The Comet Assay
Tails of the (Un)expected
Use of the in vivo comet assay in pharmaceutical development

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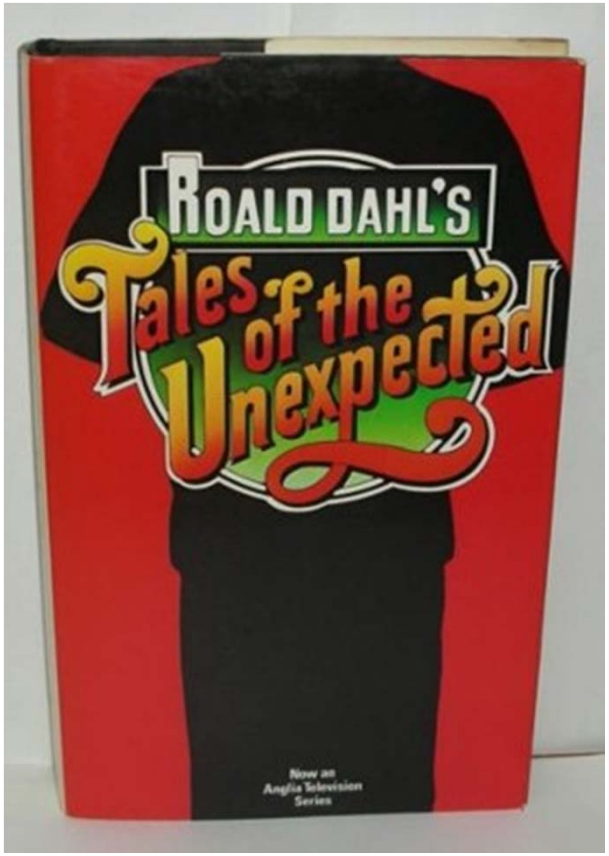
Janssen R&D – Johnson & Johnson Pharmaceutical Companies

Discovery Sciences – Genetic Toxicology

ICAW 2015 – BEMS satellite meeting – 1 sep 2015

Content

- Pharmaceutical development
- Guidelines
- Janssen R&D Genotox strategy
- Study designs aspects
- JaCVAM validation
- EFPIA survey
- Conclusions



