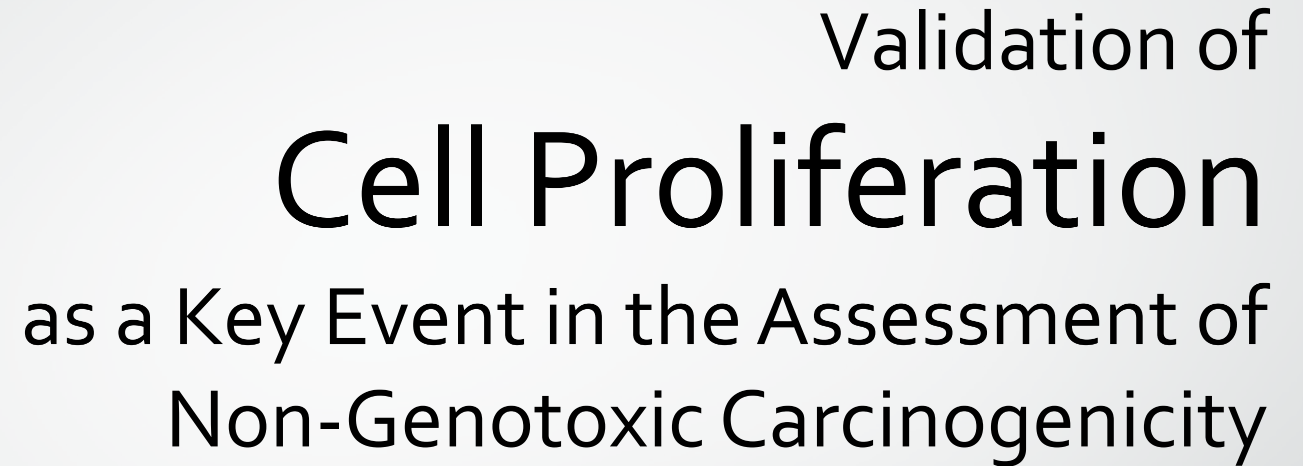

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Section 1: Method Description

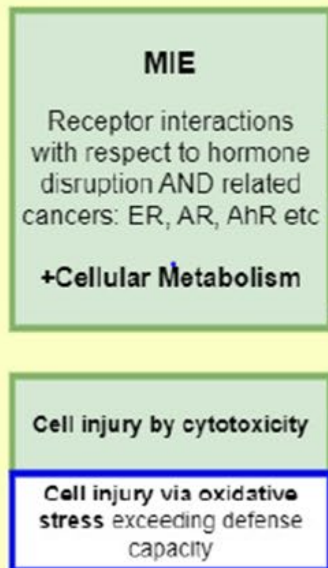
Method Description

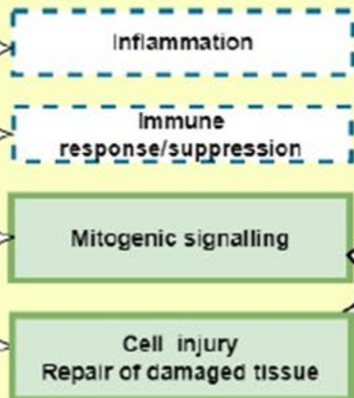
- Carcinogens can either be direct genotoxicants (mutagens or clastogens) or lead to tumor formation via non-genotoxic modes of action
- While molecular initiating events can be different, most non-genotoxic carcinogens would induce increased rates of cell proliferation which in turn increases the chance for mutation (initiated cells), as DNA replication is imperfect
- OECD has a Working Group on Non-Genotoxic Carcinogen Hazard Assessment, for the Integrated Approach to Testing and Assessment (IATA), cell proliferation is a major key event

