Mutamind Analysis of Drug Substance Related Nitrosamines

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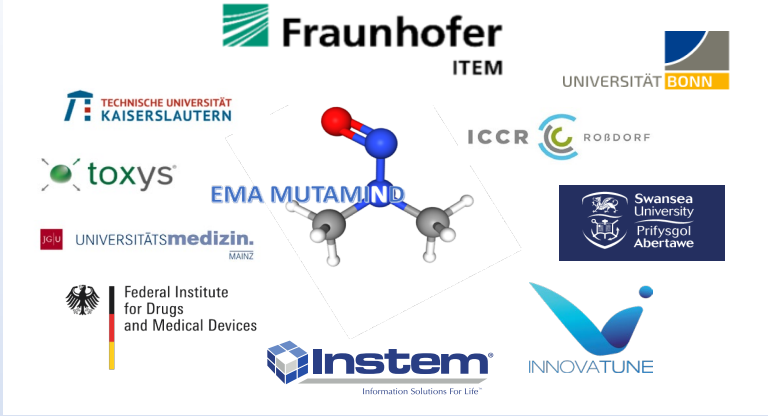
The Mutamind Team



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Focus of Mutamind

N-nitrosamines (NAs) are a topic of high concern for pharmaceuticals due to detection of NA impurities in certain drugs. **Three** separate EMA-sponsored Mutamind subprojects were set up to better understand the different processes involved in mutagenicity of NAs, with a special focus on bulky, drug-related compounds:

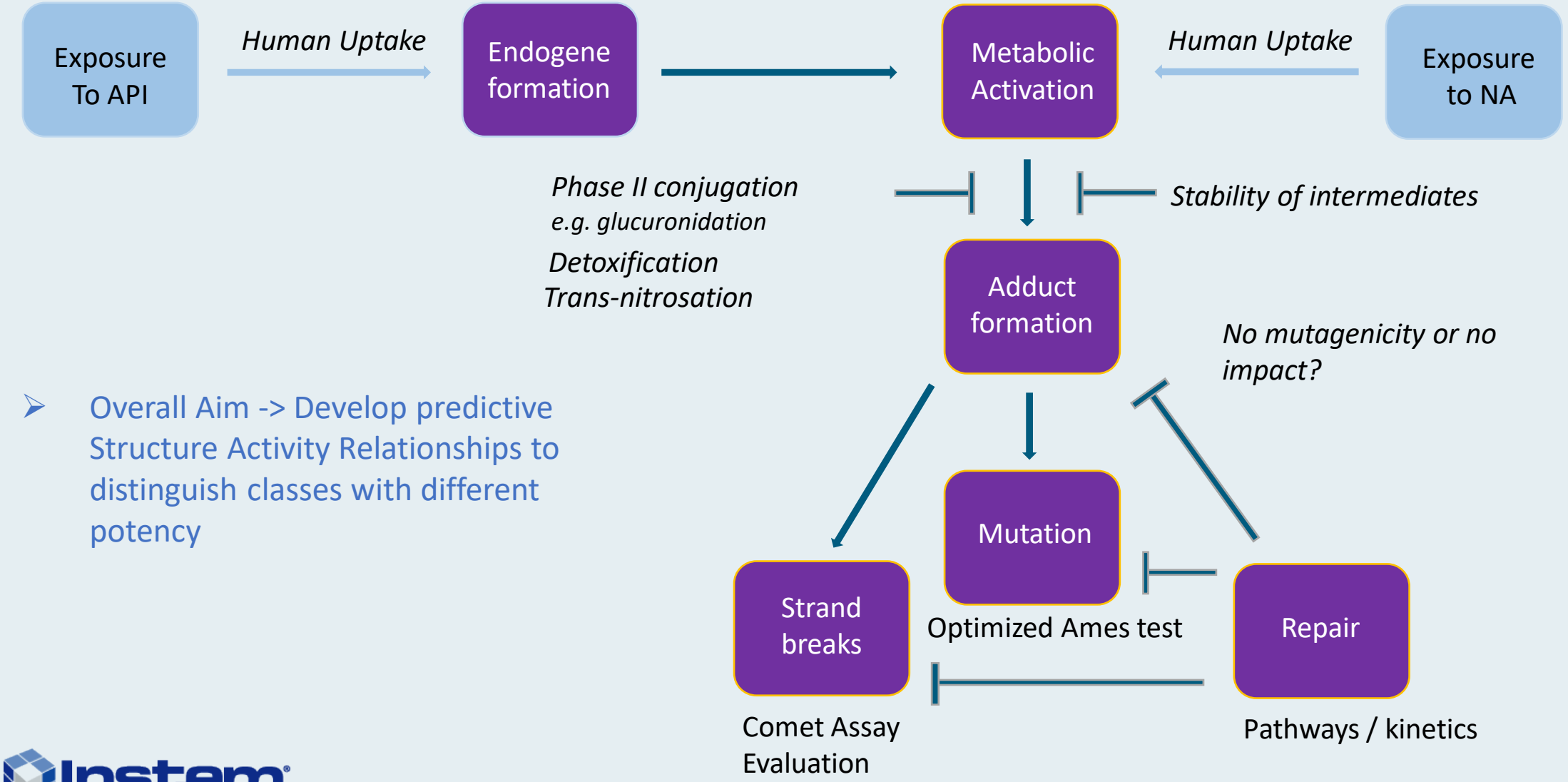
i. Formation of NAs from active pharmaceutical ingredients (APIs) under realistic conditions in different regions of the gastrointestinal tract (GIT)
ii. Metabolic activation of drug-derived NAs with identification of involved enzymes
iii. DNA adduct formation types, kinetics and repair
iv. Optimization of *in vitro* mutagenicity assays

