lee et al., 2020) was shown to promote the maintenance of CYP activities. PHH cultured in 3D spheroids, maintained activities of CYP1A2, CYP3A4, CYP2D6, CYP2C8 and CYP2C9 for 21 days in culture (Vorrink et al., 2017). Today, cryopreserved PHH are commercially available and can be purchased along with information on batch-specific CYP activities. However, in addition to the susceptibility to changes in culture conditions, cryopreserved hepatocytes have the disadvantage of very high costs for purchase and culture. For that reason, there are attempts to develop hepatocyte models based on cancer cell lines such as HepG2 or HepaRG. Gerets and co-workers (Gerets et al., 2012) characterized HepG2 and HepaRG cells and compared them to PHH regarding CYP expression and activity as well as sensitivity to detect hepatotoxicants. As expected, CYP expression and inducibility was lowest in HepG2 cells whereas HepaRG cells showed better comparability to PHH upon induction. However, principal component analysis showed a clear separation of all three cellular models. More recently, Yokoyama et al. (2018) studied metabolic capacities of HepG2, HepaRG and cryopreserved PHH. In the latter study, phase I enzyme activities I.e., CYP1A1, CYP2B6, CYP2C9 and CYP2C19 were not detectable in HepG2 cells, while CYP2D6 and CYP3A4 activities were very low. However, HepG2 cells expressed phase II enzyme activities with sulfotransferase activity being in the same range as in HepaRG cells and PHH. In contrast, enzyme activities, as expressed by HepaRG, were generally in the range of PHH or even higher. (Gupta et al., 2021) used RNA sequencing to compare various human liver cell models to native human liver tissue. The authors concluded that PHH are an adequate system for short term studies (24h) while HepG2 showed the lowest similarity with liver tissue.

Primary rat hepatocytes

Primary rat hepatocytes are an appropriate and valuable model for the *in vitro* Comet assay, bearing in mind that the *in vivo* Comet assay is routinely performed in rats in a regulatory capacity (OECD, 2016). Furthermore, the *in vivo* Comet assay was validated in comprehensive studies using liver tissue

and other tissues from rats (Rothfuss et al., 2010; Uno et al., 2015). Primary hepatocytes obtained from rodents (rat or mouse) as well as from human donors are the gold standard in pre-clinical safety testing to study the ADME profile of substances and to assess potential hepatotoxic effects (Fraczek, Bolleyn, Vanhaecke, Rogiers, & Vinken, 2013; Vilas-Boas et al., 2019). Primary hepatocytes are characterized by their metabolic competence, i.e., the expression of all relevant enzymes for phase I and phase II metabolism. Furthermore, primary hepatocyte express numerous influx and efflux transporters for the uptake and elimination of substances and their metabolites (Gupta et al., 2021). It is known that many drug-metabolizing enzymes can vary between species (*e.g.,* human versus rodent) in their isoform composition, expression and activity levels. Of particular importance for the metabolic activation of NAs are CYP2E1, CYP2A6 (rat orthologue: CYP2A1/CYP2A2) and CYP3A4 (rat orthologue: CYP3A1/CYP3A2) (Cross & Ponting, 2021; C. S. Yang et al., 1990). Regarding CYP2E1, few differences between human, mouse and rat orthologues exist. Rat CYP2E1 shows 80% sequence homology to human CYP2E1 and the substrate spectrum (*e.g.,* nitrosamines, acetaminophen, ethanol) widely overlaps, making rats the best model to study CYP2E1-dependent metabolism (M. Martignoni et al., 2006). The rat orthologues of human CYP2A6 comprise CYP2A1, 2A2 and 2A3. The latter is only expressed in extrahepatic tissues, whereas the others are expressed in the liver in a gender-specific manner (CYP2A1 – female dominant; CYP2A2 – male dominant) (Marcella Martignoni et al., 2006). Rat CYP2A1/2A2 display about 60% sequence homology to human CYP2A6. Additionally, endogenous steroids such as testosterone are substrates of rat CYP2A1/2A2, which is not the case for human CYP2A6. Regarding CYP3A, the situation is even more complex. In humans, four CYP3A isoforms are expressed: CYP3A4, -3A5, -3A7 and 3-A43, with highest abundance of CYP3A4 in the liver. In rats, six CYP3A forms were identified, including CYP3A1, -3A2, -3A9, -3A18, -3A23 and -3A62 (Marcella Martignoni et al., 2006). Rat CYP3A1 and CYP3A2 are the orthologues of human CYP3A4 and are expressed only in the liver (Marcella Martignoni et al., 2006). It should be mentioned that some differences in substrate specificity and catalytic activity of human CYP3A4 versus rat CYP3A1/-3A2 were observed using prototypical substrates such as nifedipine (Carr et al., 2006).

Human HepG2 cells

While established human hepatoma cell lines have retained some metabolic competence of primary liver cells, they are usually not comparable to primary cells with respect to amount of constitutive expression and induction of phase I and phase II enzymes. Wilkening and Bader (2003) investigated expression of CYPs 1A1, 1A2, 2B6, 2C9, 2D6, 2E1, 3A4, and 3A7 as well as phase II enzymes EPHX1, UGT1A6, GSTM1, SULT1A1, NAT1, and NQO1 in HepG2 over the first 10 culture passages and PHH over 1 week of culture using RTPCR. Extremely low expression in HepG2 was found for CYPs 1A2, 2C9, 2E1, and 3A4, whereas CYPs 1A1, 2B6, 2D6, 3A7 as well as UGT1A6, and NQO1 were well expressed in both cell types with significant passage- and time-dependent expression changes, respectively. CYP2D6, EPHX1, and GSTM1 were the most stably expressed enzymes in HepG2 and primary cells. In a further study Wilkening, Stahl, and Bader (2003), NDMA was not found positive in HepG2 cells using the *in vitro* Comet assay in contrast to B[a]P, which confirms negligible expression of CYP2E1 in HepG2 cells. In contrast, NDMA was clearly positive in primary hepatocytes in the *in vitro* Comet assays. In a CYP2E1 overexpressing HepG2 cell line (HepG2 E47), however, NDMA was also found clearly positive. Similar results were reported by Yokoyama et al. (2018). While HepG2 cells retained some liver cell typical metabolism, the deficiencies will be characterized in this project prior to use and rat liver S9 will be employed to complement the cell model to efficiently activate NAs, as CYP2E1 represents a very relevant isoform. The advantages of HepG2 cells, however, are their easy cultivation and handling,

ubiquitous availability and low price, compared to other human liver cell models. This facilitates the use with easy to reproduce standard protocols.

