SPECIFIC CONTRACT
No. 01 (SC01)
implementing framework contract No. EMA/2020/46/L1.02

Objectives of Deliverable 3a - Study Design and Study Protocols

Deliverable 3a describes the experimental approach to fill data gaps relevant for the development of quantitative structure relationships and an expert system in this project.

The project follows a tiered testing strategy as outlined in Figure 1.

Step 1: Compound selection: About 30 candidate Nitrosamine (NA) compounds were selected according to predefined selection criteria (described in full detail in deliverable D1).
Step 2: Coordinated **pre-testing** on solubility as well as stability of test compounds to optimize conditions. Identification of and impurities to filter out challenging compounds.
Step 3: Identify **metabolic activation** and **adduct formation** as well as the **stability of metabolites** and of **DNA adducts** by screening of about 20 NAs -> select 8 representative NAs, which differ with regard to their DNA adduct profile
Step 4: Measure **repair mechanisms** for the 8 NAs identified in Step 3.
Step 5: On the basis of existing information and the results from the new research (Step 3 and 4), develop approaches to the **risk assessment of nitrosamines** without robust *in vivo* carcinogenicity data

Deliverable 3a provides detailed study protocols, which include the description of the approaches and methods applied in Step 2-4. As qualitative and quantitative approaches will be performed, we will assure that the number of doses or time points will be appropriate for benchmark dose modelling. To minimize variability in assays, test compounds will be ordered by one partner and shared with all other testing parties. Cell lines tested by several partners will be shared to reduce as much as possible variance in responses.

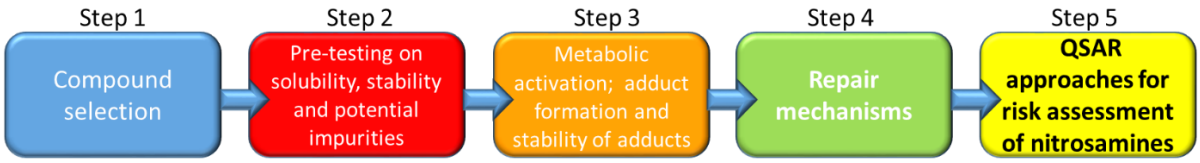

Figure 1: Schematic presentation of the workflow in project „Quantitative Structure Activity Relationships (QSAR) for nitrosamine risk assessment" Specific Contract No. 01 (SC01). The study protocol will address the experimental approaches needed to perform Step 2 to 4.

Content

List of Figures and Tables:

Detailed description of the planned testing approaches and methods

The details on the study protocols of all methods is provided in the following chapters. An overview on the methods and read-outs is summarized in Annex 1.

1. Pretesting, ordering and distribution

1.1 Coordinated pre-test on solubility

The solubility of NAs, to be reconstituted by uncritical solvents for cell and/or enzyme stability, is crucial for all experiments. Highly concentrated stock solutions with totally solved compounds will result in lower end concentrations of organic solvent in the presence of cell cultures or metabolic enzymes. Therefore, NA stock solutions of 1 mg/mL will be prepared with DMSO, H2O, acetonitrile, methanol and DMF to estimate the solubility. In case of insufficient solubility at ambient temperature, solubilisation will be retested at 37°C and at lower concentrations (100 and 10 µg/mL). All samples will be evaluated by considering opacity, reprecipitation of the solution, and color change. The solubility assays aim to define conditions that allow to use final organic solvent concentration of 1% and less in the incubation of cell systems (as described below). In case of sufficient solubility under aqueous conditions respective buffers or bidestilled water should be preferred.

1.2 Stability and impurity testing of the pure compounds

Prior to the laboratory experiments it is of utmost importance to exclude nonspecific or adverse effects on cell viability, metabolism or NA reactivity to obtain valid results. Therefore, all NAs are screened for impurities by means of LC-MS full scan analysis. A determination range of 70 – 1000 Da is applied on standard stock solutions of NAs with 1 µg/mL to reveal and identify undesired signals. One limitation is the unavailability to screen for inorganic compounds like heavy metals or other salts. Additionally, stability experiments will be performed by incubating the assay specific NAs [1 µg/mL] with selected matrices like HepG2 cultivation buffer (e.g. DMEM, 10% FCS, 10% glutamine, Penstrep) and organic solvents like DMSO, acetontrile, N,N-dimethylformamide, etc. at representative conditions (37°C and ambient temperature). At respective timepoints with t[h] = 0, 1, 2, 4, 8, 16, 24, and 48 aliquots of each stability sample will be subjected to LC-MS analysis to evaluate the time-dependent decrease of signal intensity, which directly correlates with nonspecific degradation and nonspecific binding. Instability of NAs will result in lesser concentrations in the incubation procedures, and finally to lower relative DNA alkylation rates.

2. Metabolic competence, DNA adduct formation and stability of reactive intermediates

Four analytical testing strategies are proposed to characterize the metabolic quality of cell lines as well as enzymatic liver derived concentrates as well as to elucidate the metabolic fate of NAs after enzymatic toxification.

1. Identification and characterization of enzymes and tissue derived enzymatic preparations, relevant for the metabolism of the defined NAs/NA classes, to form related DNA adducts.

2. Characterization of metabolic competence of the different liver cells and tissue models (e.g. human liver slices) by means of CYP450 mRNA gene expression (see chapter 3)

3. Characterization of the stability of metabolites and of DNA adducts in the different *in vitro* human liver model systems.

4. Investigate the repair of NA induced DNA adducts in DNA repair deficient and proficient liver cell models in a concentration- and time-dependent manner by means of LC-MS/MS (see chapter 4.1 and 4.2)

2.1 Characterization of metabolic competence of different liver cells and model tissue by means of CYP450 specific degradation of substrates

The upcoming analytical approaches mainly depend on LC-MS measurements and real-time qPCR experiments. The latter covers the above-mentioned point 2 to detect CYP450 mRNA gene expression rates and is further described under point 3. To assess the metabolic competence with regard to CYP activity in the used cell types and enzyme preparations (induced rat and hamster liver S9 mix, HepG2 cells, murine primary hepatocytes), a respective assay is suggested for this study. The assay will include multiple testing strategies to meet success criteria for point 1, 3, and 4. Specific substrates for CYP enzyme subclasses are incubated with the cells or cell-derived systems. Subsequently, the metabolic degradation and the conversion rate are determined by LC-MS. Table 1 provides an overview of the substrates planned for use. It is expected that orthologous CYP subclasses from mouse and rat will convert these substrates in the same way, since there is a homology of the amino acid sequence of about 75%. The information on the orthological equivalence of enzymes between humans, rats and mice was taken from published work (Robottom-Ferreira, Aquino et al. 2003; Woodland, Huang et al. 2008; Li, Ross-Viola et al. 2009; Abu-Bakar, Hakkola et al. 2013; Hammer, Schmidt et al. 2021). In order to investigate the testing of metabolic competence, in particular the discussed mechanism of transnitrosation, it is planned to develop a method based on the experiment by Yanagimoto, Toyota et al. 2007 (Yanagimoto, Toyota et al. 2007). In this assay, the transnitrosation of aliphatic nitrosamines on glutathione is observed, which occurs spontaneously mainly under acidic conditions. This assay is not yet established at the BfArM, but will be tested in the study. In case of transnitrosation, a time-dependent determination of the nitrosoglutathione concentration will be performed, similar to the DNA alkylation assay. Observation of a transnitrosation reaction would change the classification of a particular nitrosamine to the extent that other new nitrosamines with possible toxicity potential could be identified. This assay is more of a discovery and elucidation of principle mechanisms of nitrosamines than a test for mutagenicity. Nevertheless, the eventual detection of transnitrosation reactions may help to reassess toxicity profiles of nitrosamines.

A possible influence by phase II metabolism, especially glucuronidation, is expected only for tolbutamide, mephenytoin, dextromethorphan, and nicotine. However, in all metabolic competence assays, essential cofactors such as 3′-phosphoadenosine-5′-phosphosulfate (PAPS) or uridine-5-diphosphoglucuronic acid (UDPGA) are not used in excess to provoke phase II metabolism. Many substrates must also first undergo phase I metabolism to be further metabolized via phase II as glucuronide (nicotine is an exception). The focus should therefore remain on phase I metabolism.