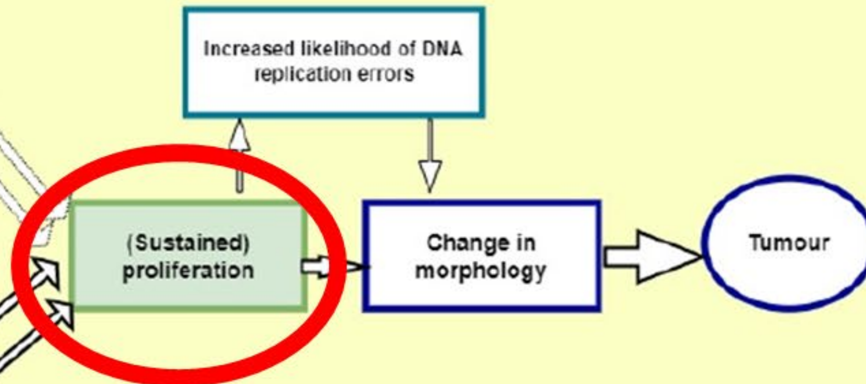
Genotoxicity negative

Existing (Regulatory) data:

- Endocrine activity screening,
- Systemic toxicity (toxicological target)
- and carc. literature
- omics databases
- Structural and read across information
- QSARs and expert systems







- In silico:
- SARs/QSARs/docking studies/expert systems
- TK data, e.g. Metapath
- Generic PBK modelling
- 28 day *in vivo*
- 90 day *in vivo* +range finding studies

Phase I: CYP induction
in vitro HepaRG CYP induction test method and/or primary rodent hepatcytes; *in vivo* assay
Phase II: other enzymes
Oxidative stress biomarkers:
Toxtracker test method

Cell proliferation specific test methods

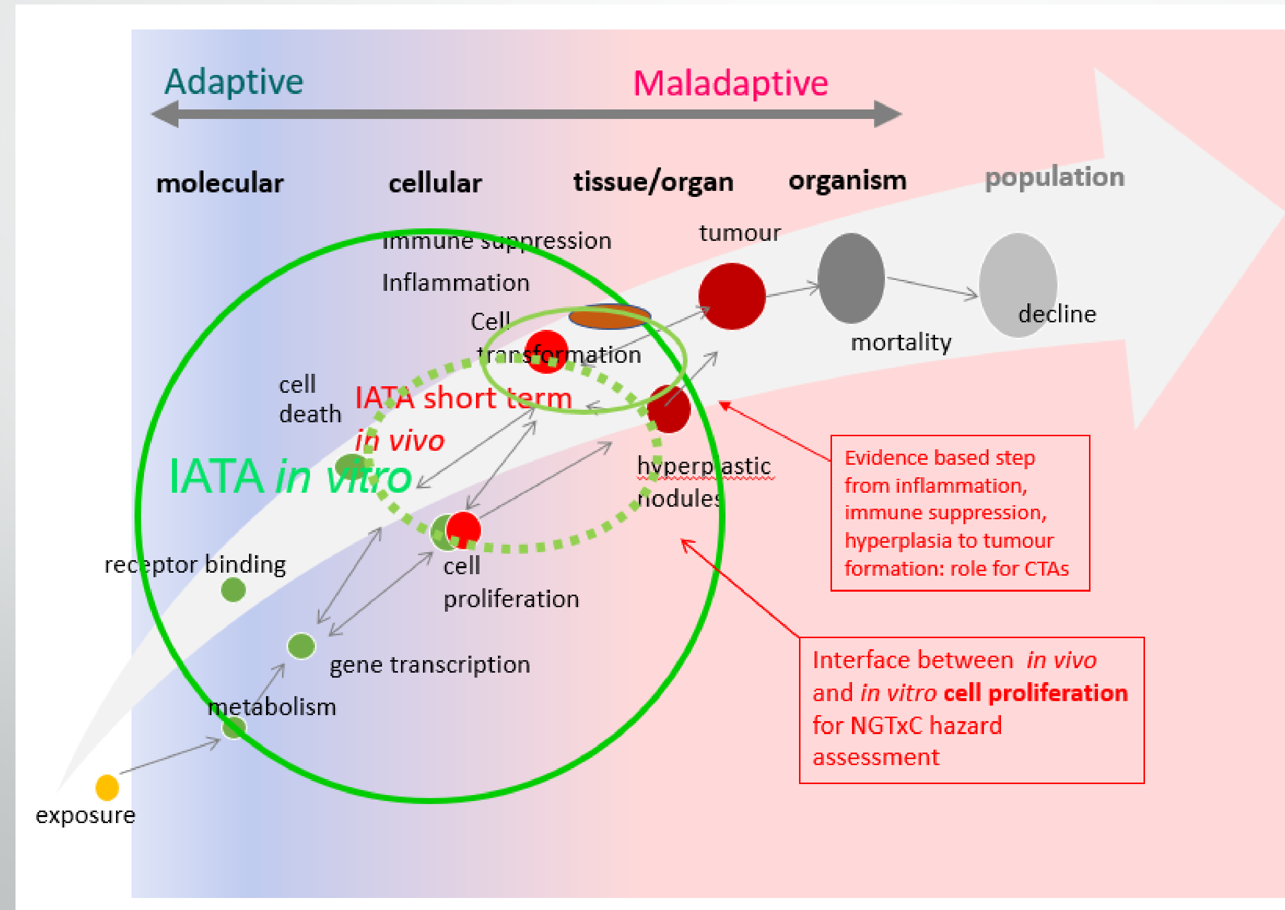
- Identified target tissue and cells thereof, i.e. liver, lung, GIT
- Utilised recommended histology methodology
- Ki67; BrdU
- Transience: kinetics and dynamics on 1st then 2nd organ
- Timing: 1-2; 3-4; 5-15 days

