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Project acronym: ONTOX

Project title: ontology-driven and artificial intelligence-based repeated dose toxicity testing of chemicals for next generation risk assessment

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Deliverable number:	D8.1/D26
Deliverable title:	Report on existing *in vitro* assays to detect MIEs and KEs in the qAOP networks on tubular necrosis and crystallopathy
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Contents

1. Introduction and objectives

Two case studies using state-of-the-art kidney-based *in vitro* systems have been addressed: tubular necrosis and crystallopathy. Kidney tubular necrosis is the most common type of intrinsic kidney injury and is predominantly caused by ischemia or direct toxic injury (Barnett & Cummings, 2018). Urinary tract obstruction, such as crystallopathy, leads to postrenal kidney injury by impairing renal blood flow and inducing inflammatory processes. But supersaturation of solutes within tubular cells can lead to crystallopathy as well (Cohen, 2018). The objective of this deliverable was to develop and evaluate *in vitro* assays to detect newly identified MIEs and KEs for tubular necrosis and crystallopathy following the establishment of the ontologies. These *in vitro* assays will be evaluated following the OECD guidelines for validation of *in vitro* test methods (*OECD* 2005) with focus on reproducibility, chemical applicability domain and pathological relevance as well as biological coverage.

The first system used for tubular injury modelling, includes 2-dimensional (2D) and 3D cultures of human renal proximal tubule cells as they have shown similarities to the kidney physiology in vivo, retaining their phenotype, morphology, viability and kidney tubule-specific functions including expression of relevant uptake and efflux transporters and have demonstrated suitability in PBPK modelling (Jansen et al. 2016; Van der Made et al. 2019).

Kidney tubular crystallopathy was set up *in vitro* utilizing uric acid which has a low solubility and its crystallization in the kidney can cause an inflammatory response that is associated with the initiation and progression of chronic kidney disease and end-stage renal failure. Crystal formation is dependent on pH, which was evaluated in conditionally immortalized primary tubular epithelial cells (ciPTECs) by studying the interchangeable effects of prolonged exposure to uric acid and medium pH on the inflammasome activation and downstream inflammatory processes.

2. Results

2.1 *In vitro* assays for nephrotoxicity screening

A battery of *in vitro* assays has been set up, each focusing on a MIE or KE within the spectrum of mechanisms depicted in the qAOP networks on acute tubular necrosis and crystallopathy. An overview of the assays is presented in Table 1.

Table 1: Overview of in vitro assays for both case studies leading to kidney failure.

Case study	**Key event**	**Assay**	**Endpoint**	**References**
Both	Cytotoxicity	Prestoblue	Live-cell metabolism and viability	(Maass et al., 2019)
Both	Cytotoxicity	Lactate dehydrogenase UV absorbance/colorimetric assay	LDH leakage	(Vormann et al., 2021)
Both	Mitochondrial dysfunction	JC10 Mitochondrial Membrane Potential Assay Kit	Fluorescent JC-10 = Red (healthy), green (dead)	(Mossoba et al., 2017)
Both	Oxidative stress	2',7'-dichlorodihydrofluorescein-diacetate fluorescence assay	Fluorescent DCF in supernatant	(Pujalte et al., 2011)
Both	Cell death	Annexin V-FITC / PI staining	Fluorescent detection of apoptotic/necrotic cells	(Yuan et al., 2021)
Both	Inflammation	ELISA	TNFα, IL-6, IL-8, IL-1β	(Lu et al., 2021)
Crystall -opathy	Crystal formation	Microscopy	Visual image of crystalline formation	(Liu et al., 2019)
Crystall opathy	Inflammasome activation	qPCR	NLRP3 mRNA and protein expression	(Chun et al., 2016)

Principles of the assays with read-outs that are compatible with high-throughput and, eventually, automatic processing leading to robust and reproducible results according to the OECD guidelines for validation of *in vitro* test methods (*OECD* 2005) are given below.

2.2. Assay Overview

- **Prestoblue**

Fluorescent detection of cell viability using a resazurin-based solution.