Table 1: *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)*	Orthologue(s) (mice)*	Substrate (human)	Metabolite (human)
CYP3A4	CYP3A9	CYP3A11***/ A13	Terfenadine (Jurima-Romet, Wright et al. 1998)	Hydroxyterfenadine
CYP2D6	CYP2D3	CYP2D9/2D10	Dextromethorphan (Kerry, Somogyi et al. 1994)	Dextrorphan
CYP1A2	CYP1A2	CYP1A2	Caffeine (Notarianni, Oliver et al. 1995)	Paraxanthine
CYP2E1	CYP2E1	CYP2E1	Chlorzoxazone (Bachmann and Sarver 1996)	Hydroxychlorzoxazone

CYP2C9	CYP2C12	CYP2C38	Tolbutamide (Zhang, Peng et al. 2022)	Hydroxytolbutamide
CYP2B6	CYP2B1/2B2	CYP2B10	Bupropion (Turpeinen, Nieminen et al. 2004)	Hydroxybupropion
CYP2C19	CYP2C13/2C55	CYP2C39/2C55	Mephenytoin (Lasker, Wester et al. 1998)	Hydroxymephenytoin
CYP2A6	CYP2A1/2A2/2A3 **	CYP2A5****	Nicotine (Yamazaki, Inoue et al. 1999)	Cotinine/Norcotinine

* According to Hammer, Schmidt et al. (2021) except: ** (Robottom-Ferreira, Aquino et al. 2003; Martignoni 2006) *** (Martignoni 2006; Martignoni, Groothuis et al. 2006) **** (Poca, Parente et al. 2017)

The main difference between microsome preparations and S9 preparations lies on the one hand in the method of production and on the other hand in the resulting composition with regard to metabolizing enzymes. Due to the ultracentrifugation in the production of microsomes, these preparations contain only enzymes of the endoplasmic reticulum. These are primarily CYP450 enzymes and glucuronyltransferase as a phase II enzyme. However, in order to consider all metabolic pathways for the degradation of e.g. a drug, S9 preparations are recommended for PK/ADME studies, as these contain both cytosolic and endoplasmic reticulum enzymes. Important cytosolic phase II enzymes are e.g. the family of sulfotransferases. Other enzymes influencing metabolism and detoxification processes, such as catechol methyltransferase or glutathione transferase, are also part of the S9 mix. In this study, the experimental conditions and cofactors are selected so that only CYP450 enzymes are investigated. Important cofactors for the investigation of e.g. phase II enzymes such as UDPGA or PAPS are not added.

The production of the S9 homogenate from rats was carried out for our studies as follows. S9 liver homogenate was prepared from induced male Wistar rats with a body weight of about 200 – 320 g (Elliott, Combes et al. 1992). The animals were obtained from a certified animal breeding company (e.g., Janvier Labs, France). After arrival at the test facility there was an acclimatization period of at least 5 days in the animal house. Then the animals were administered orally the inducers of liver enzymes on three consecutive days (phenobarbitone in deionized water and ß-naphthoflavone in corn oil 80 mg/kg body weight, each; volume 1 mL/kg body weight). One day after the last application the animals were humanely killed. The livers were prepared, washed with ice cold 0.15 M potassium chloride solution, pooled and homogenized on ice. This was followed by centrifugation at 9000 g for 25 minutes at 4°C. The obtained homogenate was diluted with 0.15 M potassium chloride solution (ratio: 1 part homogenate + 3 parts KCl). Then the homogenate was aliquoted in sterile tubes and stored at $\leq$ -65°C. Each batch was qualified for sterility and protein content. Additionally, the S9 liver homogenate was routinely tested for its capability to activate the known mutagens benzo[a]pyrene and 2-aminoanthracene in the bacterial reverse mutation test with the *Salmonella typhimurium* strain TA 98.

Characterizing S9 mix metabolic competence

A protocol established at the BfArM for characterizing the enzyme activity of microsomes and S9 mix preparations is used for testing the rat and hamster S9 mix. Since the quality of the preparations can be expected to be consistent due to manufacturing controls during production, the enzyme preparations are initially examined once before the AMES test and Comet assay are started. If a repeat assay is required, the conversion rate of substrates is re-assayed.

The final sample composition consists of:

- 183 µL 100mM PBS, containing 5 mM $MgCl_2$ (pH7.4)
- 5 µL rat/hamster S9 mix [20 mg protein/mL]
- 10 µL 20 mM NADPH tetrasodium salt in PBS

- 2 µL test substrate

Final compound concentrations are as follows in Table 2.

After a pre-incubation of 5 min, NADPH is finally added to the preparation. All substrates are incubated individually to avoid inhibition or side reactions. The incubation time is 60 min at 37°C and 550 rpm on the thermomixer. Due to the importance of activity detection for CYP2E1 and its involvement in the toxicity of NDMA, NDEA and smaller nitrosamines, two substrates are tested for this enzyme. It should be noted that all substrates are specific substances for the detection of human CYP450 enzymes. However, in the *in vitro* model, both rat and hamster liver enzymes should be able to metabolize these substrates, and correspondingly CYP-specific nitrosamines, by orthologous enzymes.

Table 2 Overview of final compound concentration.

CYP substrate	**Metabolite**	**CYP enzyme**	**Final concentration (substrate)**	**Michaelis-Menten constant [µM]**
Caffeine	Paraxanthine	1A2	200 µM	240 (Tassaneeyakul, Birkett et al. 1994)
Bupropion	Hydroxybupropion	2B6	20 µM	0.39 (Chen, Pan et al. 2012)
Tolbutamide	4-Hydroxytolbutamide	2C9	80 µM	105 (Guo, Wang et al. 2005)
S-Mephenytoin	4-Hydroxymephenytoin	2C19	100 µM	38.4 (Shirasaka, Chaudhry et al. 2016)
Dextro-methorphan	Dextrorphan	2D6	5 µM	0.44 (Obach and Reed-Hagen 2002)
Terfenadine	Hydroxyterfenadine	3A4	100 µM	1.78 (Kishimoto, Hiroi et al. 1997)
Nicotine	Cotinine/Norcotinine	2A6	60 µM	11 (Yamazaki, Inoue et al. 1999)
Zopiclone*	Zopiclone-N-oxide	2E1	50 µM	-
Chlorzoxazone	6-Hydroxychlorzoxazone	2E1	120 µM	410 (Yamamura, Koyama et al. 2015)

* Zopiclone is more sensitive than chlorzoxazone, but less specific. Here specificity might be more important. Therefore both compounds will be tested.(Yamazaki, Inoue et al. 1999)

Characterizing HepG2 cells and primary hepatocytes metabolic competence

The characterization of the metabolic competence of vital cell systems should take place under optimal conditions and cell stress, such as unnecessary thawing and defrosting cycles, should be avoided. Therefore, it is planned to perform these experiments directly in the cell cultivators. Following the conditions of the Comet assay (see section 4), which uses primary hepatocytes and HepG2 cells, the nitrosamines to be tested will be replaced by the above-mentioned CYP450 substrates and incubated (Table 1). The incubation time, incubation buffer and conditions should correspond as closely as possible to the final assay conditions. After incubation, both the cell supernatant and the cells are sent to the BfArM and processed by solid phase extraction and probed 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. In order to avoid costs, this characterization is performed for all metabolically competent systems at the beginning to ensure the general suitability within the Comet assays.

Study output – metabolic competence

The examination of the metabolic capability guarantees a sufficient CYP450 activity to toxify nitrosamines by e.g. alpha hydroxylation and thus to increase their alkylating reactivity. Using the metabolic ratio, it is possible to find out semi-quantitatively which enzymatic system shows the highest activity for a certain CYP450 enzyme when equimolar amounts of substrate are used. The metabolic ratio is calculated as follows:

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

After a certain incubation time has elapsed, this can be related to the percentage conversion and the amount of protein used. It follows as a result output:

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

Nitrosamines metabolized by certain CYP450 subclasses can be incubated with the predicted enzyme activity using appropriate preparations to ensure a high conversion rate. Peak areas are reported by the MS detector as arbitrary units in counts-per-second. The metabolic activity can be compared semiquantitatively 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 these studies, since quantitative data, e.g. for structure-activity relationships or calculations of pharmacological intrinsic activity, are not in the foreground. Statistical analyses will be performed only to a limited extent, since the number of samples will be too small for this purpose. For each approach, of course, 3 technical replicates will be performed. In the further course of the study, biological replicates will be measured again after a few months to confirm metabolic capacity and quality. Statistical standard calculations such as mean, standard deviation, relative standard deviation, etc. are the main focus here (Table 3).