(Existing) Cell proliferation *in vivo* (28 and 90 day TGs) Ki67, BrdU

Exposure — Sustained exposure

Strupp et al., Int. J. Mol. Sci. 2023, 24, 13246

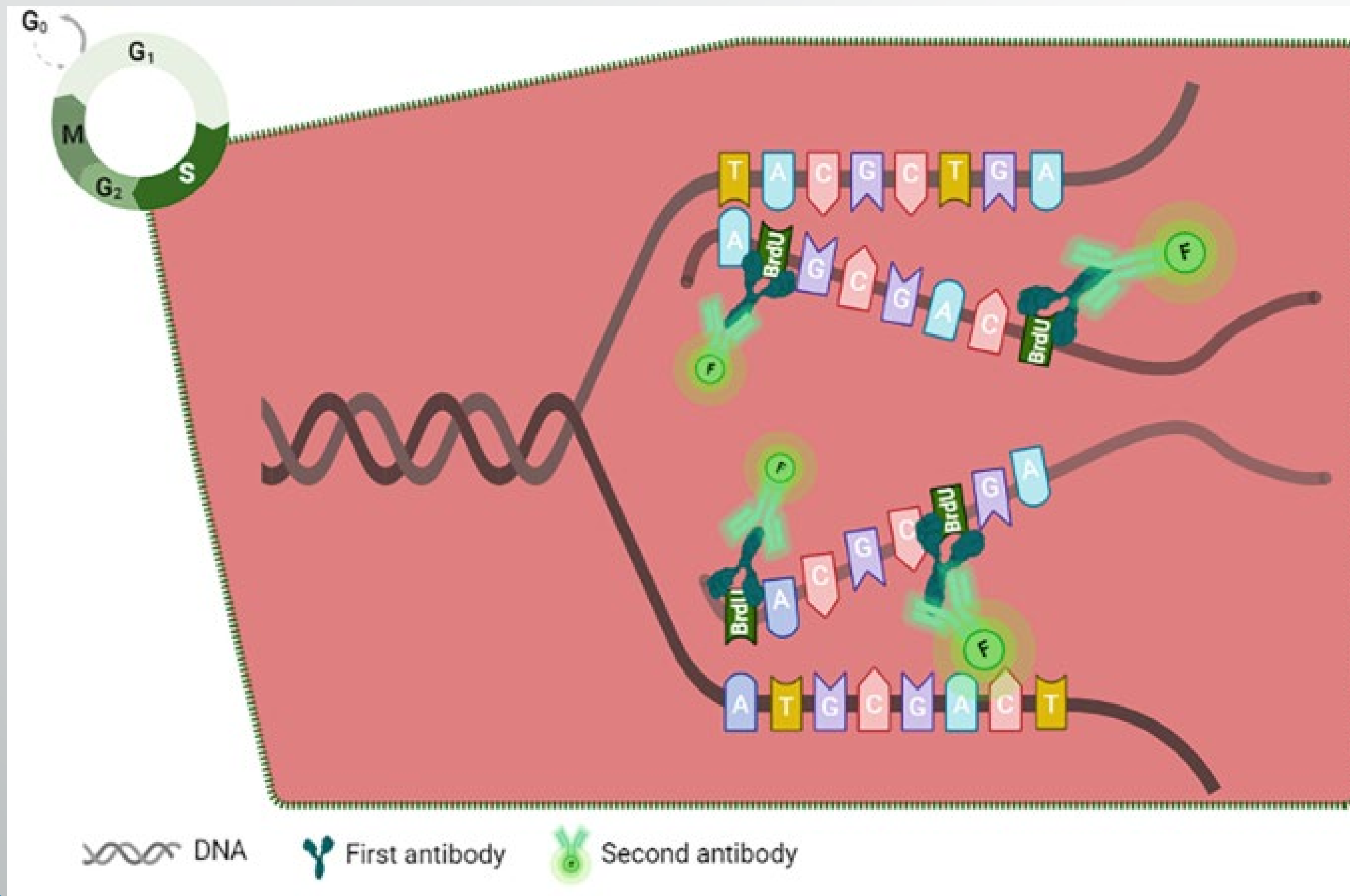
Transition from adaptive to maladaptive, critical role of cell proliferation KE and subsequent KE of cell transformation