5.4 Basic protocol for *in vitro* Comet assay testing with primary rat hepatocytes

Isolation and plating of primary rat hepatocytes: Wistar rats (Janvier Labs) will be used to isolate primary hepatocytes by *in situ* liver perfusion. To this end, rats are anesthetized with pentobarbital, followed by hepatocyte isolation using a two-step EGTA/collagenase-perfusion protocol as described previously (Gao, Rutz, & Schrenk, 2020; Schrenk, Karger, Lipp, & Bock, 1992; Seglen, 1976). Cells will only be used for culture and NA testing, if the viability, as determined by trypan blue exclusion, will exceed 85%. For subsequent genotoxicity and cytotoxicity testing, isolated hepatocytes (200,000 per well) will be seeded in a volume pf 0.5 ml cell culture medium (DMEM low glucose, supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin solution) per well on rat tail collagen-coated 24-well plates at 37 °C in humidified atmosphere with 5% CO_2. Cells will be left for 3 h to enable cell attachment. Subsequently, the medium will be replaced by 0.5 ml of fresh medium per well containing the NAs of interest or the positive and negative references.

Characterization of metabolic competence of primary rat hepatocytes: The isolated primary rat hepatocytes will be characterized regarding their metabolic competence by means of CYP-isoform specific substrates and mass spectrometric analysis, which will be performed by BfArM, as described under 3.2.

Cytotoxicity testing and concentration finding: To determine cellular sensitivity and for concentration-range finding for the *in vitro* Comet assay, the primary rat hepatocytes will initially be exposed to increasing concentrations of the respective NA (seven concentrations including solvent control) for 24 h as the most often used incubation time for NAs in liver cell models (see ***Table 2***). Four hours of incubation might alternatively be used, when considering opposing DNA repair in the Comet assay experiments and the results of pre-experiments. Literature data will serve to guide the selection of concentration ranges (see 5.2 and ***Table 2***). Viability will then be assessed by resazurine reduction test (endpoint: metabolic activity), as described previously (Arnold et al., 2022; Gao et al., 2020). Initially, experiments will be performed with NDMA (prototype of ubiquitous small NAs) and NNN (tobacco-specific NA, prototype of bulky NAs) as data-rich substances. Up to eight NAs selected as priority 1 compounds (see 2.1) will subsequently be tested. The obtained data will be used to derive EC_{50} and EC_{20} values, which will allow for grouping of the tested NAs with regard to their cytotoxic potential. Furthermore, both parameters will be crucial to determine the concentration-range for the subsequent genotoxicity study i.e., the *in vitro* alkaline Comet assay. Parallel cytotoxicity testing will also be needed for data interpretation in the alkaline Comet assay experiments.

Genotoxicity testing: The *in vitro* alkaline Comet assay will be used to analyze DNA strand break induction by the selected NAs. Cells will be exposed to increasing concentrations of the selected NAs (five concentrations plus solvent control, maximum concentration EC_{50}) for 4 or 24 h, depending on pre-experiments on the most appropriate incubation time for NAs. Only concentrations will be used for the main experiment, which cause less than 50 % cytotoxicity according to the ICH S2 (R1) guidance (EMA, 2012) document. As positive controls, the alkylating agent methyl methanesulfonate (MMS) and the anticancer pro-drug cyclophosphamide monohydrate (CP) will be included. After the incubation period, the medium will be aspirated, and cells will be dislodged by TrypLE digestion. The alkaline Comet assay will then be conducted as recently published (Dorsam et al., 2018) and detailed in ***Table***

11. The obtained data sets will subsequently be subjected to benchmark dose (BMD) modeling using PROAST software to enable amongst others comparative analysis with the other two liver cell models.

5.5 Basic protocol for *in vitro* Comet assay testing with primary human hepatocytes

Plating and culture of primary human hepatocytes: Cryoplateable human hepatocytes will be purchased from a commercial provider (strongly adhering to ethical rules; e.g., BioIVT, XENOTECH or ThermoFisher) and should, preferentially, be appropriately characterized in terms of viability, yield, and metabolic activity. Furthermore, the cells should be pooled from different donors (both females and males) to cover a broad panel of enzyme polymorphisms with regard to NA-relevant metabolic enzymes (see 3.1). If feasible (availability of a sufficient large batch from one provider), one batch will be used for both the characterization of metabolic competence (see 3.2) and *in vitro* (geno)toxicity testing of in total 10 NAs. After thawing, hepatocytes (about 350,000) will be seeded in 0.5 - 1 ml medium (depending on the optimized medium of the provider to maximally support cell differentiation) per well on collagen-coated 24-well plates and cultured for 2 - 4 h at 37°C in a humidified atmosphere with 5% CO_2. Following cell attachment, the medium will be replaced by 0.5 - 1 ml fresh cell culture medium per well containing the NAs of interest or the positive and negative references. To ensure appropriate cell integrity and optimal culture conditions, the entire process is performed according to the provider's instructions, using all recommended components.

Characterization of metabolic competence of primary human hepatocytes: As already described for the primary rat hepatocytes the human hepatocytes will also be characterized with regard to metabolic competence by means of CYP-isoform specific substrates and mass spectrometric analysis, which will be performed by BfArM, as described under 3.2.

Cytotoxicity and genotoxicity testing: Based on the results obtained from concentration-range finding with primary rat hepatocytes and dose-finding experiments in HepG2 cells, as surrogate models (only a limited number of PHHs can be afforded in the present project) will be exposed to increasing concentrations of the selected NAs (five concentrations plus solvent control) for 24 h. Four hours of incubation might alternatively be used, when considering opposing DNA repair in the Comet assay experiments and the results of pre-experiments. The most appropriate incubation time will be subject of pre-experiments, preferably by estimation in HepG2 cells. For the main experiment, only concentrations will be used, which mediate less than 50 % cytotoxicity, according to the ICH S2 (R1) guidance (EMA, 2012) document. As positive controls, the direct mutagenic agent ethyl methanesulfonate (EMS; directly clastogenic) and the anticancer pro-drug cyclophosphamide monohydrate (CP; clastogenic after metabolic activation; indicator for metabolic competence of the PHH) will be included. After the incubation period, the medium will be removed, the cells will be detached carefully and subjected to the alkaline Comet assay. The alkaline Comet assay will then be conducted as detailed in ***Table 11***. As indicators for cytotoxicity, lactate dehydrogenase (LDH) release and cell number will be measured after incubation to exclude excess cytotoxicity with artificial DNA damage. The obtained data will be used to derive EC_{50} and EC_{20} values, which will allow for grouping of the tested NAs with regard to their cytotoxic potential. The panel of cytotoxicity endpoints might be subject of change and harmonization between laboratories for better comparison. The obtained data sets will subsequently be subjected to benchmark dose (BMD) modeling using PROAST software to enable amongst others comparative analysis with the other two liver cell models.