Table 3: Exemplary table showing the metabolic activity of individual CYP450 enzymes. The unit is defined as the percentage of degradation per hour per mg of protein. All listed values are hypothetical examples and do not correspond to measured results

	Primary rat hepatocytes	HepG2 cell line	S9 mix
CYP2E1	1.9	0.9	13.1
CYP2A6	2.2	1.3	9.8

2.2 DNA alkylation assay

Principle

Physiological nucleic acid alkylation, in particular methylation, is a biological process which is caused by e.g. DNA-methyltransferases. These key processes trigger the occurrence of epigenetic variants and are also involved in gene expression rates, aging, as well as carcinogenesis (Cheng 1995). In contrast to physiological alkylation, the exposition towards harmful alkylating agents, like polycyclic aromatic compounds (PCA) or N-nitrosamines (NA) lead to spontaneous and unspecific DNA alkylation uncapable to be further recognized by cell or DNA repair mechanisms or respective repair enzymes. As a reason, DNA mutations, in form of single nucleotide polymorphisms may occur and are discussed as one crucial factor for carcinogenesis. Up until today, several publications were released in the field of adductomics, investigating the interrelationship between DNA alkylation processes and the successive cellular nucleic acid repair mechanisms (Balbo, Hecht et al. 2014, Balbo, Turesky et al. 2014, Chang, Cooke et al. 2018). The evaluation of detrimental effects on DNA is a crucial step to assess a compounds‘ toxicity. Many chemical subgroups, which are classified as carcinogenic, like many NA or

PCA, have to be metabolically activated prior to DNA damage. Here, the large group of the cytochrome P450 (CYP450) enzymatic system is involved in many pre-toxification processes. Therefore, the common strategy is an incubation of potent compounds with CYP450 competent preparations like liver S9 fractions or even vital liver cell incubations (HepG2, primary hepatocytes, etc.). Most the performed steps during the here presented DNA alkylation assay are deduced from already published protocols. An overview of the assay is depicted in Figure 2.

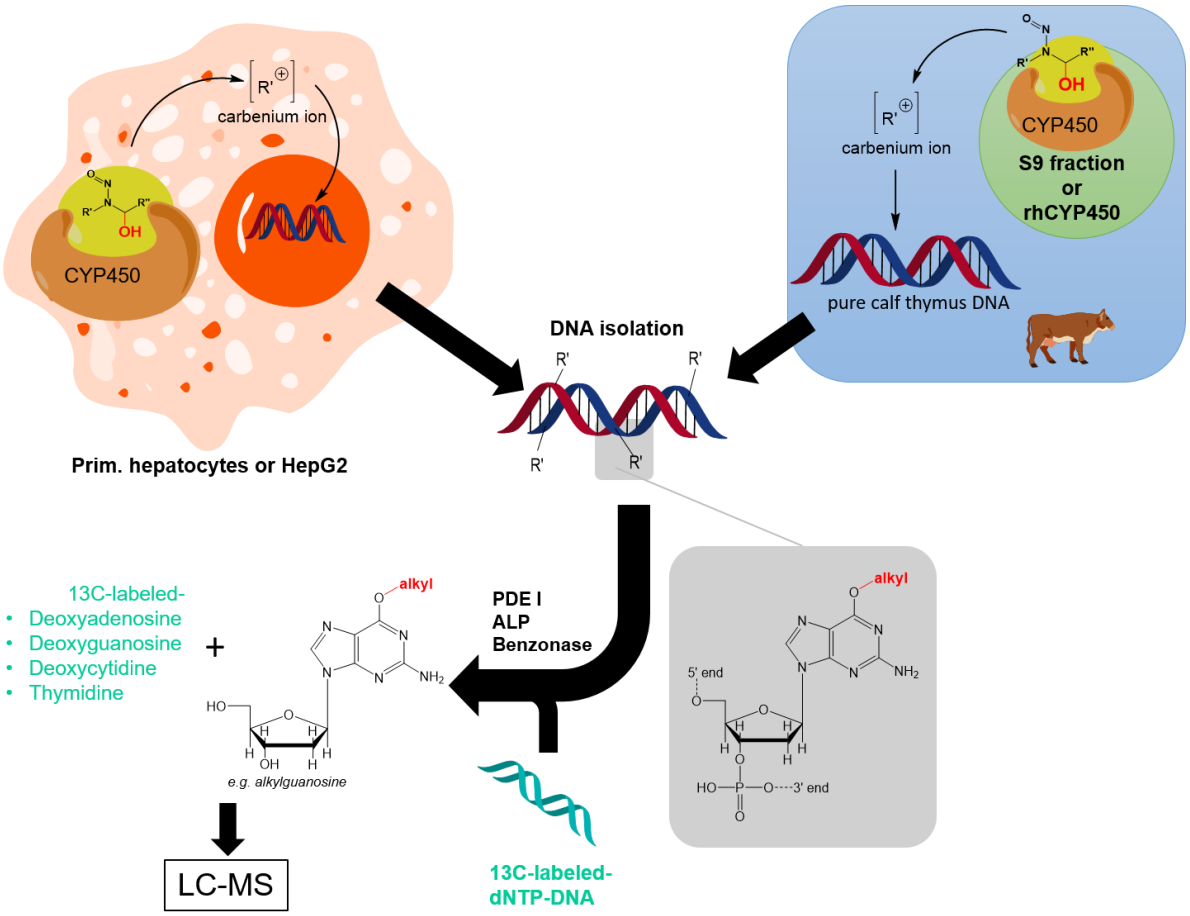

Figure 2: Overview of the DNA alkylation assay by using primary hepatocytes or HepG2 cells, representing vital cell systems. Pure calf thymus DNA is used in the presence of active induced rat liver S9 fractions to obtain first data revealing N-nitrosamine toxification and reactivity.https://www.xenotech.com/nonclinical-studies/adme/

Experimental part DNA-alkylation assay (overview)

In the experimental approach will follow the below listed five steps:

1. **Incubation part**
    a. Incubation of NAs with CYP450 competent cell cultures (HepG2, primary hepatocytes) under vital conditions (please see part 4)
    b. Incubation of NAs with induced rat and hamster liver S9 fractions in the presence of adjusted concentrations calf thymus DNA (BfArM)
    c. Incubation of NAs with human and induced rat/hamster liver microsomes in the presence of adjusted concentrations calf thymus DNA

d. Incubation of NAs with recombinant human CYP450 enzymes in the presence of adjusted concentrations calf thymus DNA (BfArM)

2. **DNA isolation**
    a. Lysis of equal cell numbers and/or direct extraction and purification of DNA; RNA removal by prior RNase; Qiagen DNeasy Blood & Tissue Kits
    b. Adjustment of DNA to equal concentrations and preparation of equal volumes
3. **Addition of stable isotopically labeled DNA as internal standard**
    a. Respective amounts of isolated and purified DNA from cell cultures or ctDNA concentrations are fortified with ISTD
4. **Enzymatic DNA digestion for nucleoside analysis**
5. **Nucleoside purification and LC-MS analysis**

Incubation part

a) Please see protocol HepG2 and primary hepatocytes incubation (please refer to section 4)

b) Refering to the publications of Pohjola, Lappi et al (2003) and Kuljukka-rabb, Kuusimäki et al. (2000) the assay, to predict the general DNA alkylation tendency of NAs, will be performed in the following composition (Kuljukka-rabb, Kuusimäki et al. 2000; Pohjola, Lappi et al. 2003):

- 120 µM test substrate / positive control (benzo[a]pyrene, aflatoxin b1)
- 0.5 mg/mL S9 protein
- 1 mg/mL calf thymus DNA (ctDNA)
- 400 µM NADPH + regenerating system

All experiments are performed with 0.2 M phosphate buffered saline (PBS), pH 7.4 at 37°C for 4h. For the negative control samples ‚substrate blank' and ‚enzyme blank' the volumes of substrate and S9 protein are exchanged with equal volumes of PBS, respectively to exclude false positive results.

Similarly, to measure the activity of the S9 mix, all volumes of the stock solutions are added except for NADPH. After a pre-incubation of 5 min, the NADPH regeneration system is added. Here, too, the maximum concentration of organic solvents must not exceed 1%. This is achieved by preparing suitably concentrated solutions. The total volume of the preparations is 500 µL. After 4 hours, the reaction is interrupted by adding 10% ammonium acetate buffer pH 3.5 and the DNA is purified. At the moment, it is planned to perform only one final determination and to subsequently semiquantify the alkylative yield by referring to the same quantity of the internal standard.

c) Up today, no protocols exist for the direct determination of DNA alkylation by N-nitrosamines solely using recombinant CYP450 enzymes and ctDNA. During this study, it is planned to implement a respective protocol by means of CYP-specific Corning® Supersomes™ in the presence of ctDNA. These formulations consists of a CYP450 microsomal system capable to present membrane-bound subgroups of metabolic enzymes. With regards to point b) within the incubation part, same conditions will be used in the first experiments and will be constantly optimized. On one hand, this approach simplifies the identification of specific CYP450 subgroups, which are responsible for individual metabolic degradation of NAs, on the other hand, this assay is not cell-dependent and can serve as proof-of-principle assay for future studies. Bellec et al. (1996) reported a selection of CYP450 which play a key role for the metabolism of different NAs including CYP1A1, 1A2, 2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 2E1 and 3A4 (Bellec, Dreano et al. 1996). It is planned to rely on the enzyme selection from this publication. Additionally, all enzymes are available as standardized supersomal preparation by Corning®. The DNA purification and digestion procedure will be identical to the following protocol.