Mutamind projects

- 40 NAs were selected for study in the following sub-projects:

1. Quantitative Structure Activity Relationships **(QSAR project)**
2. *In vitro* mutagenicity methodology for *N*-nitrosamines **(*in vitro* project)**
3. Endogenous formation of *N*-nitrosamines from a drug substance **(endogenous project)**

Mutamind project overview



- Overall Aim -> Develop predictive Structure Activity Relationships to distinguish classes with different potency

1. QSAR

Project 1: Quantitative Structure Activity Relationships (QSAR)

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Aim:

- Investigate metabolic activation, DNA adduct formation and DNA repair mechanisms to distinguish NA classes with distinct mutagenic potency.

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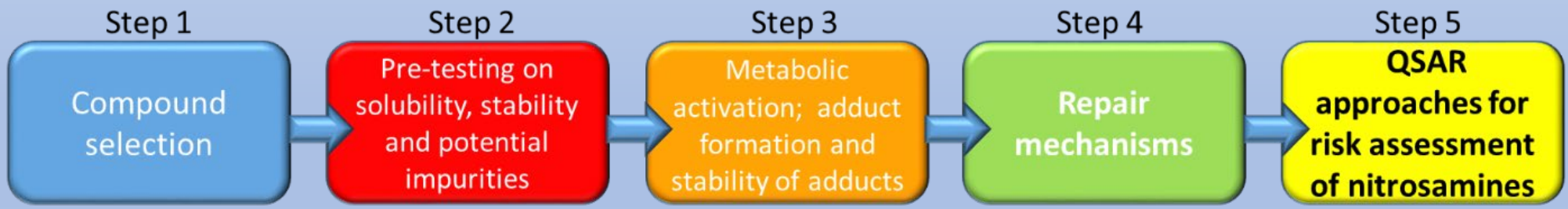
QSAR project study design and protocols

- Filling data gaps relevant for development of QSAR relationships
  - **Step 1** - **Compound selection:** Selection of 30 candidate Nitrosamine (NA) compounds according to predefined selection criteria.
  - **Step 2 - Pre-testing:** Pre-testing solubility and stability of test compounds to optimize conditions. Identification of impurities to filter out challenging compounds.
  - **Step 3- Analyze:** Identification of **metabolic activation** and **DNA adduct formation** and stability of metabolites and DNA adducts by screening 20 NAs -> Selection of 8 representative NAs, which differ regarding their DNA adduct profile.
  - **Step 4 - DNA Repair**: Measurement of **DNA repair mechanisms** for the 8 NAs identified in Step 3.
  - **Step 5- Safety Assessment:** Development of approaches to assess safety of NAs without robust *in vivo* carcinogenicity data.