Company info

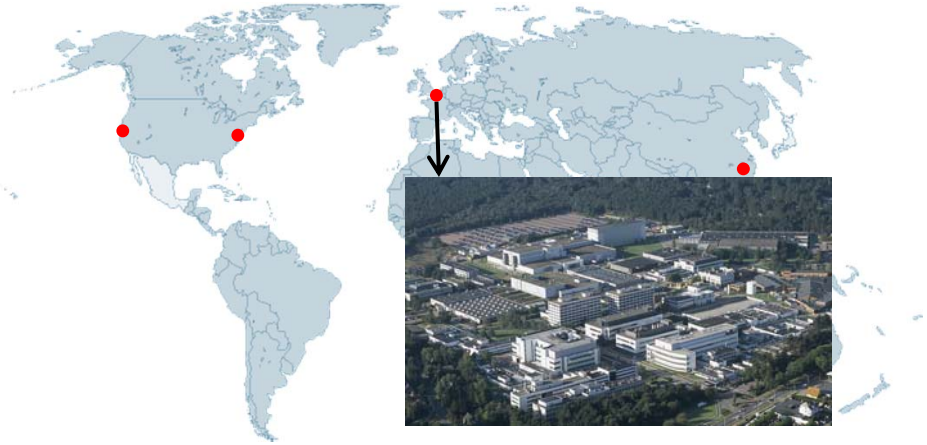
Johnson & Johnson

Pharmaceuticals | Consumer Health | Medical Devices & Diagnostics

janssen PHARMACEUTICAL COMPANIES OF Johnson & Johnson

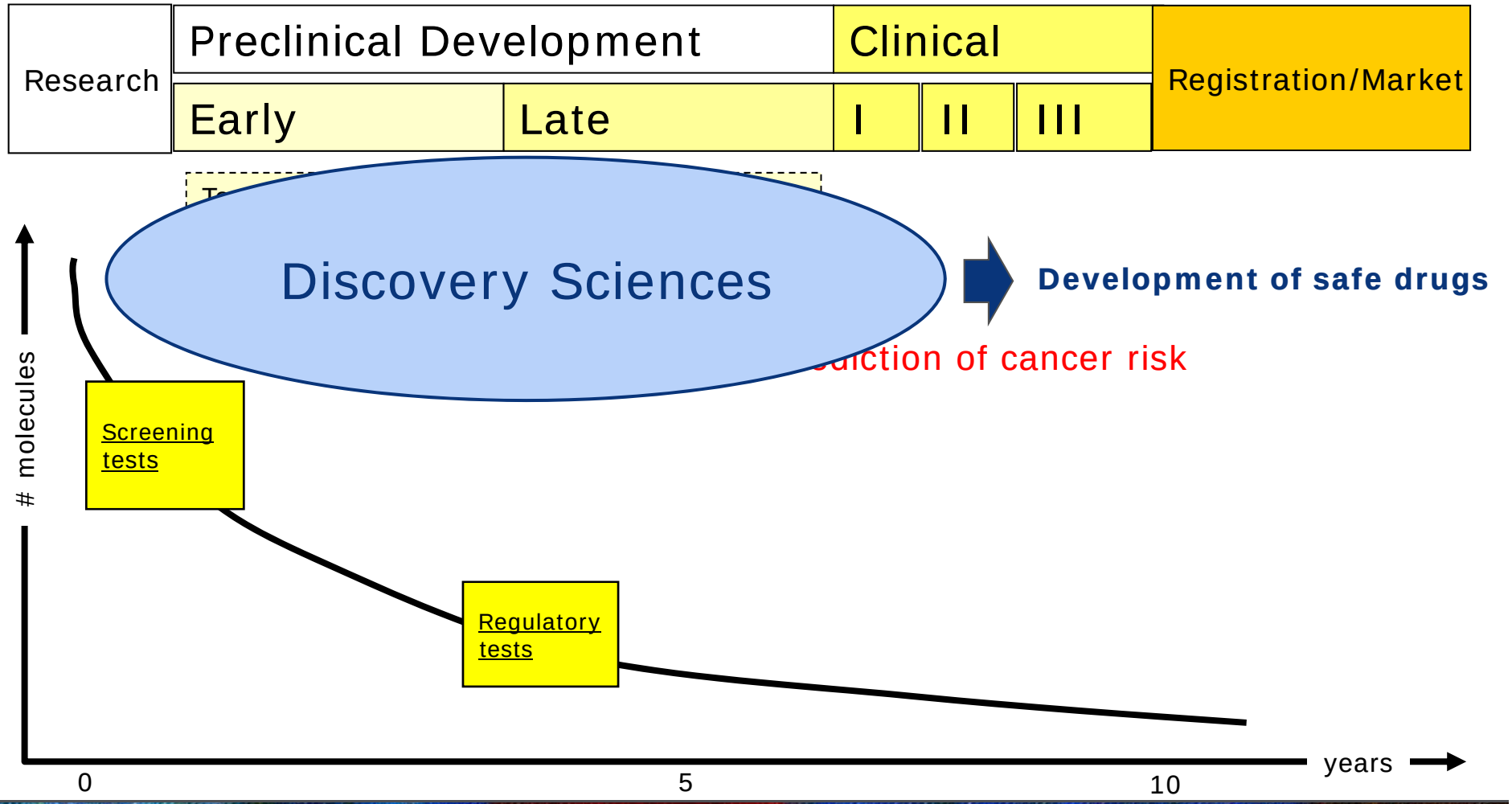
Small molecules | Large molecules | Vaccines

Discovery Sciences

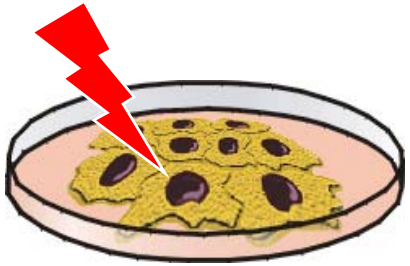


Genetic Toxicology
Beerse (BELGIUM)