DNA isolation and addition of ISTD

The DNA isolation and purification procedures for all cell dependent assays are described under the HepG2/primary hepatocytes incubation parts (please refer to section 4). The prepared 13C labeled ISTD is added to 20 µg of obtained DNA from HepG2 cells and primary hepatocytes and subjected to enzymatic DNA digestion. For the direct alkylation assay with ctDNA, respective aliquots of approximately 20 µg processed ctDNA is fortified with ISTD and further purified via Qiagen DNeasy® Kit. Subsequently, after elution from the resin, DNA is also digested as mentioned in the DNA digestion protocol.

Enzymatic DNA digestion for nucleoside analysis

To guarantee adequate assay conditions and obtain sufficient nucleosides from isolated DNA, a short literature review was performed to evaluate and improve the already existing method for nucleoside analysis. Six publications were selected, which offers a detailed description of the digestion protocol and the usage of respective enzymes (Quinlivan and Gregory 2008; Balbo, Hecht et al. 2014; Guo, Villalta et al. 2017; Zhu, Zhou et al. 2017; Geisen, Aloisi et al. 2021). The results of the literature review and the respective amounts of utilized enzymes are listed in Table 4.

Table 4: Overview of the used enzymes in each approach.

Author *	DNAse I [U]	Phospho-diesterase I [mU]	Phospho-diesterase II [mU]	Alkaline Phosphatas [U]	Benzonase [U]	Nuclease P1 [U]	DNA degradase plus®	DNA [µg]
Balbo	2x 350	225	32.5	750	-	-	-	500
Guo	-	3.2	-	0.04	300	0.1	-	20
Geisen & University of Florida	-	60	-	40	50	-	-	20
Quinlivan	-	300	-	200	250	-	-	10
Zhu	2	-	-	-	-	-	10	5

* (Quinlivan and Gregory 2008; Balbo, Hecht et al. 2014; Guo, Villalta et al. 2017; Zhu, Zhou et al. 2017; Geisen, Aloisi et al. 2021)

Most utilized enzymes were phosphodiesterase I, alkaline phosphatase, and benzonase. All enzymes also have same pH optima in the range between pH 8 and 9. As a reason, a standardized reaction buffer for DNA digestion (DNA digestion buffer) was prepared with the following composition:

- 10mM Tris
- 50mM NaCl
- 10mM $MgCl_2$

The buffer were adapted to pH 8 and the incubation time lasts approximately 16-18h/overnight. All enzyme concentrations were chosen with regards to the protocol from Geisen et al. With 3 mU phosphodiesterase I / µg DNA, 2 U alkaline phosphatase / µg DNA, and 2.5 U benzonase / µg DNA. Due to limitations for the DNA amount, only 10 µg of DNA were processed in 50 µL of the DNA digestion buffer.

In prior experiments and quality controls, this modified version of the digestion protocol from Geisen et al. could be successfully implemented at the BfArM. In temozolomide (TMZ) treated cell cultures, where TMZ is used as DNA methylating agent, DNA was isolated and further processed in accordance to the protocol of (Geisen, Aloisi et al. 2021). In case of lower amounts of DNA, the addition of reagents were adapted to guarantee equal conditions.

Nucleoside purification and LC-MS analysis

After the digestion of artificially labeled and native nucleic acids, remnants of proteins, undigested DNA fragments, and excesses of buffer salt, which may negatively impact analyte ionization, have to be removed from nucleosides prior to LC-MS analysis. Thereby, solid phase extraction (SPE) is implemented into our protocol for purification.

Study output – DNA alkylation assay

The DNA alkylation assay can be seen as an approach to qualitatively and at most semi-quantitatively determine the alkylation tendency of metabolized and reactive nitrosamines. Primarily, the assay serves to corroborate the results of the Ames test as well as the Comet assay and to provide possible explanations in case of classification as a mutagenic substance. Little or no information on mutagenic potential is available for any of the nitrosamines tested in this study. Data evaluation primarily provides for observation of characteristic signals, particularly mass-to-charge ratios or m/z. These can be further characterized based on conversion back to molar masses and assigned to the pre-calculated masses of the expected alkylated nucleosides. This part of the evaluation is a qualitative assessment of the results and observation of the alkylating processes on DNA. These data are included in the results evaluation as mass spectra. An example spectrum for the methylation of guanosine following (Churchwell, Beland et al. 2006) is given in Figure 3.

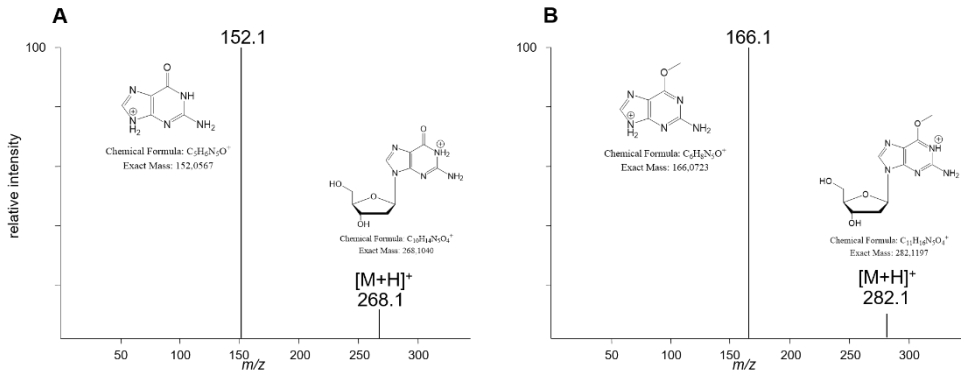

Figure 3: Exemplary figure with A, the underivatized native guanosine, and B, the methylated guanosine after reaction with alkylating agents.

In practice, the original MS/MS spectrum will most likely be much more complex and show more product ions. Also, depending on the position of the alkylation on the molecule, different product ions will be observed with the same initial mass. For each nitrosamine used in the study, other nucleoside derivatives with different masses or *m/z* values and product ions are expected after cleavage of the reactive residue. The possible interpretation and structural elucidation is an essential part of the study. Thus, a mutagenicity and alkylation profile is established for each nitrosamine in conjunction with the results of the AMES test and COMET assay.

For the semi-quantitative estimation of the degree of alkylation, the intensity of the observed signals per alkylated nucleoside is correlated with the signals of the added internal standards. The following is an example equation for the degree of alkylation of a particular nitrosamine or its residue to the stable isotopically labeled nucleoside cytosine of the ISTD mix.

$$Alkylation\ grade = \frac{\sum peak\ areas\ cytosin\ adducts_{nitrosamine\ 1}}{peak\ area\ ISTD_{cytosin}}$$

Since equal amounts of DNA and, after interruption of the enzyme activity, equal amounts of internal standards are added per batch, the final analytical yield only depends on the enzymatic digestion. Alignment of all intensities for the internal standards across samples allows the degree of alkylation of individual nitrosamines to be compared. It is assumed that the signal response per amount of analyte is more or less the same for each alkyl nucleoside of a purine or pyrimidine base group. Otherwise, a semi-quantitative comparison is not possible.

Quantitative determinations are unfortunately not possible in this experiment, since direct reference substances for DNA alkylation products are commercially available only to a limited extent. There is also the problem that alkylation reactions can take place at several molecular positions for all 4 DNA nucleosides. If all the nitrosamines to be tested in this study are also taken into account, the measurement capacity and the budget for the number of reference substances to be purchased would be far exceeded.

2.3 LC-MS measurements

During all studies LC-MS measurements will be performed with a Sciex QTRAP® 6500 triple quadrupole mass spectrometer equipped with a Shimadzu Nexera® ultra performance liquid chromatograph. This system is capable to work under high pressure up to 400 bar allowing the use of small particle sizes in chromatographic columns to increase peak sharpness and the methods‘ selectivity. During the study, chromatographic columns with core-shell technology will be used to measure the substrates and metabolites. In the past, an Accucore C8 (Thermo Fisher Scientific), 50x3mm, 2.6 µm particle size has always proved to be the best choice, as it offers a very high resolution of the signals due to the shortening of pores. Acetonitrile in combination with 0.2% formic acid is routinely used as a flow agent. The determination of DNA adducts will be performed with predefined targeted as well as untargeted experiments. In case of targeted experiments, *m/z* values of pre-calculated nucleoside adducts will be integrated in an enhanced product ion experiment. Here, the linear ion trap of the third quadrupole is engaged to significantly increase the sensitivity and limit of detection. Structure elucidation is mandatory to confirm DNA alkylation. In case of twofold, or even threefold, alkylated nucleosides or to detect alkylated nucleosides with unpredicted residues, untargeted experiments will be performed with neutral loss experiments. Here, the loss of deoxy ribose is monitored to detect uncommon residues or respective mass shifts. In order to adequately separate nucleosides and achieve retention, it is planned to use a surface-modified column specifically for DNA analysis. One possible column could be the Phenomenex Fusion-RP® C18, which is characterized by the separation of polar substances in particular. The flow agent would be 20 mM ammonium acetate buffer, pH 4.5 and acetonitrile.