The QSAR project protocol (QSAR for Nitrosamines EUPAS 46057) is available at https://www.encepp.eu/encepp/viewResource.htm?id=48784

1. QSAR

Main topics for investigation

- Determine metabolic ratio and activity based on the conversion rate of substrates with different CYP450 enzymes.
- Investigate trans-nitrosation of aliphatic NAs on glutathione; the stability of reactive intermediates and kinetics of hydroxylated NAs.
- *Investigate the DNA adduct profiles of 20 NAs via DNA alkylation assay – select 8 representative NAs for further investigation.*
- Analyze of DNA adduct formation and repair over time (in DNA repair deficient human, murine and hamster cells) to investigate important DNA repair pathways and kinetics such as identify new DNA adducts.
- Analyze DNA strand breaks and alkali- labile sites via Comet assay: benchmark dose (BMD) modeling

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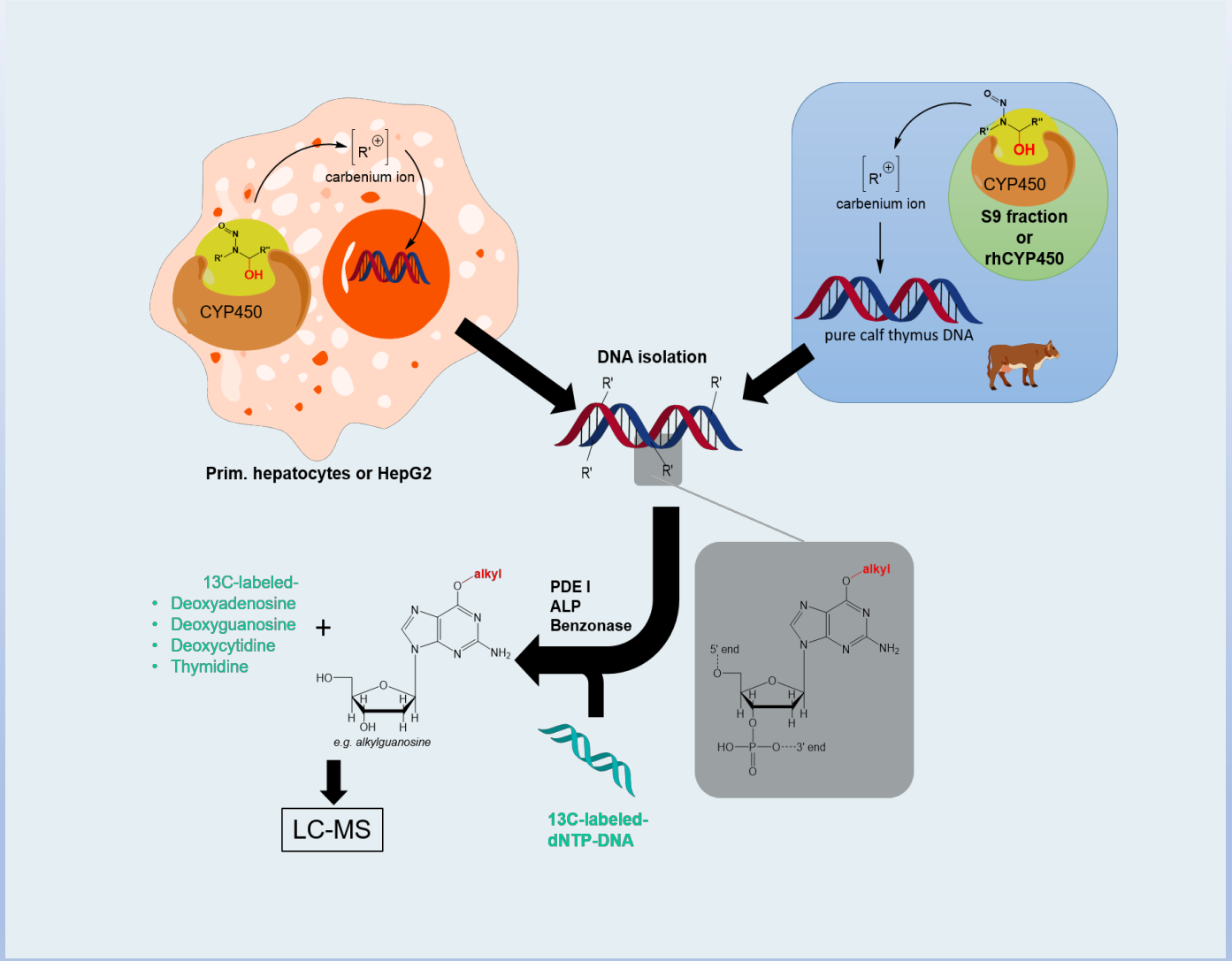
Overview of a DNA alkylation assay