Human liver slices: Optionally, a small number of NAs might be tested with human liver tissue slices to be finally compared with PHH *in vitro*. However, these experiments will strongly depend on availability of human liver tissue at the respective time and would need specific protocol development. Therefore, this model is only optionally included as *in vitro* model, and no basic protocol is presented.

5.6 Basic protocol for *in vitro* Comet assay testing with HepG2 cells

Primary human hepatocytes are regarded as gold standard for metabolically competent hepatic *in vitro* systems. However, experiments with primary cells bear the risk of applying small experimental approaches in terms of replicates and/or concentration levels due to the costs, variability between different sources regarding metabolic competence based on polymorphisms and potential limitations based on availability. The human liver cell line HepG2 will, therefore, be used as a surrogate for primary human hepatocytes. HepG2 cell will be validated for its specific metabolic competence as already described for primary cells (see 3.2) and compared to primary hepatocytes. HepG2 cells will be complemented initially with S9-mix prepared from rat S9-fraction, if necessary. The rat S9-fraction will be provided by partner ICCR for all experiments with HepG2 cells, including the round-robin study. The S9-fraction will be characterized regarding metabolic activity as described under 3.2. Depending on the outcome of metabolic competence testing of rat and hamster S9-fractions and on preliminary results of Ames testing with both types of S9-fractions, hamster S9-mix might also be used for specific compounds in *in vitro* Comet assays with HepG2 cells.

HepG2 Cell Culture: HepG2 cells were originally provided by the DSMZ (Brunswig, Germany) and a working batch was generated, deeply characterized and distributed for the project by Fraunhofer ITEM. The same characterized cell batch will be used for the whole project. Cells are cultured in DMEM (PAN-Biotech, Aidenbach, Germany) supplemented with 10% FCS (e.g., PAN-Biotech) and e.g., 0.01% Gentamicin as antibiotic agent (Invitrogen) at 37°C in a humidified atmosphere with 5% CO_2.

Cytotoxicity testing: To determine cellular sensitivity the standard MTT test will initially be used for concentration-range finding experiments for the *in vitro* Comet assay with HepG2 cells. The assay is named after the yellow dye tetrazolium (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and based on an enzymatic reduction, which is catalyzed only in viable cells. This reduction is dependent on mitochondrial dehydrogenase enzymes, which convert the MTT dye into a soluble purple compound named formazan. Five thousand HepG2 cells will be seeded in 96-well plates and incubated for 24 h at 37 °C and 5% CO_2 prior to treatment. Cells will be treated with seven serial dilutions of the NAs in the presence of S9-mix in fresh DMEM supplemented with 10% FCS and 0.01% Gentamicin initially for 4 h at 37°C and 5% CO_2 to avoid nonspecific effects of the S9-mix, but later on it might be tested whether 24 h of incubation in the presence of S9-mix are also possible without excess cytotoxicity of the S9-mix. Medium will then be replaced by 120 µl of MTT solution per well (5 mg/mL MTT in PBS) and incubated for 1 h at 37°C and 5% CO_2. MTT solution is then replaced by 100 µl of DMSO per well. To solve the purple formazan crystals, the plate is shaken for 2 min and absorbance will be measured using in a Tecan® Microplate Reader at 590 nm. Concentration effect curves will be fitted to data points by nonlinear regression analysis using the four-parameter logistic equation (GraphPad™ Prism). Literature data will serve to guide the selection of concentration ranges (see 5.2 and ***Table 2***).

Comet assay testing: The basic *in vitro* Comet assay protocol will be performed according to Singh, McCoy, Tice, and Schneider (1988) with some modifications. Cells will be seeded at a density of 2 x 10^5 cells/well in 24-well plates (down-scaling might be done to save material in the case of highly expensive compounds) and cultured for 24 h prior to treatment. Test compound concentrations are determined in the cytotoxicity dose range finding and will usually be the IC_{50} as top dose with four additional

descending concentrations spaced by a factor of e.g., 3.3 (root10) or adapted depending on the cytotoxicity dose response. Cells are treated with the respective NA compound in the presence of S9-mix (S9-fraction with all needed co-factors) at five different concentrations, as defined in the prior testing for cytotoxicity plus solvent control for 4 or 24 h (depending on the pre-test on cytotoxicity) and processed for endpoint analysis immediately after treatment. Cell suspensions are prepared by washing the cells with PBS and treating with Accutase for 5-10 min. Cells will then be pelleted by centrifugation (100 x g, 5 min) and resuspended in 200 µl DMEM supplemented with 10% FCS and 0.01% gentamicin. Twenty µl of the cell solution will be mixed with 180 µl of molten 0.8% low melting agarose (Biozym, Hess. Oldendorf Germany). Fifty µl of this solution will be poured on a glass slide, pre-coated with 1.5% normal agarose (Biozym; precoated slides are allowed to dry overnight), covered with a 22 x 22 mm cover slip and placed at 4°C until the agarose has hardened. Cover slips are removed, and slides will be incubated for 1 h in ice cold lysis buffer (10 mM Tris, 100 mM EDTA 2.5 M NaCl adjusted to pH 10 with NaOH, 178 ml of this solution are supplemented with 20 ml DMSO and 2 ml Triton X-100 prior to use). After lysis, slides are exposed to ice cold alkaline electrophoresis buffer (300 mM NaOH, 1 mM EDTA, pH >13) for 20 min and subjected to electrophoresis for 30 min at 0.8 V/cm, 300 mA at 4°C. Finally, the alkaline pH will be neutralized with Tris buffer and the slides will be rinsed with water, dehydrated in ice cold ethanol and dried. Dried slides are later stained with 50 µl propidium iodide (20 µg/ml in PBS w/o Ca^{2+}, Mg^{2+}). Fifty nuclei per slide and three slides per concentration will be evaluated under a Zeiss fluorescence microscope (Zeiss, Göttingen, Germany) and analyzed by using the image analysis system COMET IV (Instem, UK), as also given in ***Table 11***. However, number of cells and slides evaluated might be a subject to change, when harmonizing protocols.