2.4 Preparation of the internal standard (ISTD)

For semiquantitative approaches, the utilized DNA amounts should be fortified with equal amounts of a representative ISTD prior to enzymatic digest to examine enzymatic conversion and degradation. It is planned to synthesize isotopically labeled DNA templates by performing polymerase chain reaction (PCR) with 13C-labeled deoxynucleosidetriphosphates (13C-dNTPs) instead of common dNTPs. Therefore, a human serum albumin specific primer set will originate and exponentially amplify an approximately 1kb long gene sequence. In this way, up to 1 mg of artificially labeled DNA will be synthesized and freed from excesses of dNTPs by ultracentrifugation with 10 kDa molecular weight cut-off filter. Therefore, the PCR products will be given to the filter units and washed thrice with high purity commercially available DNA wash buffer. The filter residue is recovered by centrifugation and reconstituted with 500 µL DNA storage buffer for later use. After determining the DNA concentration via a nano drop® device, the final ISTD stock solution will be prepared containing 10 µg 13C-DNA / mL. The incubation with the DNA degrading enzymes bezonase, PDE I, and AP will lead to a release of labeled nucleosides which highly depends on each enzymes activity and final amount. The peak response of detected alkylated NTPs are then further evaluated by means of the peak responses or

released amounts of 13C-deoxynucleosides as well as the non-alkylated deoxynucleosides. An overview of ISTD synthesis is summarized in Figure 4.

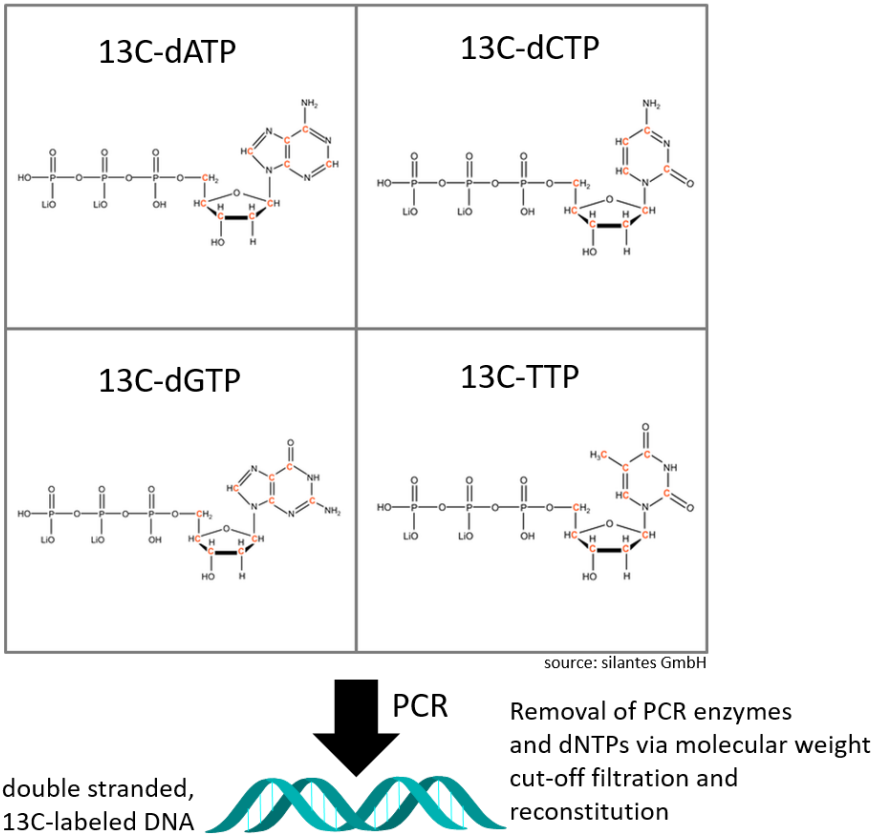

Figure 4: Synthesis of the ISTD by means of 13C-labeled deoxynucleoside triphosphates. Each 13C-dNTP contains carbon 13 instead of carbon 12 which results in mass shifts of 10 amu for 13C-dATP/dGTP/TTP and 9 amu for 13C-dCTP, respectively. These mass shifts can be distinguished from native DNA signals and are therefore suitable as ISTD.

3. Characterization of metabolic competence of different liver cells and model tissue by means of CYP450 mRNA gene expression

Standardized protocols at the BfArM will be utilized within the study for gene expression measurements. All detailed steps and reagents are as follows.

Liver cells and model tissue:

- Primary rat hepatocytes
- Primary human hepatocytes
- HepG2 cells
- Optional - to be decided in the course of the project: cryopreserved human liver organ slices

Kits and reagents:

- RNAprotect (Qiagen)
- RNeasy mini Kit (Qiagen)
- QiaShredder colums (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™ (TermoFisher)
- Bioanalyzer™ (Agilent)
- Thermocycler
- LightCycler 480™ (Roche)

Protocol

1. **RNA isolation:**

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

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

- *Add 1/100 volume of ß-mercaptoethanol to the required volume of RLT Buffer (e.g.: 1ml RLT + 10 µl ß-mercaptoethanol).*
- *For < 5 x 10^6 cells: 350 µl RLT buffer (+ 3.5 µl ß-Mercapto ETOH).*
- *For < 1 x 10^7 cells: 600 µl RLT buffer (+ 6 µl ß-Mercapto ETOH).*
- *Mix cells with RLT buffer and pipette complete lysate onto a QiaShredder column. (Centrifuge for 2 min at 13,000 g).*
- *Pipette 1 volume of 70% ethanol onto the lysate in the Collection Tube and mix. (e.g.: 350 µl lysate + 350 µl ETOH).*
- *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)*
- *Discard Collection Tube, previously dumping the liquid into a sealable waste container*
- *Set column on new Collection Tube*
- *Pipette 700 µl RW1 onto the column and centrifuge for 30 sec. Centrifuge at 8000 g*
- *Discard Collection Tube, previously dumping the liquid into a sealable waste container*
- *Set column on new Collection Tube*

- *Pipette 500 µl RPE onto the column and centrifuge for 30 sec. Centrifuge at 8000 g*
- *Discard Collection Tube, previously dumping the liquid into a sealable waste container*
- *Set column on new Collection Tube*
- *Pipette 500 µl RPE onto the column and centrifuge at 8000 g for 2 min.*
- *Place the column on a new collection tube and centrifuge at 13,000 g for 1 min.*
- *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*
- *freeze RNA at -80 °C*

2. <u>RNA quantification and quality determination</u>

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

3. <u>cDNA synthesis via reverse transcriptase PCR</u>

cDNA is synthesized using QuantiTect® Reverse Transcription Kit according to manufacturer's protocol:

- *Thaw all reagents and centrifuge briefly, then place on ice*
- *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*
- *Incubate for 2 min at 42°C, then store on ice.*
- *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*
- *Mix carefully (do not vortex!) and, if necessary, centrifuge briefly.*
- *In a thermal cycler, call up RT program: 15 min. 42°C, 3 min. 95°C, then stop the reaction on ice!*

4. <u>Real-time qPCR</u>

qPCR is performed using QuantiTect® SYBR® Green PCR Kit on a LightCycler 480™ according to the manufacturer's protocol. Analysis is performed in technical triplicates using QuantiTect Primer Assays:

- *Thaw QuantiTect SYBR Green Mastermix (2x), PCR-grade Water, cDNA and QuantiTect Primerassay (10x).*
- *Dilute the cDNA with PCR-grade Water 1:10.*
- *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*
- *Pipet mix into a white 96 well PCR plate and seal with the appropriate foil.*
- *Centrifuge the PCR plate at 3000 g for 2-3 min and store it in the dark at 4°C until measurement*
- *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:*

<u>PCR Initial Heat Inactivation 95°C 15 min</u>

Denaturation 94°C 15 s

Annealing 55 °C 30 s *40 cycles*

Extension *72 °C 30 s*

5. Data analyses

CYP450 gene expression is evaluated from measured Ct values and normalized to the reference genes 18S rRNA or GAPDH. Additionally, beside the 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.