Method Description

- Cell proliferation is assessed both *in vitro* and *in vivo* since the 1980s , but regulatory endorsement varies substantially as methods are not validated
- Key markers of proliferation have been established: BrdU incorporation, Ki-67 expression and genomics markers of proliferation
  - BrdU/EDU: an artificial nucleotide is built into newly synthesized DNA, and detected by immunohistochemistry/fluorescence on histopathology slides or cell cultures
  - Ki-67: a cell cycle marker which is upregulated for 4-6 hrs as long as proliferative stimulus is persisting (PCR or immunohistochemistry)
  - Genomics markers: a set of proliferation-linked genes that is upregulated as long as the proliferative stimulus persists

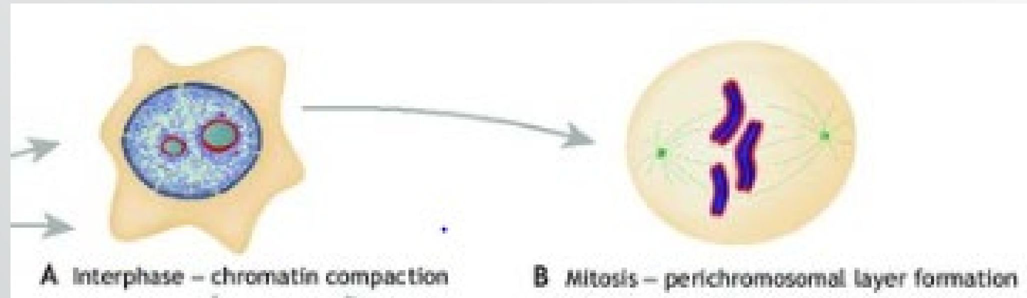
Method Description: BrdU (or EdU)



From: Yu, J., Wang, Z., Wang, Y. (2022). BrdU Incorporation Assay to Analyze the Entry into S Phase. In: Wang, Z. (eds) Cell-Cycle Synchronization. Methods in Molecular Biology, vol 2579. Humana, New York, NY. https://doi.org/10.1007/978-1-0716-2736-5_16