5.7 Basic methodological parameters and optimization

The alkaline version of the *in vitro* Comet assay will be used by all partners. The protocols/standard operation procedures (SOPs) of the different laboratories, planned to use for the present project, were collected and compiled in (***Table 11***). The specific methodological parameters were assessed as essential, desirable and not required, based on the MIRCA recommendations (Moller et al., 2020) and a Meeting Report from the 11th International Comet Assay Workshop (Koppen et al., 2017) to compare in a prioritized way the three different methods, and to evaluate potential parameters for later harmonization in the planned round-robin study with HepG2 cells. It was agreed on to initially test two data-rich NAs (NDMA and NNN), with each laboratory using his own established method to make use of historical reference data, and respecting the different cell models used, which make it difficult to completely harmonize the different methods. After pre-experiments the results will be compared and discussed with regard to potential harmonization of the candidate parameters. The three methods are highly comparable with only slight differences e.g., regarding standard cytotoxicity endpoints, agarose concentration for slide coating and cell embedding, unwinding, electrophoresis times, cell numbers and volumes (for details see ***Table 11***). All these parameters might be candidates for optimization. If possible, the same batch of relevant chemicals will be used. Standard templates will be developed for collection of data and experimental information, and slide analysis will consider the principles given in the OECD Guideline for the Testing of Chemicals No. 489 (*In Vivo* Mammalian Alkaline Comet Assay) (OECD, 2016) regarding main measure, number of comets evaluated and centrality measures for summing up the nuclei analyzed per slide. The same number of concentrations will be used respecting ICH S2 (R1) regarding maximum cytotoxicity (50%), the use of concentrations with low, medium and high adverse potential and the updated *in vitro* OECD genotoxicity guidelines with regard to limit concentrations (2 mg/ml or 10 mM, whichever is the lowest). Statistical analyses and BMD modeling will finally be discussed and agreed upon.

Table 11: *In vitro* alkaline Comet assay methods of the three laboratories Fraunhofer ITEM, TU Kaiserslautern and BfArM

Parameter	Reporting requirement	Fraunhofer ITEM	TU Kaiserslautern	BfArM
In vitro cell model:				
Type	essential	primary human hepatocytes	mouse and rat hepatocytes	Hep-G2
Provider	not required	to be decided on	own breeding and Janvier Labs	DSMZ (preparation of a working batch, Fraunhofer ITEM)
Culture conditions	not required	depending on provider	DMEM + 10 % FCS + 1 % Penicilline/Streptomycin	DMEM + 10 % FCS + 1 % Penicilline/Streptomycin
Passage number range	not required	not applicable	not applicable	2 to maximally 12 passages after thawing
Cell processing after detachment	essential	centrifugation at RT, direct resuspension in agarose and slide preparation (red light)	centrifugation at 4°C, resuspension and placement on ice	centrifugation at RT, resuspension and placement on ice
Storage of cells	essential	wet chamber, at 4°C	wet chamber at 4°C or fixed and dried	wet chamber at 4°C or fixed and dried
Study design:				
Incubation vessel	not required	24- or 48-well plates	12- or 24-well plate	6-well plate, adaption possible
Cell exposure method	essential	addition of fresh medium + compound	addition of fresh medium + compound	addition of fresh medium + compound
Planned number of concentrations	not required	max. 5 concentrations	max. 6 concentrations	max. 6 concentrations
Incubation time (h)	not required	24 h, eventually also 4 h	24 h, eventually also 4 h	24 h, eventually also 4 h
Cytotoxicity endpoint(s)	not required	Cell number, LDH, ASL, ALT	Resazurin Assay	MTT
Detachment method	not required	will depend on the provider	TrypLE express	Accutase
Assay control	essential	negative/vehicle controls, EMS -S9-mix, CP +S9-mix	negative/vehicle controls, MMS -S9-mix, CP +S9-mix	negative/vehicle controls, streptonigrin -S9-mix, CP +S9-mix
Slide preparation:				
Slide pre-coating (yes/no)	desirable	yes (1.5% normal melting agarose)	yes (1,2% normal melting agarose)	yes (1.5% normal melting agarose)
Concentration of Low Melting Agarose (LMA)	essential	0,75%	0.5%	0.8%
Cells per slide	not required	100000-200000	50000 - 75000	50000
Volume cell suspension per slide (µl)	not required	80 µl	10 µl	50 µl
Sandwich method (yes/no)	desirable	yes	no	no
Cell lysis & Electrophresis				
Lysis buffer composition and pH	essential	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris base, 1% Triton X-100, 10% DMSO, pH 10	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris-Base, % Triton X-100, 10% DMSO, pH 10	2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Tris base, 1% Na-Lauroylsarcosine, 10% DMSO, 1% Triton X-100, pH 10
Lysis time (e.g. 4 h, in h)	desirable	1 h (up to overnight)	1 h	1 h
Lysis temperature	desirable	4°C (in the dark)	4°C (in the dark)	4 °C (in the dark)
Electrophoresis buffer composition and pH	essential	1 mM Na_2EDTA, 300 mM NaOH, pH >13 (4°C)	1 mM Na_2EDTA, 300 mM NaOH, pH >13 (4°C)	1 mM Na_2EDTA, 300 mM NaOH, pH >13 (4°C)
Electrophoresis temperature	essential	electrophoresis on ice	4°C	4°C
Unwinding time (minutes)	essential	20-60	25	20
Duration of electrophoresis (minutes)	essential	20-60	15	30
Voltage (V/cm)	essential	24 (small chamber) and 32 (large chamber)	25	19-21
Amperage (mA)	essential	320	300	300
Randomized placement of slides in reservoir (yes/no)	not required	yes	yes	yes
Staining & Analysis				
Staining (PI=propidium iodide; EB=ethidium bromide)	essential	EB	PI	PI
Type of primary descriptor (tail moment, tail intensity, etc.)	essential	tail intensity	tail intensity or tail moment	tail intensity and tail moment
Analysis system with version	essential	Comet Assay III or IV Software (Perceptive Instruments/Instem)	Comet Assay IV Software (Perceptive Instruments/Instem)	Comet Assay III or IV Software (Perceptive Instruments/Instem)
Microscopic magnification (in x)	desirable	40	20 x	20 x
Number of comets scored	essential	150 per slide/treatment	50 per slide, 3 slides per treatment	50 per slide, 3 slides per treatment
Measure of central value of comets (mean/median)	essential	mean or median per slide (data distribution), mean per treatment	mean per slide and mean per treatment	median per slide and mean per tretament
Coding of slides (yes/no)	not required	yes	no	no
One evaluator per experiment (yes/no)	not required	yes	no	no
No. of evaluators per project partner	not required	3	2	2
Statistical analysis of results	essential	to be discussed	to be discussed	to be discussed