4. Selection of eight representative NAs

The DNA adduct formation data will help distinguish NAs with different DNA adduct profiles. The working hypothesis of this project is, that repair mechanisms differ depending on the location of the DNA adduct as well as its steric hindrance (bulky versus small adducts). Repair mechanisms for eight representative NAs will be further investigated considering the following selection criteria:

1. mutagen/not-mutagen adduct

2. predominately bulky adducts, focus on API derived NAs, include one small well-known positive control

3. NA differing with regard to carcinogenic potency (low to high)

4. NAs that are not already covered by the second project on optimized testing procedures, as Comet assays will be done in both projects. Same positive/negative control as in second project, and also same amount of solvent

5. Capacity, velocity and accuracy of relevant cellular repair mechanisms and impact of DNA repair on genotoxicity and cytotoxicity

Several DNA repair pathways are involved in the repair of the various DNA alkylation adducts induced by NAs, namely O^6-methylguanine DNA methyltransferase (MGMT), base excision repair (BER) and XPA-mediated nucleotide excision repair (NER) particularly relevant for bulky DNA alkylation adducts (Figure 5 and Figure 6). The ALKBH family, which repairs only a minor fraction of small DNA alkylation adducts, will not be studied in this project.

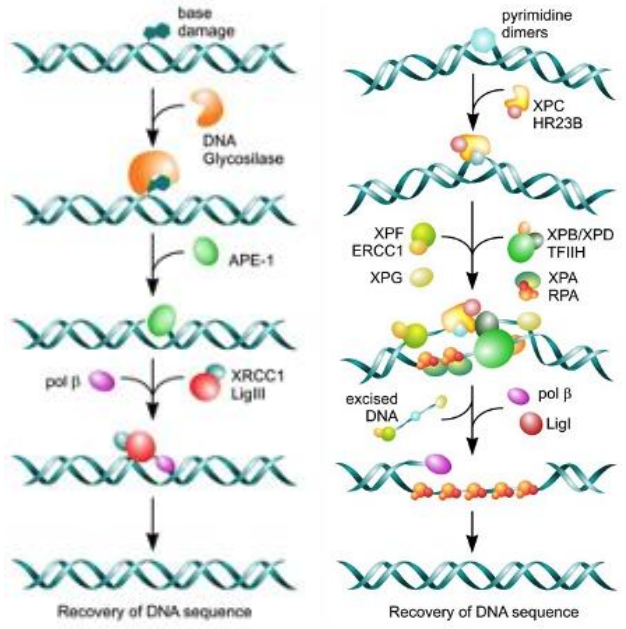

Figure 5: Overview of the BER (left) and NER (right) pathways (Fahrer and Kaina 2017).

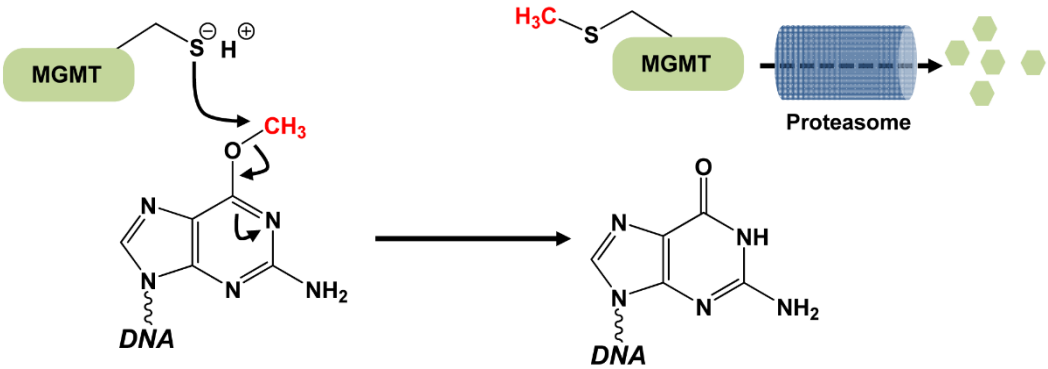

Figure 6: Direct damage reversal by MGMT (Fahrer and Kaina 2017).

To measure the impact of the different DNA repair pathways, we will investigate the following biological endpoints in both DNA repair-proficient and -deficient cells systems after treatment with NAs:

1. Cellular sensitivity will be determined by a cell viability assay
2. Formation of DNA strand breaks and alkali-labile sites will be analyzed by the alkaline Comet assay
3. DNA adduct analysis will be performed together with the collaboration partner BfArM

Up to 8 NAs will be selected for *in vitro* testing. This panel will widely overlap with the panel selected for the *in vitro* Comet assay (project 02_in vitro) and will include all relevant structural classes (simple, bulky – aryl, bulky – cyclic and bulky – substituted/substituted dialky NAs). If possible, stock solution of the NAs will be prepared as developed in chapter 1. Pretesting, ordering and distribution. As reference substances, the data-rich NAs NDMA (prototype of ubiquitous small NAs) and NNN (tobacco-specific NA as prototype of bulky NAs) will be used, which will also be tested in the second EMA project on the development of *in vitro* test systems. The experiments will be performed in rodent (chapter 4.1) and human (chapter 4.2) DNA-repair proficient and deficient cells.

5.1 Capacity, velocity and accuracy of relevant DNA repair mechanisms and impact of DNA repair in primary mouse hepatocytes

- **Isolation of murine primary hepatocytes and cell seeding**

Primary hepatocytes will be isolated from MGMT-, MPG- and XPA-deficient mouse models (de Vries, van Oostrom et al. 1995; Wirtz, Nagel et al. 2010; Fahrer, Frisch et al. 2015) as well as from DNA repair competent wildtype mice. The expression of the DNA repair genes *XPA, MPG* and *MGMT* within these mouse models will be verified by qPCR. As an alternative for DNA repair deficient hepatocytes, wildtype hepatocytes can be used and pre-treated with DNA repair inhibitors (MGMT inhibitor, BER inhibitor) prior to incubation with the selected NAs.

To this end, the animals will be anesthetized with pentobarbital administered via intraperitoneal injection and livers will be perfused using a two-step procedure as described previously (Seglen 1976; Schrenk, Karger et al. 1992). Viabilities of hepatocyte preparations will be assessed by trypan blue exclusion. Only hepatocytes with a viability ≥ 85 % will be used in further experiments. For genotoxicity and cytotoxicity studies, isolated hepatocytes (200,000 per well) will be seeded in 0.5 ml medium (DMEM-LG supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin solution) per well on rat tail collagen-coated 24-well plates at 37 °C under an atmosphere of 5% CO_2 and left for 3 h to attach. Subsequently, the medium will be replaced by 0.5 ml fresh medium/well containing the NAs of interest. For DNA adduct analysis, isolated hepatocytes (2,000,000) will be seeded in 5 ml medium (DMEM-LG supplemented with 10% fetal calf serum and 1% Penicillin/Streptomycin solution) on rat tail collagen-coated 6 cm dishes at 37 °C under an atmosphere of 5% CO_2 and left for 3 h to attach. Treatment will be performed as described above.

- **Characterization of metabolic competence of primary murine hepatocytes**

The isolated primary murine hepatocytes will be characterized regarding their metabolic competence by means of CYP-isoform specific substrates and mass spectrometric analysis. This will be performed by the collaboration partner BfArM, who is also responsible for the data evaluation (see chapter 2.1).

- **Determination of cellular sensitivity**

To determine the cellular sensitivity, primary murine hepatocytes differing in their DNA repair status will be exposed to increasing concentrations of the respective NA (seven concentrations including solvent control) for 24 h. Viability will then be assessed by the "CellTiter-Glo Luminiscent Cell Viability Assay" or the resazurine reduction test (also called Alamar Blue assay) according to our

previous work (Gao, Rutz et al. 2020; Arnold, Demuth et al. 2022) and pre-experiments performed with NDMA and NNN. These experiments will yield quantitative data, which will be presented in a bar diagram using GraphPad Prism software. The output is the cell viability at a given NA concentration normalized to the solvent control, i.e., cell viability [% of control]. The data will be shown as mean + standard error of the mean. The data sets will be analyzed by Student's t test or One-way ANOVA. Furthermore, the data will be used to derive EC_{50} and EC_{20} by means of GraphPad Prism software (Dorsam, Goder et al. 2015) Briefly, the used NA concentrations will be transformed into the log scale and plotted against the cell viability. The curve will then be fitted by nonlinear regression with variable slope, providing the EC_{50} and EC_{20} values. This evaluation will allow grouping the NAs with regard to their cytotoxic potential and to identify engaged DNA repair pathways. Furthermore, both parameters will be used to determine the concentration range for the subsequent genotoxicity studies (alkaline Comet assay, DNA adduct formation).