Development of a new drug …



Genotoxicity Test Systems



DNA reactive

Non-DNA reactive

- Comet assay ← *DNA-damage*

Spindle figure

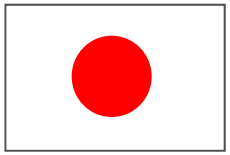
DNA replication

Gene Mutations	Structural Chromosome Mutations	Numerical Chromosome Mutations
• AMES Test	• Mouse Lymphoma Test (MLT)	• Micronucleus Test (MNT)
• Mouse Lymphoma Test (MLT)	• Micronucleus Test (MNT)	
• Pig-a Mutation Assay	• Chromosome Aberration Test (CAT)	

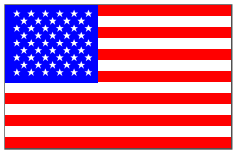
The International Conference on Harmonisation

... of Technical Requirements for the Registration of **Pharmaceuticals** for Human Use (ICH)

European Union (**EU**)



US Food and Drug Administration (**FDA**)

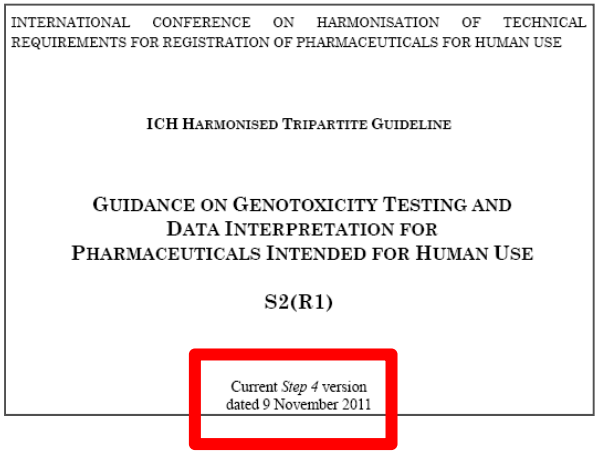


Japanese Ministry of Health, Labour and Welfare (**MHLW**)

European Federation of Pharmaceutical Industries and Associations (**EFPIA**)

Japan Pharmaceutical Manufacturers Association (**JPMA**)

Pharmaceutical Research and Manufacturers of America (**PhRMA**)