Principle and workflow to determine alkylated nucleosides after metabolic activation of NAs by means of LC-MS

1. QSAR

Experimental design DNA-alkylation assay (overview)

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- 1. Incubation and assessment of metabolic confidence
  - Incubation of NAs with CYP450 competent cell cultures (HepG2, primary hepatocytes) under vital conditions
  - Incubation of NAs with induced rat and hamster liver S9 fractions in the presence of adjusted concentrations **calf thymus DNA**
  - Incubation of NAs with **human** and induced rat/hamster liver microsomes in the presence of adjusted concentrations **calf thymus DNA**
  - Incubation of NAs with recombinant **human** CYP450 enzymes in the presence of adjusted concentrations **calf thymus DNA**
- 2. DNA isolation
  - Lysis of equal cell numbers and/or direct extraction and purification of DNA; RNA removal by prior RNase; Qiagen DNeasy Blood & Tissue Kits
  - Adjustment of DNA to equal concentrations and preparation of equal volumes
- 3. Addition of stable isotopically labeled DNA as internal standard
  - 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

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2. In vitro

Project 2: *in vitro* mutagenicity methodology for *N*-nitrosamines

Aim:

- To optimize Ames test conditions for mutagenicity testing of NAs and to evaluate the *in vitro* alkaline Comet assay with liver cell models (HepG2 cells, primary rat and human hepatocytes) for detection of carcinogenic NAs.

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2. In vitro

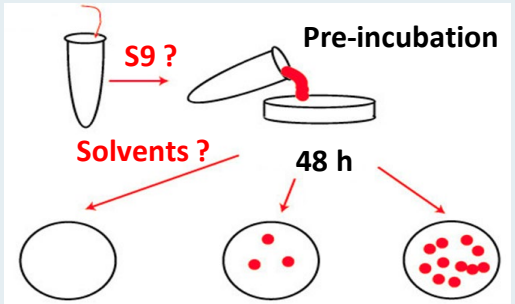
Main topics for investigation

- Literature search on Ames and Comet assay testing of NAs
- Stability, purity and solubility testing of selected NAs
- Detection of enzymes relevant for metabolism of selected NA classes with development of a detection method based upon adduct detection on calf thymus DNA by LC-MS/MS
- Metabolic competence of rat/hamster S9 fractions and *in vitro* liver cell models (determination of metabolic ratio and activity by conversion rate of specific substrates for relevant CYP450 enzymes and qPCR) with impact of solvents
- Optimizing Ames test conditions and testing of different NAs and API-related NAs
- *In vitro* alkaline Comet assay testing of 10 NAs/API-related NAs with 3 different liver cell models, complemented by a final round-robin study with HepG2 cells.

2. In vitro

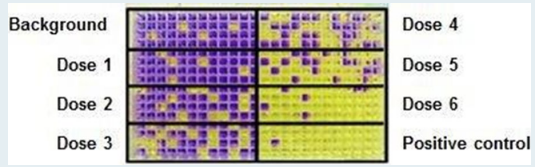
Methods evaluated for *in vitro* genotoxicity of NAs

Ames test

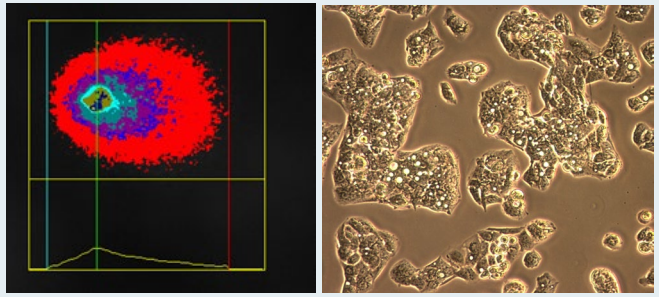


TA1535, TA100, EC WP2 *uvr*A pKM101

Ames fluctuation test



In vitro alkaline Comet assay



Data Integration, IATA, BMD

Liver cell models: HepG2, primary rat and human hepatocytes

2. In vitro

Ames NA testing protocol (finding optimal test conditions)