- **Assessment of DNA strand break induction using the alkaline Comet Assay**

To analyze DNA strand break induction by NAs and the relevance of DNA repair, the alkaline Comet assay will be used. Cells will be exposed to increasing concentrations of the selected NAs (five concentrations including solvent control, maximum concentration EC_{50}). The alkaline Comet assay will be conducted as recently published (Dorsam, Seiwert et al. 2018) and detailed in Table 5. These experiments will yield quantitative data, which will be presented in a bar diagram using Graph Pad Prims software. The output is the tail intensity [%] as a measure of DNA damage at a given NA concentration. The data will be depicted as mean + standard error of the mean and will be analyzed by Student's t test or One-way ANOVA. Furthermore, the data sets will be subjected to benchmark dose (BMD) modeling by PROAST or BMD-Express software. PROAST is the preferred model, as it allows to compare individual modelling approaches using best fit analyses with model averaging. These analyses will allow determining a critical effect concentration, i.e., a BMC_{50} that corresponds to a 50 % increase over background level, and the corresponding BMCL [benchmark concentration lower boundary] and BMCU [benchmark concentration upper boundary] levels. These values will be used for ranking the NAs according to their genotoxic potency and to identify involved DNA repair mechanism. As an alternative method, the γ-H2AX assay is established in our lab, which serves as surrogate marker for DNA strand breaks. To this end, induction of γ-H2AX can be detected by SDS-PAGE and Western blot detection or immunofluorescence microscopy as reported (Mimmler, Peter et al. 2016; Seiwert, Neitzel et al. 2017).

- **Analysis of DNA adduct formation and repair**

As further genotoxic endpoint, formation of DNA adducts and their repair will be studied using time course experiments. To this end, cells will be exposed for 2 h to the selected NA at a concentration at the EC_{20} value to avoid overt cytotoxicity. Cells will then be harvested after 0, 4, 8, 12 and 24 h according to our previous study with temozolomide induced O^6-MeG adducts (Kraus, McKeague et al. 2019). Genomic DNA will be isolated and sent to the collaboration partner BfArM, which will be responsible for the DNA adduct analysis by mass spectrometry and the subsequent data evaluation (see chapter 2.3). The obtained data will be crucial to determine the relevance of the DNA repair pathways and to identify persistent DNA adducts.

- **Experiments in DNA repair proficient and deficient hamster cells lines**

In addition to the primary murine hepatocytes, hamster cell lines deficient for specific DNA repair pathways will be included in the testing strategy. This also ensures that we have data from an established proliferating cell system for comparison. Due to the lack of metabolic competence in the hamster cell lines, S9-mix will be used that is provided by ICCR (see also below, chapter 4.2). The hamster cell lines comprise CHO-9 cells with defects in the BER (EM9) and NER pathway (UV5 and UV61) (Thompson, Brookman et al. 1982; Yang, Zdzienicka et al. 1991). The cells will be provided and employed by Toxys using clonogenic survival as primary assay to determine which pathways are important for the effects of NAs. The clonogenic survival assay runs as follows:

- First, suitable compound doses for the cytotoxicity assay will be established in a broad dose range finding test using Alamar Blue. Native cells (AA8, V79) will be seeded in 96-well plates at a low cell density (cell density that results in a confluent cell culture after three/four days of cell growth). Twenty-four hours after seeding, asynchronous growing cells will be exposed for 48 and, if possible, 72 h to increasing concentrations of the test compounds in serial 2-fold dilutions in presence of S9.
- The clonogenic survival assay will be performed with a low, medium and high compound test concentration. DNA repair mutant cell lines will be exposed to the same concentrations as the related wild-type parental cell line. Cells are seeded at low density in 6-well plates. Twenty-four hours after seeding, cells will be exposed to the test samples in presence of S9, as well as various reference compounds. After approximately 7-10 days, cell colonies are stained and counted. The clonal cell survival is expressed as the number of cell clones after exposure relative to unexposed cells.

Apart from these cells Toxys has hamster and mouse cell lines covering further DNA repair pathways should this become interesting as the project progresses.

- Based on the parent hamster cell lines the Comet assay including S9 will be performed as detailed in Table 5 for comparison to the Comet assay for the primary cells (see above). The data will be subject to BMD analysis as described above.

Furthermore, isogenic MGMT-proficient and deficient CHO-9 hamster cells (Kaina, Fritz et al. 1991) are available to analyze the genotoxic and cytotoxic potential of selected NAs as described (Uni KL). The potential use of these additional cell lines in the study will be discussed with EMA as the project progresses and if resources allow.

-

5.2 Capacity, velocity and accuracy of relevant DNA repair mechanisms and impact of DNA repair in human liver cells

- **Generation and characterization of DNA repair deficient human liver cells**

Human DNA repair deficient HepG2 cells are not existing and will be established within the project. For generation of these cell lines, HepG2 cells were already distributed from Dr. Ziemann to the members of the consortium to allow compatibility of the results obtained. In these cells, *MGMT*, *MPG* and *XPA* will be knocked out by Crispr/Cas9 technology. Therefore, transfection efficiency in HepG2 cells will be optimized using GFP-expressing plasmid. Thereafter, gene specific Crispr/Cas9 plasmids, obtained from Santa Cruz, will be transfected into HepG2 cells and puromycin resistant cell clones will be isolated. The expression of *XPA*, *MPG* and *MGMT* within these clones will be tested by qPCR and immunodetection. Cell clones showing successful knockout of the DNA repair factors will be selected for further testing of the NAs as described above. Since HepG2 cells are difficult to transfect, an additional human cell line (MCF7) will be used as backup to generate repair proficient and deficient human cells in case of a non-successful knockout in HepG2 cells. The intrinsic metabolic capacity of HepG2 and MCF7 cells will be measured in cooperation with the BfArM. Furthermore, S9-mix will be isolated, tested and distributed to the different groups by the collaboration partner ICCR.

- **Seeding of cells and treatment**

For genotoxicity and cytotoxicity studies, cells (200,000 per well) will be seeded in 0.5 ml medium (HepG2: DMEM supplemented with 10% fetal calf serum, 2mM glutamine and 1% Penicillin/Streptomycin solution / MCF7: DMEM-F12, 5% fetal calf serum) per well on 24-well plates at 37 °C under an atmosphere of 5% CO_2 and left for 6 h to attach. Subsequently, the medium will be replaced by 0.5 ml fresh medium/well containing the NA of interest. For DNA adduct analysis, cells (1,000,000) will be seeded in 5 ml medium on 6 cm dishes at 37 °C under an atmosphere of 5% CO_2 and left for 6 h to attach. Treatment will be performed as described above.

- **Establishment of genotoxicity and cytotoxicity assays**

In parallel to the generation of DNA repair deficient cells, toxicity as measured by the "CellTiter-Glo Luminiscent Cell Viability Assay" or the resazurine reduction test (Gao, Rutz et al. 2020; Arnold, Demuth et al. 2022), and DNA strand-break formation using alkaline Comet assay, will be established with the NAs NNN and NDMA in the presence and absence of the MGMT inhibitor O^6-Benzylguanine (O^6-BG) in HepG2 and MCF7 cells (HepG2 *vs.* HepG2-O^6BG / MCF7 *vs.* MCF7-O^6BG). Therefore, NNN and NDMA will be pre-incubated in S9-mix for 10 min to achieve full metabolic activation. The NAs and the S9-mix will then be added to the cells for 4h and thereafter, the medium will be exchanged to avoid S9-mix mediated toxicity. O^6-BG will be added 1 h before addition of the NAs at a concentration of 10µM. Concerning the cellular sensitivity assay seven different concentrations of the given compounds will be used and sensitivity will be measured 24 h after exposure. Concentration-response curves will be used for establishing the EC_{20} and EC_{50} of the tested NAs. Concerning the formation of AP sites and DNA strand breaks five concentrations up to the EC_{50} concentration including solvent control, will be used and the alkaline comet assay will be performed 24 h after exposure. The results show genotoxic potency of the tested NAs and will be used for generating BMDs. In addition, these cell systems will be treated for 2 h with the selected NAs at the EC_{20} and 0, 4, 8, 12 and 24h later

genomic DNA is isolated and transferred to the BfArM for DNA adduct measurements by mass spectrometry.

- **Testing of NAs with regard to their genotoxicity and cytotoxicity**

During the actual testing phase, cell viability, as well as formation of AP sites and DNA strand breaks will be compared using up to 8 selected NAs in the following systems:

1. HepG2 *vs.* HepG2-O^6BG or alternatively MCF7 *vs.* MCF7-O^6BG
2. HepG2 *vs.* HepG2-MGMTko *vs.* HepG2-MPGko *vs.* HepG2-XPAko or alternatively MCF7 *vs.* MCF7-MGMTko *vs.* MCF7-MPGko *vs.* MCF7-XPAko

For the testing, the selected NAs will be pre-incubated in S9-mix for 10 min to achieve full metabolic activation. The NAs and the S9-mix will then be added to the cells for 4h and thereafter, the medium will be exchanged to avoid S9-mix mediated toxicity. Concerning the cellular sensitivity assay seven different concentrations of the given compounds will be used and sensitivity will be measured 24 h after exposure. Concentration-response curves will be used for establishing the EC_{20} and EC_{50} of the tested NAs. Concerning the formation of AP sites and DNA strand breaks five concentrations up to the EC_{50} concentration including solvent control, will be used and the alkaline comet assay will be performed 24 h after exposure as detailed in Table 5 and harmonized with the HepG2 protocol developed in the 02_in vitro project. The results show genotoxic potency of the tested NAs depending on the DNA repair status and data will be used for BMD analysis (see chapter 4.1). As an alternative method, the γ-H2AX assay is established in our lab, which serves as surrogate marker for DNA strand breaks. This assay can be used as an alternative endpoint if there are technical problems or other issues with the Comet assay in the used cell models. Thus, formation of γ-H2AX can be detected by SDS-PAGE and Western blot detection or by immunofluorescence microscopy in case that we will face technical problems or other issues concerning the comet assay.