INTERNATIONAL CONFERENCE ON HARMONISATION OF TECHNICAL REQUIREMENTS FOR REGISTRATION OF PHARMACEUTICALS FOR HUMAN USE

ICH HARMONISED TRIPARTITE GUIDELINE

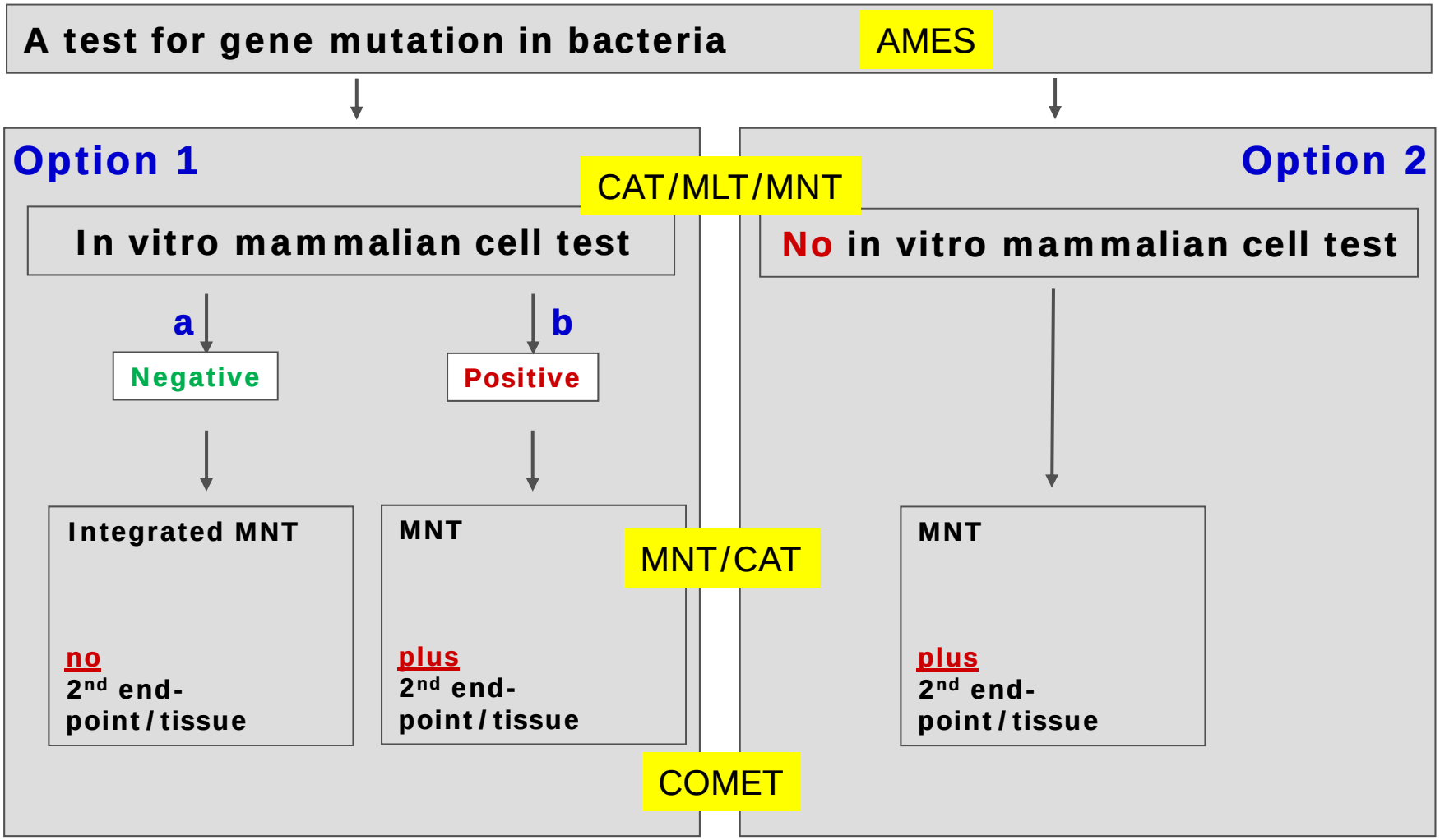
GUIDANCE ON GENOTOXICITY TESTING AND DATA INTERPRETATION FOR PHARMACEUTICALS INTENDED FOR HUMAN USE

S2(R1)

Current *Step 4* version dated 9 November 2011

- Strategies for avoiding in vitro false-positives (In vitro mammalian assay)
  - top concentration: 1 mM (or 500 µg/ml)
  - the maximum concentration should be the lowest concentration at which minimal precipitate is visible in cultures, even if toxicity occurs above the solubility limit (*Aardema et al., 2011*)
- In vitro MN test as 3rd option for mammalian cell tests
- Integration of in vivo MN test in repeat-dose toxicity studies
- Use of rat blood for micronucleus test (flow cytometric analysis of rat peripheral blood reticulocytes)
- Confirmatory testing in the case of clearly negative or positive in vitro test results is not usually warranted.

The revised ICH S2 guideline (2011)