Product overview: https://www.thermofisher.com/order/catalog/product/A13261

- **LDH**

Bioluminescent depiction of LDH activity in the supernatant, representing its release from damaged cells.
Product overview: https://www.thermofisher.com/order/catalog/product/C20300

- **JC10**

Fluorescent detection of mitochondrial membrane potential changes in cells by the cationic, lipophilic JC-10 dye.
Product overview: https://www.aatbio.com/products/cell-meter-jc-10-mitochondrion-membrane-potential-assay-kit-optimized-for-microplate-assays#jump_document

- **ROS**

Quantification of fluorescence of dihydroethidium or fluorescein derivatives.
Product overview: https://www.thermofisher.com/order/catalog/product/C6827

- **Annexin V FITC / PI staining**

Quantification of fluorescently detected apoptotic (annexin V-FITC) and necrotic cell populations (PI).
Product overview: https://www.thermofisher.com/order/catalog/product/88-8005-74

- **ELISA**

Detection of cytokine concentration in the supernatant, to determine the release of proinflammatory markers released from damaged cells. Product overview:
TNFα
https://www.thermofisher.com/elisa/product/TNF-alpha-Human-Uncoated-ELISA-Kit/88-7346-88
IL-6
https://www.thermofisher.com/elisa/product/IL-6-Human-Uncoated-ELISA-Kit/88-7066-88
IL-8
https://www.thermofisher.com/elisa/product/IL-8-Human-Uncoated-ELISA-Kit/88-8086-88
IL-1β
https://www.thermofisher.com/elisa/product/IL-1-beta-Human-Uncoated-ELISA-Kit/88-7261-88

- **Crystal formation**

Quantification and characterisation of formed crystalline structures using brightfield microscopy and image analysis software.

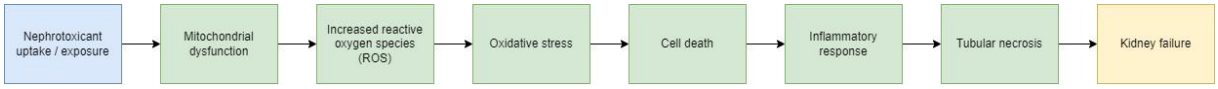
- **Inflammasome activation**

Detection and quantification of mRNA and protein expression of the NLRP3 inflammasome and additional constituents

2.3 AOP development

For the two case studies, linear AOPs have been drafted summarizing the MIE and KE's leading to kidney failure.

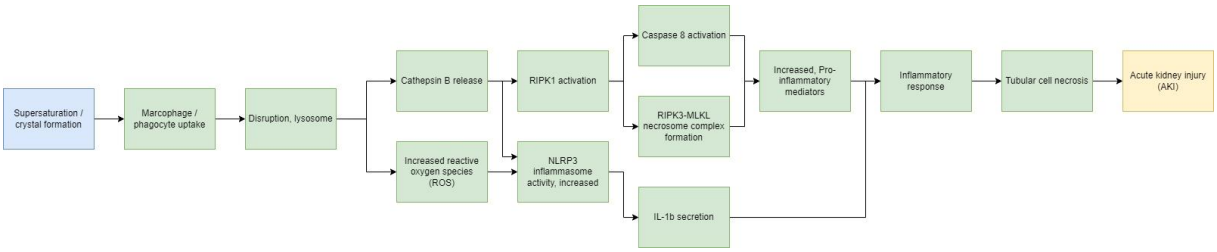
2.3.1 Tubular Necrosis



Key event overview:

- Mitochondrial dysfunction
- Increased reactive oxygen species (ROS)
- Oxidative stress
- Cell death
- Inflammation
- Tubular necrosis

2.3.2 Crystallopathy AOP



Key event overview:

- Crystal formation
- Increased reactive oxygen species (ROS)
- RIPK1 activation
- NLRP3 inflammasome activity
- Cell death
- IL-1β secretion
- Inflammation
- Tubular cell necrosis

3. Conclusions and follow-up

The *in vitro* systems for the two case studies are in place and assays suitable to monitor MIEs and KEs have been selected and are being validated. SOPs for assays are under development and will be finalized and harmonized with W7 and WP9.

Follow-up studies will include full validation of the *in vitro* assays using selected chemicals, positive and negative controls.

4. Delays, issues and contingency

Not applicable.

5. References

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