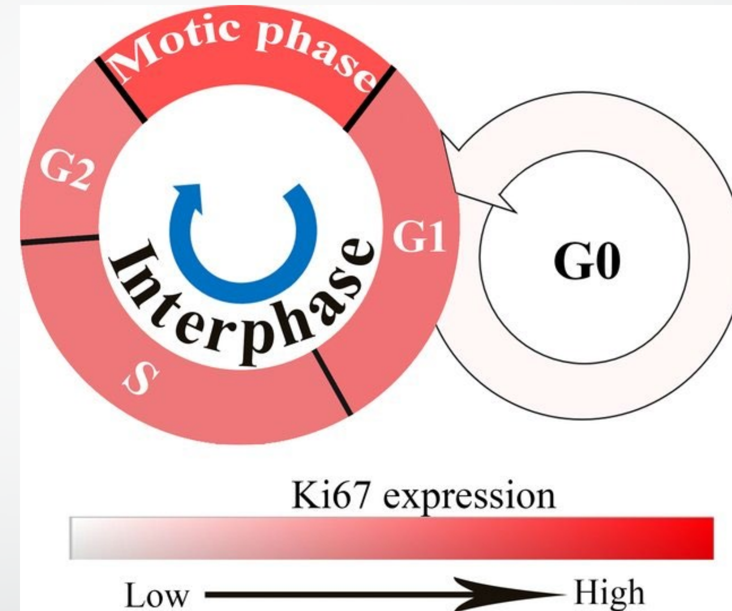
EdU: Soldatow V et al. Development of an in vitro high content imaging assay for quantitative assessment of CAR-dependent mouse, rat, and human primary hepatocyte proliferation. Toxicol In Vitro. 2016

Method Description: Ki-67



From: Andrez-Sanchez et al. J Cell Sci (2022) 135 (11): jcs258932.

Ki-67 protein is a cellular marker strictly associated with cell proliferation. It can be assessed at mRNA (PCR) and protein level (immunohistochemistry).



From: Yuan (2015) PLoS ONE 10(4): e0122734. https://doi.org/10.1371/journal.pone.0122734

Cellular content of Ki-67 protein markedly increases during cell progression through S phase of the cell cycle.

Method Description

- There are no limitations in applicability domain *in vivo* (all species, all organs)
- *In vitro* proliferation is also principally unlimited, however
  - primary cell cultures capable to proliferate are not yet standardly established for all organs and
  - solubility of the test item in cell culture media is the same potential limiting factor as for most in vitro methods
  - Important to focus on true proliferation, i.e. DNA de novo synthesis, rather than cell counts

Method Description

- It is proposed to formally validate these methods, both *in vivo* and *in vitro*, and propose a respective OECD test guideline

- OECD xxx A: Cell Proliferation Assessment (*In Vivo* Methods)
  - Substantial data on several prototypic activators is available
- OECD xxx B: Cell Proliferation Assessment (*In Vitro* Methods)
  - Substantial data for primary hepatocytes is available that may allow retrospective validation

- The same set of markers would be used in both methods
- The results would be integrated with other assay results into an IATA and a modular (defined) approach
  - References: Jacobs et al., Arch Toxicol. 2020 94(8):2899-2923; Louekari and Jacobs, Arch Toxicol. 2024, 98: 2463-2485

Section 2: Context of Use

Context of Use

- Genotoxicants can already be confidently identified *in vitro* and reliable methods are available
- The proposed method would serve a key role in the OECD IATA for assessment of non-genotoxic carcinogens (pharmaceuticals, medical devices, cosmetics, (agro)chemicals, food contaminants/-contact materials)
- Ultimate goal is to refine international regulatory carcinogen assessment, in particular to reduce the use of the rodent cancer bioassay as a vertebrate-rich study with low predictive power for human relevant non-genotoxic carcinogens
- It would allow both hazard conclusions and points of departure for risk assessment and is being submitted here for this purpose in the context of agrochemical regulatory submissions, and potentially can be applied for industrial chemicals
- The validation exercise is necessary for adoption as an OECD Test Guideline