5.8 Round-robin study with HepG2 cells

Finally, alkaline *in vitro* Comet assay testing of NAs will be complemented by a cross validation approach for selected NAs amongst all three involved partners (ITEM, TU Kaiserslautern and BfArM) and TUK), using the same batch of HepG2 cells + S9 fraction (S9-mix protocol) and the optimized *in vitro* Comet assay SOP for HepG2 cells to look for inter-laboratory reproducibility of the generated data. For this purpose, cell culture and treatment as well as the *in vitro* Comet assay protocol will be harmonized as far as needed, including usage of the same batches of e.g., media, enzymes and buffer for cell culture, the same solvents and NA batches as well as, may be, agaroses for the *in vitro* Comet assay. It will, furthermore, be discussed, whether the compounds will be provided by BfArM together with appropriate reference items, both encoded, to exclude analyzer bias, based on knowledge of treatment. Additionally, the same S9 homogenate batch will be provided by ICCR for cell incubation. Final results will be statistically analyzed for inter-laboratory variability.

5.9 Data output

The present project will finally estimate performance of both the optimized Ames and the *in vitro* Comet assay with liver cell models by evaluating both known positive controls others than NAs and the carcinogenicity positive and negative NA compounds. For performance estimation of genotoxicity tests with regard to prediction of rodent carcinogenicity, the parameter reproducibility (will be addressed for the *in vitro* Comet assay by a round-robin study with HepG2 cells and for the Ames test and the *in vitro* Comet assay using technical and/or biological replicates), sensitivity (proportion of genotoxic carcinogens that generated positive results) and specificity (proportion of non-genotoxic compounds that generated negative results) are normally calculated. Please find definitions and the respective equations in ***Figure 2***.*Fehler! Verweisquelle konnte nicht gefunden werden.*

*** Give indications regarding "false negative" results.**
 # Give indications regarding "false positive" results.

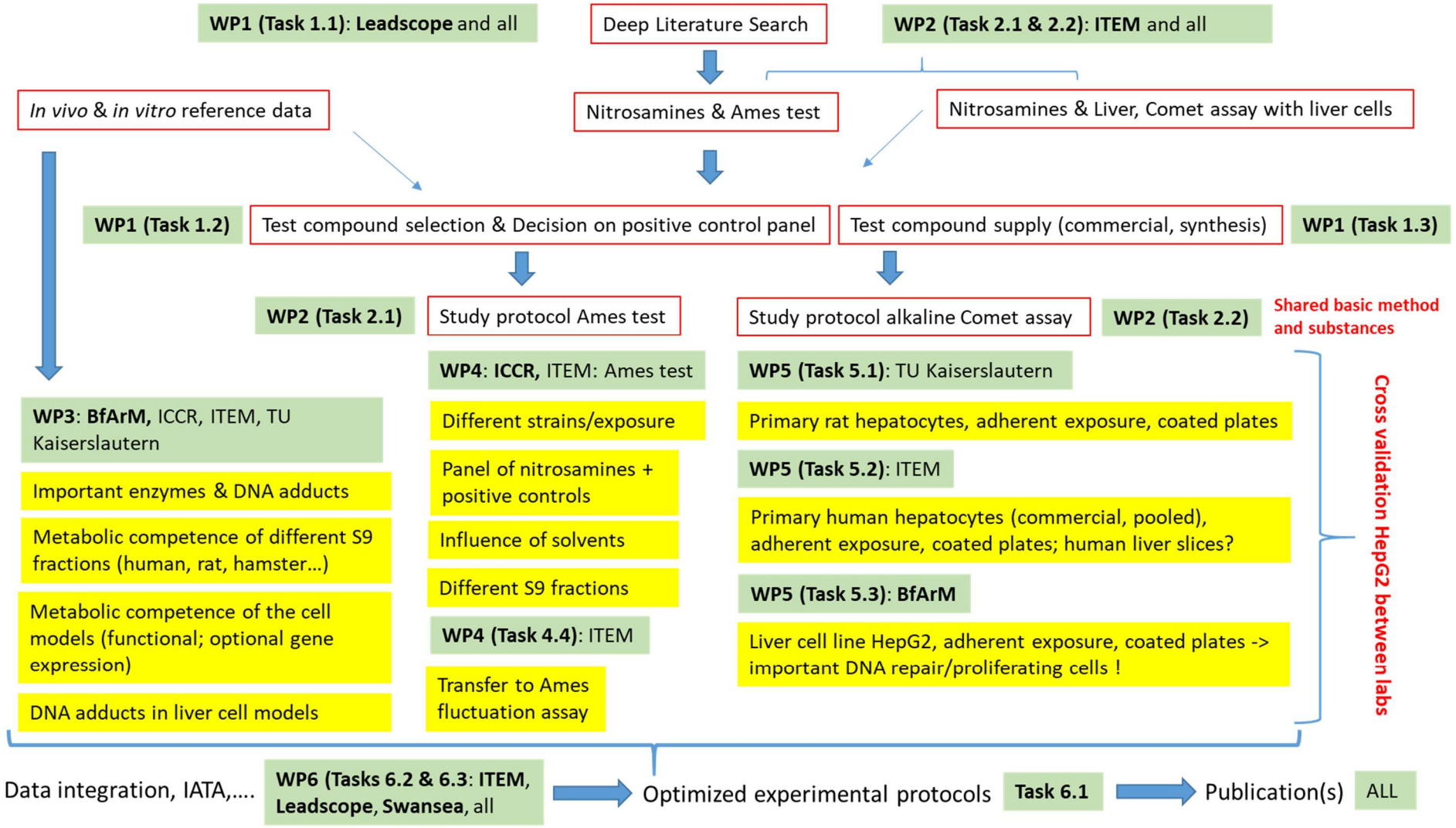
	Carcinogen	Non-Carcinogen
Genotoxicity +	A	B
Genotoxicity -	C	D

Term	Definition	Assessment
Sensitivity*	% correctly identified carcinogens	A/(A+C) * 100
***Specificity**#*	% correctly identified non-carcinogens	D/(B+D) * 100
Concordance	% correctly identified carcinogens and non-carcinogens	(A + D)/(A+B+C+D) * 100
***Positive predictivity**#*	% carcinogens among positive genotoxicity results	A/(A+B) *100
Negative predictivity*	% non carcinogens among negative genotoxicity results	D/(C+D) * 100