In addition, these cell systems will be treated with the selected NAs at EC_{20} and 2, 4, 8, 12 and 24h later genomic DNA isolated and transferred to the BfArM for DNA adduct measurements by mass spectrometry (see chapter 2.3).

Table 5: In vitro alkaline Comet assay methods of the three laboratories Toxys, TU Kaiserslautern and University Medical Center Mainz.

Parameter	Toxys	TU Kaiserslautern	Univerisity Medical Center Mainz
In vitro cell model:			
Type	Chinese hamster ovary cells (AA8, EM9, UV5, UV61)	mouse hepatocytes (WT, MGMT k.o., MPG k.o., XPA k.o.)	Hep-G2, Hep-G2-MGMT-ko, Hep-G2-MPG-ko, Hep-G2-XPA-ko
Provider	own stock (Toxys)	own breeding and Janvier Labs	DSMZ (preparation of a working batch, Fraunhofer ITEM)
Culture conditions	DMEM/F12	DMEM + 10 % FCS + 1 % Penicilline/Streptomycin	DMEM + 10 % FCS + 1 % Penicilline/Streptomycin
Passage number range	not applicable	not applicable	not applicable
Cell processing after detachment	centrifugation at RT, direct resuspension in agarose, slide preparation	centrifugation at 4°C, resuspension and placement on ice	centrifugation at RT, resuspension and placement on ice
Storage of cells	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	24- or 48-well plates	12- or 24-well plate	6-well plate, adaption possible
Cell exposure method	addition of fresh medium + compound	addition of fresh medium + compound	addition of fresh medium + compound
Planned number of concentrations	max. 5 concentrations	max. 5 concentrations	max. 5 concentrations
Incubation time (h)	24 h, eventually also 4 h	24 h, eventually also 4 h	24 h, eventually also 4 h
Cytotoxicity endpoint(s)	Resazurin Assay, cell number	Resazurin Assay	MTT, Resazurin Assay
Detachment method	Trypsin-EDTA	TrypLE express	Trypsin-EDTA
Assay control	negative/vehicle controls, EMS -S9-mix, CP +S9-mix	negative/vehicle controls, MMS -S9-mix, CP +S9-mix	negative/vehicle controls, MMS -S9-mix, CP +S9-mix
Slide preparation:			
Slide pre-coating (yes/no)	yes (1.5% normal melting agarose)	yes (1,2% normal melting agarose)	yes (1,2% normal melting agarose)
Concentration of Low Melting Agarose (LMA)	0,75%	0.5%	0.5%
Cells per slide	100000-200000	50000 - 75000	50000
Volume cell suspension per slide (µl)	40 µl	10 µl	120 µl
Sandwich method (yes/no)	no	no	no
Cell lysis & Electrophresis			
Lysis buffer composition and pH	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 % Triton X-100, pH 10
Lysis time (e.g. 4 h, in h)	1 h (up to overnight)	1 h	1 h
Lysis temperature	4°C (in the dark)	4°C (in the dark)	4 °C (in the dark)
Electrophoresis buffer composition and pH	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	electrophoresis on ice	4°C	4°C
Unwinding time (minutes)	40	25	25
Duration of electrophoresis (minutes)	30	15	15
Voltage (V/cm)	25	25	23
Amperage (mA)	300	300	300
Randomized placement of slides in reservoir (yes/no)	yes	yes	yes
Staining & Analysis			
Staining	Sybr Gold	Propidium iodide	Propidium iodide
Type of primary descriptor (tail intensity, etc.)	tail intensity	tail intensity or tail moment	tail intensity and tail moment
Analysis system with version	Harmony 5.1 software on Operetta imager	Comet Assay IV Software (Perceptive Instruments/Instem)	Comet Assay IV Software (Perceptive Instruments/Instem)
Microscopic magnification (in x)	20x	20 x	20 x
Number of comets scored	50 per slide, 3 slides per treatment	50 per slide, 3 slides per treatment	50 per slide, 3 slides per treatment
Measure of central value of comets (mean/median)	median per slide and mean per tretament	mean per slide and mean per treatment	mean per slide and mean per tretament
Coding of slides (yes/no)	no	no	no
One evaluator per experiment (yes/no)	no	no	no
No. of evaluators per project partner	2	2	2
Statistical analysis of results	to be discussed	to be discussed	to be discussed

Annex I

Overview on experimental approaches and outputs in 01_QSAR project.

Topic	Experimental approach (high level summary)	Output
Pretesting of compounds	**Solubility screening assays** to develop methods, which allow reduction of solvent concentration to less than 1%.	Solubility will be determined based on the read-outs opacity, reprecipitation of the solution, and color change
	Impurity screening to control quality of delivered test compounds	LC-MS full scan analysis is used to identify additional peaks and their masses compared to the originally solved test substance. Semiquantitative assessment based on signal intensity.
	Time course **stability experiments** in relevant matrices; sample taken after t[h] = 0, 1, 2, 4, 8, 16, 24, and 48h of incubation	LC-MS full scan analysis is used to determine the time-dependent decrease of signal intensity of the test compound, which directly correlates with nonspecific degradation and nonspecific binding, quantification of loss over time for test substance.
Metabolic competence	**Activity of phase I enzymes** by incubation with selected enzyme substrates; relevant assay system include: rat and hamster S9 mix and microsomes, HepG2 cells and primary hepatocytes	LC-MS measurements to determine metabolic ratio and metabolic activity based on the conversion rate of substrates
	Transnitrosation of aliphatic nitrosamines on glutathione, which occurs spontaneously mainly under acidic conditions according to method described by Yanagimoto, Toyota et al. 2007	Formation of nitroso glutathione quantified by LC-MS measurements. Identification of transnitrosation activity from a respective nitrosamine to form other nitrosamines with unknown toxic classification (qualitative).
	CYP450 gene expression in primary rat hepatocytes; primary human hepatocytes and HepG2 cells using real-time q-PCR	Expression levels of different enzymes
Stability of reactive intermediates	S9 incubation of nitrosamines, ultrafiltration and direct measurement of the filtrate	Time-dependent semiquantitative determination of hydroxylated nitrosamines; as far as these species are stable in general or for LC-MS determination
DNA adduct formation	**DNA alkylation assay** - incubation of NAs with CYP450 HepG2, primary hepatocytes.	

Topic	Experimental approach (high level summary)	Output
	DNA alkylation assay - incubation of NAs with induced rat and hamster liver S9 fractions in the presence of adjusted concentrations calf thymus DNA	LC-MS analysis to qualitatively determine DNA alkylation products; as far as possible also the degree of alkylation by comparing of the observed products to internal standards.
	DNA alkylation assay - incubation of NAs with human and induced rat/hamster liver microsomes in the presence of adjusted concentrations calf thymus DNA	
	DNA alkylation assay - incubation of NAs with recombinant human CYP450 enzymes in the presence of adjusted concentrations calf thymus DNA	
Investigate the repair of NA induced DNA adducts in DNA repair deficient human, murine and hamster cells in a concentration- and time-dependent manner	**Cell viability assay** to determine cellular sensitivity using different assays such as the CellTiter-Glo Luminiscent Cell Viability Assay" or the resazurine reduction test (also called Alamar Blue assay)	Cell viability at a given NA concentration normalized to the solvent control, i.e., cell viability [% of control]. The data will be depicted as mean + standard error of the mean and will be analyzed by Student's t test or One-way ANOVA. EC_{50} and EC_{20} will be provided.
	Alkaline Comet assay to analyze DNA strand breaks and alkali-labile sites	The output is the tail intensity [%] as a measure of DNA damage at a given NA concentration. The data will be depicted as mean + standard error of the mean and will be analyzed by Student's t test or One-way ANOVA. Furthermore, the data sets will be subjected to benchmark dose (BMD) modeling by PROAST or BMD-Express software.
	Time course experiments to investigate the **formation and repair of DNA adducts** (after 0, 4, 8, 12 and 24 h of incubation)	LC-MS analysis to identify relevant DNA repair pathways and/or repair kinetics as well as the so far unknown DNA adducts

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