Section 3: Biological Relevance

Biological Relevance

A. Mechanistic understanding

- Adverse Outcome Pathways for non-genotoxic carcinogens have been postulated since the 1950s (Armitage at al., Br. J. Cancer 1954, 8, 1–12), but key work in the 1970s and 1980s was needed to get to understand the relationship (Knudson et al., Proc. Natl. Acad. Sci. USA 1971, 68, 820–823; Moolgavkar, J. Natl. Cancer Inst. 1981, 66, 1037–1052)
- The concept was properly described in the 1980s/1990s (Greenfield et al., Carcinogenesis 1984, 5, 437–445; Cohen et al., Science 1990, 249, 1007–1011)
- Latest review: Cohen 2024, Front. Oncol. 14:1394584

Biological Relevance

A. Mechanistic understanding

- Uptake into regulatory domain: US EPA Guideline for Carcinogen Risk Assessment from 2005 (EPA/630/P-03/001F), and Scientific and Regulatory Policy Committee in 2015 (Wolf et al., Toxicol. Pathol. 2015, 43, 760–775)
- Recently reviewed in context of an IATA:

Review

Increased Cell Proliferation as a Key Event in Chemical Carcinogenesis: Application in an Integrated Approach for the Testing and Assessment of Non-Genotoxic Carcinogenesis

Christian Strupp [1,*], Marco Corvaro [2], Samuel M. Cohen [3], J. Christopher Corton [4], Kumiko Ogawa [5], Lysiane Richert [6] and Miriam N. Jacobs [7]

- And integrated into a modular approach:

Archives of Toxicology (2024) 98:2463–2485
https://doi.org/10.1007/s00204-024-03753-y

REGULATORY TOXICOLOGY

A modular strategy for the testing and assessment of non-genotoxic carcinogens

Kimmo Louekari[1] · Miriam N. Jacobs[2]

Int. J. Mol. Sci. 2023, 24, 13246; Arch Toxicol. 2024, 98: 2463-2485

Biological Relevance

A. Mechanistic understanding

- Notes:
  - Cell proliferation will need to be integrated into an IATA – not any proliferation is an immediate alert for carcinogenicity
  - Some mechanisms are species-specific, and comparative cell proliferation from different species (including human *in vitro*) can help establish an understanding if and where adverse outcome pathways may diverge
  - What counts is the total number of proliferations, not an increase in rate at a specific time
  - It is critical to focus on a specific target cell type
  - Can be used to establish thresholds preventive for non-genotoxic carcinogenicity

Biological Relevance

B. Reference compounds

- Will need a balanced set of positive and negative compounds
- Ample data is already available on different target organs and modes of action; few prototypical listed here
- A thorough review and analysis of available laboratory data will be conducted at the outset of the project
  - Published as well as in-house data from all collaborators involved will be available

Prototypical Activator	Target Organ	Mechanism
Phenobarbital	Liver, thyroid	Nuclear receptor binding (CAR), metabolic phase II induction, thyroid (T)-hormone clearance, and constant feedback stimulation
Thiazopyr	Thyroid	Metabolic phase II induction, T-hormone clearance, and constant feedback stimulation
Clofibrate, Wyeth (WY)-14,643	Liver, pancreas, testis	Nuclear receptor binding (PPARα), metabolic phase II induction, T-hormone clearance, and constant feedback stimulation
Chloroform, methapyrilene	Liver, kidney	Cytotoxicity/repair
Omeprazol, chlorothalonil	Stomach, neuroendocrine	Gastrin-induced mitogenesis
Folpet, chromium	Duodenum	Cytotoxicity/repair
D-Limonene, nitrapyrin	Kidney	Alpha 2u-globulin
Sodium saccharin, ascorbate	Bladder	Crystal formation and chronic local irritation
Estrogen	Mammary gland	Constant mitogenicity
Cyclosporin A	Lymphoma, squamous cell carcinoma	Immunosuppression
Isoniazid	Lung	Mitogen
Fluensulfone, Styrene	Lung	Metabolic induction and resulting damage in Club cells