Figure 2: Definition and equations for description of genotoxicity test performance regarding prediction of rodent carcinogenicity. Adapted from EFSA "Scientific opinion on genotoxicity testing strategies applicable to food and feed safety assessment". EFSA Journal 2011; 9(9):2379

With regard to specificity, however, the validity of calculations will be limited, due to the low number of non-carcinogenic versus carcinogenic NAs in the present study. The performance of the Ames test will, additionally, be compared with the performance of the *in vitro* Comet assay, and cytotoxicity in the *in vitro* Comet assay will be correlated with DNA strand break induction. It is also planned to estimate potency of the different compounds in both the Ames test and the *in vitro* Comet assay by benchmark dose (BMD) modelling using the PROAST software. In the Ames test, BMD modelling might aid in identifying key methodological parameters for the testing of NAS, whereas in the *in vitro* Comet assay BMD modelling will be used as a tool to compare performance of the three liver cell models.

6 Schematic overview on study strategy and interrelationships



7 Appendices

7.1 Appendix 1

Results of the deep literature search for the *in vitro* and *in vivo* Comet assay with nitrosamines:

Excel File: *Appendix_1_Literature Search_Comet Assay.xlsx*

7.2 Appendix 2

Covering both detailed comparison of the *in vitro* Comet assay methods of Fraunhofer ITEM, BfArM and the TU Kaiserslautern and the detailed evaluation of the *in vitro* Comet assay methods used for nitrosamine testing with liver cell models, as identified in literature:

Excel File: *Appendix_2_Methods_in vitro Comet assay_Partner and Literature.xlsx*

8 References

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.

Araki, A., Muramatsu, M., & Matsushima, T. (1984). COMPARISON OF MUTAGENICITIES OF N-NITROSAMINES ON SALMONELLA TYPHIMURIUM TA100 AND ESCHERICHIA COLI WP2 UVRA/PKM101 USING RAT AND HAMSTER LIVER S9. *GANN Japanese Journal of Cancer Research, 75*(1), 8-16.

Arnold, C., Demuth, P., Seiwert, N., Wittmann, S., Boengler, K., Rasenberger, B., et al. (2022). The Mitochondrial Disruptor Devimistat (CPI-613) Synergizes with Genotoxic Anticancer Drugs in Colorectal Cancer Therapy in a Bim-Dependent Manner. *Mol Cancer Ther, 21*(1), 100-112.

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

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

Azqueta, A., & Collins, A. R. (2013). The essential comet assay: a comprehensive guide to measuring DNA damage and repair. *Arch Toxicol, 87*(6), 949-968.

Baird, J., Begg, C., Pritchard, M. P., & Voice, M. W. (2005). Effects of Common Organic Solvents on the Activity of Recombinant Human Cytochrome P450s Expressed in E. coli.

Bartsch, H., Malaveille, C., & Montesano, R. (1975). In Vitro Metabolism and Microsome-mediated Mutagenicity of Dialkylnitrosamines in Rat, Hamster, and Mouse Tissues. *Cancer Research, 35*, 644-651.

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.

Brater, D. C. (1991). Clinical Pharmacology of Loop Diuretics. *Drugs, 41*(Supplement 3), 14-22.

Bringezu, F., & Simon, S. (2022). Salmonella typhimurium TA100 and TA1535 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.

Busby, W. F., Jr., Ackermann, J. M., & Crespi, C. L. (1999). Effect of methanol, ethanol, dimethyl sulfoxide, and acetonitrile on in vitro activities of cDNA-expressed human cytochromes P-450. *Drug Metab Dispos, 27*(2), 246-249.

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

Carr, B., Norcross, R., Fang, Y., Lu, P., Rodrigues, A. D., Shou, M., et al. (2006). Characterization of the rhesus monkey CYP3A64 enzyme: species comparisons of CYP3A substrate specificity and kinetics using baculovirus-expressed recombinant enzymes. *Drug Metab Dispos, 34*(10), 1703-1712.

Chauret, N., Gauthier, A., & Nicoll-Griffith, D. A. (1998). Effect of Common Organic Solvents on in VitroCytochrome P450-Mediated Metabolic Activities in Human Liver Microsomes. *Drug Metabolism and Disposition, 26*(1), 1.

Cross, K. P. (2022, May 18 - May 20). *Advancing Prediction of Nitrosamine Potency.* Genetic Toxicity Association Meeting, virtual.

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.

Dobo, K. L., Kenyon, M. O., Dirat, O., Engel, M., Fleetwood, A., Martin, M., et al. (2022). Practical and Science-Based Strategy for Establishing Acceptable Intakes for Drug Product N-Nitrosamine Impurities. *Chemical Research in Toxicology, 35*(3), 475-489.

Dorsam, B., Seiwert, N., Foersch, S., Stroh, S., Nagel, G., Begaliew, D., et al. (2018). PARP-1 protects against colorectal tumor induction, but promotes inflammation-driven colorectal tumor progression. *Proc Natl Acad Sci U S A, 115*(17), E4061-E4070.

Drugbank. (2022). Drugbank.

Easterbrook, J., Lu, C., Sakai, Y., & Li, A. P. (2000). Effects of Organic Solvents on the Activities of Cytochrome P450 Isoforms, UDP-Dependent Glucuronyl Transferase, and Phenol Sulfotransferase in Human Hepatocytes. *Drug Metabolism and Disposition, 29*(2).

EMA. (2012). ICH guideline S2 (R1) on genotoxicity testing and data interpretation for pharmaceuticals intended for human use.

EMA. (2020). *EMA Assessment report on Nitrosamine impurities in human medicinal products - Procedure number: EMEA/H/A-5(3)/1490*. Retrieved from https://www.ema.europa.eu/en/documents/referral/nitrosamines-emea-h-a53-1490-assessment-report_en.pdf.

Fahrig, R., Rupp, M., Steinkamp-Zucht, A., & Bader, A. (1998). Use of Primary Rat and Human Hepatocyte Sandwich Cultures for Activation of Indirect Carcinogens: Monitoring of DNA Strand Breaks and Gene Mutations in Co-cultured Cells. *Toxicology in Vitro, 12*(4), 431-444.