- Initial test conditions for first 10 NAs:
  - Test in multiple solvents at multiple concentrations (water, DMSO, methanol, acetone)
  - Pre-incubation using 10% (v/v) phenobarbital/ßnaphthoflavone-induced rat S9-mix for 60 min
  - Tester strains TA100, TA1535, E. coli WP2 *uvrA* pKM101
  - Test with S9-mix; if negative test without metabolic activation
  - Test with 10% rat S9-mix; if negative use 30% rat S9-mix, then 30% hamster S9-mix
- Determine the optimized Ames test protocol
- Second phase: test 15 NAs using the optimized test protocol
  - If negative using 2 different solvents, test under different S9-mix and pre-incubation and plate incorporation conditions, and a third solvent if not water soluble
  - Adjust optimized protocol if needed
- Final phase: test 13 additional NAs using the optimized protocol (if budget allows)
- Analyze carcinogenicity predictivity using the optimized Ames test protocol

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2. In vitro

In vitro alkaline Comet assay NA testing protocol

- Considerations for testing 10 NAs :
  - primary rat hepatocytes,
  - primary human hepatocytes,
  - HepG2 cells,
  - optionally human liver slices
- Plating and culture of hepatocytes
- Characterization of metabolic competence
- Cytotoxicity testing
- Genotoxicity testing
- Basic *in vitro* Comet assay protocol: performed according to Singh, McCoy, Tice, and Schneider (1988) with some modifications.
- *In vitro* alkaline Comet assay testing with 3 different liver cell models, complemented by a final round-robin study with HepG2 cells at 3 different sites.

The *in vitro* project protocol (In Vitro NA Mutagenicity EUPAS 49355) is available at:
https://www.encepp.eu/encepp/viewResource.htm?id=49356

Endogenous

Project 3: endogenous formation of *N*-nitrosamines from a drug substance

Aim:

- To investigate the impact of physiological conditions on the emergence of nitrosamines from API, in particular direct introduction of the nitroso group to the API molecule.

Endogenous

Approaches to investigating endogenous nitrosation

- *In vitro* incubation of APIs with artifical GIT juices.
  - varying NO_2- concentrations and pH values analysed at increasing levels.
- What is the impact of aerobic and anaerobic microbiomal strains in the GIT to trigger nitrosation of API? Cultivating experiments in the presence of API (NO_3-/NO_2-) with representative microbiomal strains like *Helicobacter pylori*, *E. coli* and artificial intestinal flora will be carried out.
- Time dependent screenings with equal concentrations of APIs to assess susceptibility of respective secondary amines adjacent to different alkyl or aromatic moieties.
- Each of these experiments is followed by LC-MS/MS measurements.