Biological Relevance

B. Reference compounds Reviewed in the Public Literature

(several in each mentioned group)

- Liver Nuclear Receptor Agonists:
  - CAR/PXR (reviewed by Yamada et al., Crit. Rev. Toxicol. 2021, 51, 373–394)
  - PPAR-alpha agonists (reviewed by Corton et al., Crit. Rev. Toxicol. 2014, 44, 1–49 and Arch Toxicol. 2018, 92:83–119; Ozcagli et al., 2024, Front. Endocrinol. 15:1401120)
  - AhR (Becker et al., Reg. Toxicol. Pharmacol. 2015, 73, 172-190)
  - General (reviewed by Peffer et al., Regul. Toxicol. Pharmacol. 2018, 96, 106–120, Jacobs et al.,Front Toxicol. 2022, 4:880818)
- Bladder local irritants (reviewed Suzuki et al, Toxicol. Sci. 2008, 106, 350–363)
- Duodenal cytotoxicants (reviewed by Thompson et al., Toxicol. Pathol. 2017, 45, 1091–1101)
- Mouse lung cytotoxicants (reviewed byCruzan et al., Regul. Toxicol. Pharmacol. 2009, 55, 205–218 ; Strupp et al., Toxicol. Sci. 2016, 154, 296–308)

Biological Relevance

C. Comparison to existing laboratory animal methods

- *In vivo* methods allow to detect a potential for tumor formation in much shorter studies using less animals at a lower degree of severity than traditional rodent cancer bioassays
- They allow a fine titration of a point of departure for risk assessment (for example using benchmark dose analysis at the time of peak effect)
- *In vitro* methods are available for several organs and can close the gap to humans by using human tissue (human relevance)

Section 4: Technical Characterization

Technical Characterization

A. How have the sources of variability (e.g., interference, culture conditions, technique, contaminants) been evaluated?

- Main factors contributing to variability – *in vivo*
  - Establishing the time of peak effect
  - Only counting the target cell (clear differentiation from other cell types in the same organ)
  - Target tissues: tissues with quick baseline turnover or less
  - BrdU: systemic delivery (clogging of osmotic minipumps)
- Main factors – *in vitro*
  - Culture conditions (media and extracellular matrix for hepatocytes)
  - Fate of the cells (medical history, operation, cryopreservation)
  - Duration of exposure (quick or slow baseline proliferation rate)
  - Purity of the primary cell culture (contamination with quickly proliferating cell types)

Technical Characterization

B. Robustness, intra-lab reproducibility and inter lab transferability

- Ample repeats of studies with prototypic inducers of proliferation
- Even for cryopreserved primary human cells, reproducible response within and across laboratories and several prototypic inducers of cell proliferation (manuscript in preparation)
- Several laboratories competent in the assay or interested planning to exchange on and develop a standard operating procedure

Closing/Contact

- Methods established and heavily used since the 1990s
- Currently a loose network of interested stakeholders arising from OECD expert group, e.g. UKHSA, agrochemical industry, and CROs: Concept Life Sciences, KaLy-Cell and others.
- Formal validation may be organized by a subgroup of the OECD expert group on Non-Genotoxic Carcinogens
- **Funding** needed for collation and **independent statistical evaluation** of in-house and literature data and **subsequent reliable validation** based on ring trials
- If pharmaceutical companies and CROs would contribute their data, much could be potentially validated retrospectively, therefore two phases proposed:
  1. Preparatory evidence collation, chemical selection and design of validation
  2. Validation: ring trial leading to an OECD Test Guideline for inclusion in the IATA

Miriam Jacobs, Christian Strupp (cstrupp@gowanco.com)

Backup

A modular assessment framework for the OECD NGTxC IATA