Fraczek, J., Bolleyn, J., Vanhaecke, T., Rogiers, V., & Vinken, M. (2013). Primary hepatocyte cultures for pharmaco-toxicological studies: at the busy crossroad of various anti-dedifferentiation strategies. *Arch Toxicol, 87*(4), 577-610.

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.

Gao, L., Rutz, L., & Schrenk, D. (2020). Structure-dependent hepato-cytotoxic potencies of selected pyrrolizidine alkaloids in primary rat hepatocyte culture. *Food Chem Toxicol, 135*, 110923.

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.

Gerets, H. H., Tilmant, K., Gerin, B., Chanteux, H., Depelchin, B. O., Dhalluin, S., et al. (2012). Characterization of primary human hepatocytes, HepG2 cells, and HepaRG cells at the mRNA level and CYP activity in response to inducers and their predictivity for the detection of human hepatotoxins. *Cell Biol Toxicol, 28*(2), 69-87.

Gomez-Lechon, M. J., Tolosa, L., Conde, I., & Donato, M. T. (2014). Competency of different cell models to predict human hepatotoxic drugs. *Expert Opin Drug Metab Toxicol, 10*(11), 1553-1568.

Guengerich, F. P. (2000). Metabolism of chemical carcinogens. *Carcinogenesis, 21*(3), 345-351.

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

Gupta, R., Schrooders, Y., Hauser, D., van Herwijnen, M., Albrecht, W., Ter Braak, B., et al. (2021). Comparing in vitro human liver models to in vivo human liver using RNA-Seq. *Arch Toxicol, 95*(2), 573-589.

Gustafson, A., King, C., & Rey, J. A. (2013). Lorcaserin (Belviq). *Pharmacy and Therapeutics, 38*(9), 525-534.

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.

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

Heflich, R. H. (2022, May 18-20). *Optimizing the Ames Test to Enhance Assessment of the Mutagenic Potential of Nitrosamine Drug Impurities and Other Genotoxicity Studies Conducted at the FDA/NCTR.* Genetic Toxicology Association Meeting, virtual.

Hickman, D., Wang, J.-P., Wang, Y., & Unadkat, J. D. (1998). Evaluation of the Selectivity of In Vitro Probes and Suitability of Organic Solvents for the Measurement of Human Cytochrome P450 Monooxygenase Activities. *Drug Metabolism and Disposition, 26*(3), 207-215.

Hong, Y. H., Jeon, H. L., Ko, K. Y., Kim, J., Yi, J. S., Ahn, I., et al. (2018). Assessment of the predictive capacity of the optimized in vitro comet assay using HepG2 cells. *Mutat Res Genet Toxicol Environ Mutagen, 827*, 59-67.

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.

ISO. (2012). ISO 11350:2012 Water quality, *Determination of the genotoxicity of water and waste water — Salmonella/microsome fluctuation test (Ames fluctuation test)*.

Iwase, M., Kurata, N., Ehana, R., Nishimura, Y., Masamoto, T., & Yasuhara, H. (2006). Evaluation of the effects of hydrophilic organic solvents on CYP3A-mediated drug-drug interaction in vitro. *Hum Exp Toxicol, 25*(12), 715-721.

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.

Kawalek, J. C., & Andrews, A. W. (1980). The effect of solvents on drug metabolism in vitro. *Drug Metabolism and Disposition, 8*(6), 380-384.

Kay, J. E., Corrigan, J. J., Armijo, A. L., Nazari, I. S., Kohale, I. N., Torous, D. K., et al. (2021). Excision of mutagenic replication-blocking lesions suppresses cancer but promotes cytotoxicity and lethality in nitrosamine-exposed mice. *Cell Rep, 34*(11), 108864.

Kazmi, F., Yerino, P., Barbara, J. E., & Parkinson, A. (2015). Further Characterization of the Metabolism of Desloratadine and Its Cytochrome P450 and UDP-glucuronosyltransferase Inhibition Potential: Identification of Desloratadine as a Relatively Selective UGT2B10 Inhibitor. *Drug Metabolism and Disposition, 43*(9), 1294-1302.

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.

Koppen, G., Azqueta, A., Pourrut, B., Brunborg, G., Collins, A. R., & Langie, S. A. S. (2017). The next three decades of the comet assay: a report of the 11th International Comet Assay Workshop. *Mutagenesis, 32*(3), 397-408.

Lee, H. J., Ahn, J., Jung, C. R., Jeung, Y. J., Cho, H. S., Son, M. J., et al. (2020). Optimization of 3D hydrogel microenvironment for enhanced hepatic functionality of primary human hepatocytes. *Biotechnol Bioeng, 117*(6), 1864-1876.

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, D., Han, Y., Meng, X., Sun, X., Yu, Q., Li, Y., et al. (2010). Effect of regular organic solvents on cytochrome P450-mediated metabolic activities in rat liver microsomes. *Drug Metab Dispos, 38*(11), 1922-1925.

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.

Lijinsky, W., & Andrews, A. W. (1983). The superiority of hamster liver microsomal fraction for activating nitrosamines to mutagens in Salmonella typhimurium. *Mutation Research/Fundamental and Molecular Mechanisms of Mutagenesis, 111*(2), 135-144.

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., Katzenellenbogen, J., & Ames, B. N. (1981). Compatibility of organic solvents with the Salmonella/microsome test. *Mutation Research/Genetic Toxicology, 88*(4), 343-350.

Martignoni, M., Groothuis, G. M., & de Kanter, R. (2006). Species differences between mouse, rat, dog, monkey and human CYP-mediated drug metabolism, inhibition and induction. *Expert Opin Drug Metab Toxicol, 2*(6), 875-894.

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.

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.

Moller, P., Azqueta, A., Boutet-Robinet, E., Koppen, G., Bonassi, S., Milic, M., et al. (2020). Minimum Information for Reporting on the Comet Assay (MIRCA): recommendations for describing comet assay procedures and results. *Nat Protoc, 15*(12), 3817-3826.

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

Mori, Y., Yamazaki, H., Toyoshi, K., Emi, Y., Uchida, K., Tsutsumi, M., et al. (1985). Inhibitory effect of organic solvents on the mutagenicity of N-nitrosodialkylamines in Salmonella. *Mutation Research Letters, 142*(4), 153-158.

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.

Nirogi, R., Kandikere, V., Bhyrapuneni, G., Ponnamaneni, R. K., Palacharla, R. c., & Manoharan, A. (2011). Effect of Dimethyl Sulfoxide on In Vitro Cytochrome P4501A2 Mediated Phenacetin O-Deethylation in Human Liver Microsomes. *Drug Metabolism and Disposition, 39*(11), 2162-2164.