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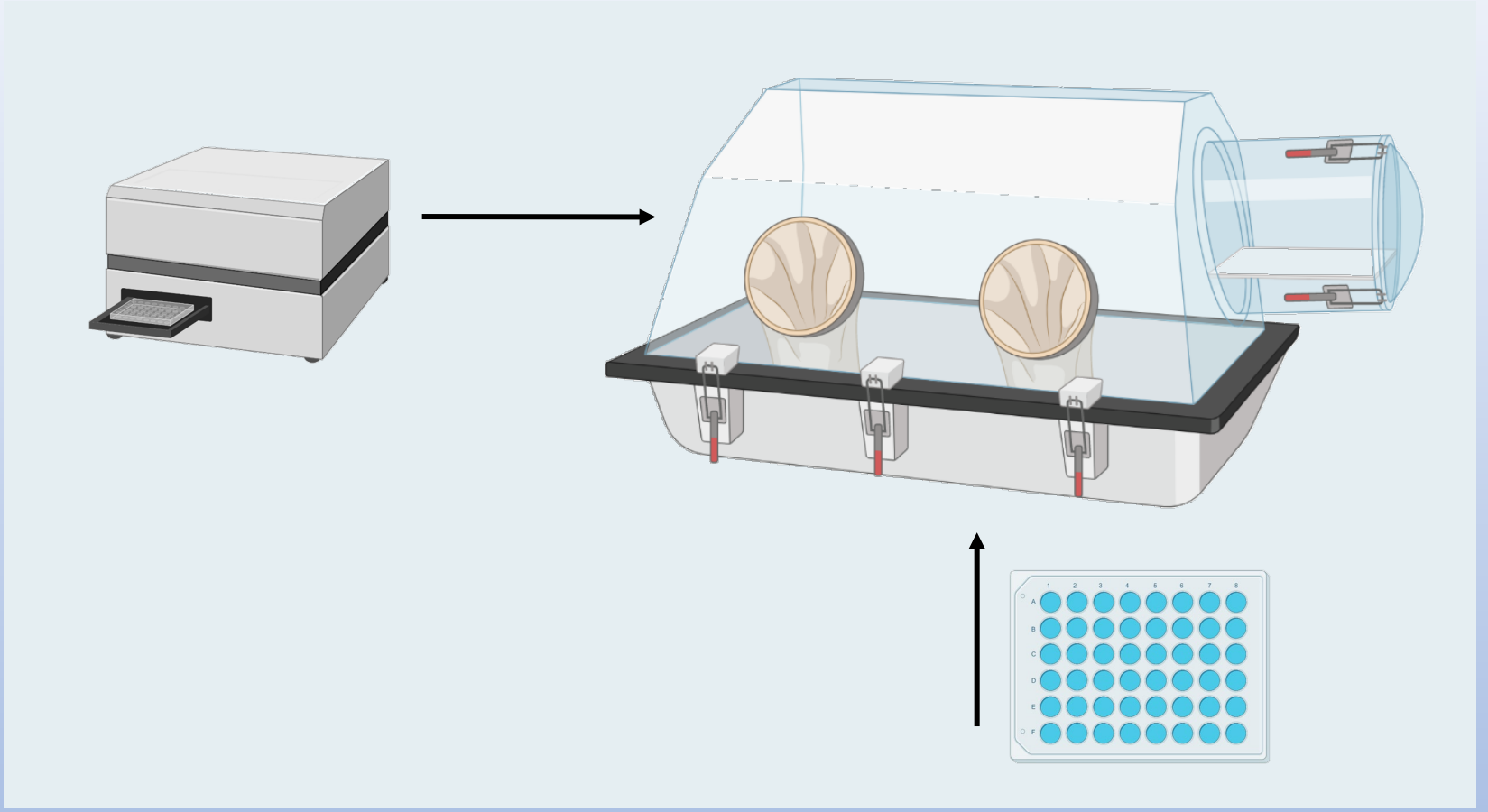
Endogenous

Equipment for the analysis of the gut microbiota



Bacteria cultivated anaerobically in an appropriate medium with selected API. Cultivation is performed in 48-well plates at 37 °C in a Microplate Reader, which allows to monitor cell growth. For cultivation under anoxic conditions, the plate reader setup is introduced into an anoxic chamber and maintained in a CO_2 / N_2 / H_2 atmosphere. Samples are taken and analyzed for nitrosation of API (M. Hövels, University of Bonn created with "Biorender")

The endogenous formation project protocol (GITox EUPAS 49089) is available at:
https://www.encepp.eu/encepp/viewResource.htm?id=49090

Mutamind status and future work

- Protocols for testing – completed for each project
- N-nitrosamine compounds
  - Purchasing and acquistion complete
  - Purity, solubility, and stability *in-process*
  - S9-mix characterization complete
- Data gap analysis (including local similarity analysis) – *complete* (see GTA 2023 poster)
- Investigation of metabolic activation, DNA adducts and repair - *in-process*
- Ames and *in vitro* Comet testing – phase 1 *in-process*
- Study of endogenous nitrosation of APIs - *in-process*
- Data analysis and QSAR approaches to Risk Assessment – *final step*
- Project completion – *end of 2023*

Thank you for your attention!

Any questions?

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