OECD. (2016). GUIDELINE FOR THE TESTING OF CHEMICALS No. 489, *In Vivo Mammalian Alkaline Comet Assay*

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.

Patil, P. G., Kamble, S. H., Shah, T. S., & Iyer, K. R. (2015). Effect of Water Miscible Organic Solvents on p-Nitrophenol Hydroxylase (CYP2E1) Activity in Rat Liver Microsomes. *Indian J Pharm Sci, 77*(3), 283-289.

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.

Raineri, R., Poiley, J. A., Andrews, A. W., Pienta, R. J., & Lijinsky, W. (1981). Greater effectiveness of hepatocyte and liver S9 preparations from hamsters than rat preparations in activating N-nitroso compounds to metabolites mutagenic to Salmonella. *Journal of the National Cancer Institute, 67*(5), 1117-1122.

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.

Reifferscheid, G., Maes, H. M., Allner, B., Badurova, J., Belkin, S., Bluhm, K., et al. (2012). International round-robin study on the Ames fluctuation test. *Environ Mol Mutagen, 53*(3), 185-197.

Rendic, S., & Guengerich, F. P. (2020). Metabolism and Interactions of Chloroquine and Hydroxychloroquine with Human Cytochrome P450 Enzymes and Drug Transporters. *Current Drug Metabolism, 21*(14), 1127-1135.

Rothfuss, A., O'Donovan, M., De Boeck, M., Brault, D., Czich, A., Custer, L., et al. (2010). Collaborative study on fifteen compounds in the rat-liver Comet assay integrated into 2- and 4-week repeat-dose studies. *Mutat Res, 702*(1), 40-69.

Schmezer, P., Brendler, S. Y., Tompa, A., & Pool, B. L. (1990). (Geno-)Toxicokinetics of DNA-Damaging N-Nitrosamines in the Rat. *Mutation Research, 234*(6), 422-422.

Schrenk, D., Karger, A., Lipp, H. P., & Bock, K. W. (1992). 2,3,7,8-Tetrachlorodibenzo-p-dioxin and ethinylestradiol as co-mitogens in cultured rat hepatocytes. *Carcinogenesis, 13*(3), 453-456.

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.

Seglen, P. O. (1976). Preparation of isolated rat liver cells. *Methods Cell Biol, 13*, 29-83.

Singh, N. P., McCoy, M. T., Tice, R. R., & Schneider, E. L. (1988). A simple technique for quantitation of low levels of DNA damage in individual cells. *Experimental Cell Research, 175*(1), 184-191.

Sulc, M., Hodek, P., & Stiborova, M. (2010). The binding affinity of carcinogenic N-nitrosodimethylamine and N-nitrosomethylaniline to cytochromes P450 2B4, 2E1 and 3A6 does not dictate the rate of their enzymatic N-demethylation. *Gen Physiol Biophys, 29*(2), 175-185.

Tice, R. R., Agurell, E., Anderson, D., Burlinson, B., Hartmann, A., Kobayashi, H., et al. (2000). Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing. *Environ Mol Mutagen, 35*(3), 206-221.

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.

Uno, Y., Kojima, H., Omori, T., Corvi, R., Honma, M., Schechtman, L. M., et al. (2015). JaCVAM-organized international validation study of the in vivo rodent alkaline comet assay for detection of genotoxic carcinogens: II. Summary of definitive validation study results. *Mutat Res Genet Toxicol Environ Mutagen, 786-788*, 45-76.

Vasquez, M. Z., & Frotschl, R. (2016). The In Vivo Comet Assay. In R. Proudlock (Ed.), *Genetic Toxicology Testing: A Laboratory Manual*: Elsevier Academic Press 2016.

Vilas-Boas, V., Cooreman, A., Gijbels, E., Van Campenhout, R., Gustafson, E., Ballet, S., et al. (2019). Primary hepatocytes and their cultures for the testing of drug-induced liver injury. *Adv Pharmacol, 85*, 1-30.

Vorrink, S. U., Ullah, S., Schmidt, S., Nandania, J., Velagapudi, V., Beck, O., et al. (2017). Endogenous and xenobiotic metabolic stability of primary human hepatocytes in long-term 3D spheroid cultures revealed by a combination of targeted and untargeted metabolomics. *FASEB J, 31*(6), 2696-2708.

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.

Wilkening, S., & Bader, A. (2003). Influence of culture time on the expression of drug-metabolizing enzymes in primary human hepatocytes and hepatoma cell line HepG2. *J Biochem Mol Toxicol, 17*(4), 207-213.

Wilkening, S., Stahl, F., & Bader, A. (2003). Comparison of primary human hepatocytes and hepatoma cell line Hepg2 with regard to their biotransformation properties. *Drug Metab Dispos, 31*(8), 1035-1042.

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.

Wills, J. W., Johnson, G. E., Doak, S. H., Soeteman-Hernandez, L. G., Slob, W., & White, P. A. (2016). Empirical analysis of BMD metrics in genetic toxicology part I: in vitro analyses to provide robust potency rankings and support MOA determinations. *Mutagenesis, 31*(3), 255-263.

Wills, J. W., Long, A. S., Johnson, G. E., Bemis, J. C., Dertinger, S. D., Slob, W., et al. (2016). Empirical analysis of BMD metrics in genetic toxicology part II: in vivo potency comparisons to promote reductions in the use of experimental animals for genetic toxicity assessment. *Mutagenesis, 31*(3), 265-275.

Xu, Q. (2021). Human Three-Dimensional Hepatic Models: Cell Type Variety and Corresponding Applications. *Front Bioeng Biotechnol, 9*, 730008.

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.

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.

Yang, K. H., Choi, Y. H., Lee, U., Lee, J. H., & Lee, M. G. (2009). Effects of cytochrome P450 inducers and inhibitors on the pharmacokinetics of intravenous furosemide in rats: involvement of CYP2C11, 2E1, 3A1 and 3A2 in furosemide metabolism. *Journal of Pharmacy and Pharmacology, 61*(1), 47-54.

Yokoyama, Y., Sasaki, Y., Terasaki, N., Kawataki, T., Takekawa, K., Iwase, Y., et al. (2018). Comparison of Drug Metabolism and Its Related Hepatotoxic Effects in HepaRG, Cryopreserved Human Hepatocytes, and HepG2 Cell Cultures. *Biol Pharm Bull, 41*(5), 722-732.