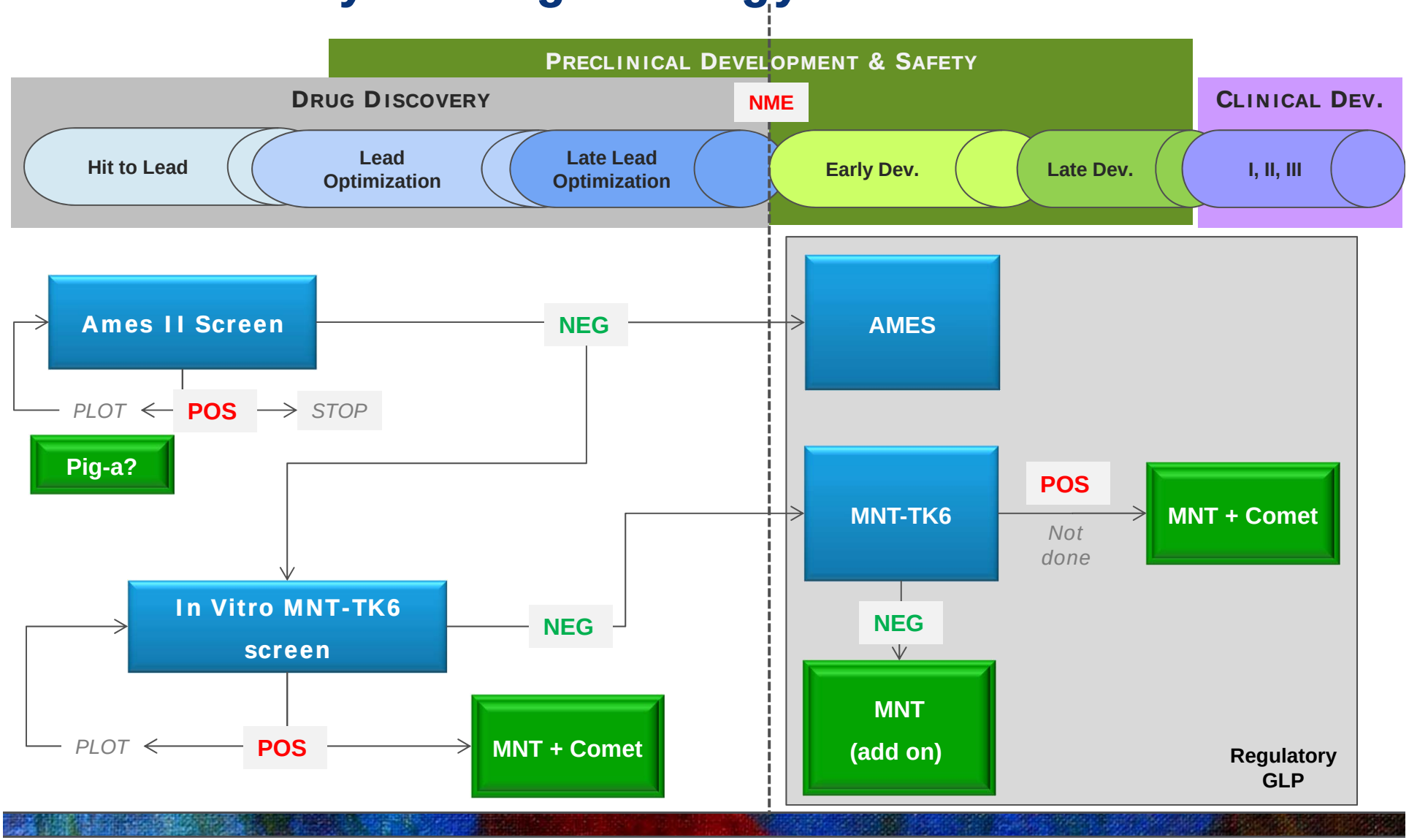


OECD Test Guidelines

Organisation for Economic Co-operation and Development

- Bacterial Reverse Mutation (Ames) Test: OECD **471**
- In vitro Mammalian Chromosome Aberration Test: OECD **473**
- In vitro Mammalian Cell Gene Mutation Test (e.g. Mouse Lymphoma Test): OECD **476**
- In vitro Mammalian Cell Micronucleus Test: OECD **487**
- In vivo Mammalian Erythrocyte Micronucleus Test: OECD **474**
- In vivo Mammalian Bone Marrow Chromosome Aberration Test: OECD **475**
- In vivo Mammalian Alkaline Comet Assay: OECD **489**

Genotoxicity testing strategy – Janssen R&D



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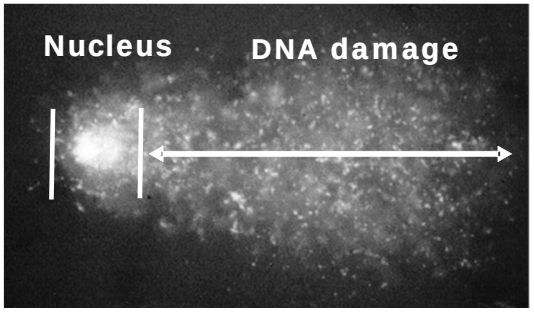
* *PLOT: preclinical lead optimization technologies (SAR, metabolism, agar Ames, specific strains, apoptosis, aneugen vs. clastogen, ROS, …)*

Genotoxicity testing strategy – Janssen R&D

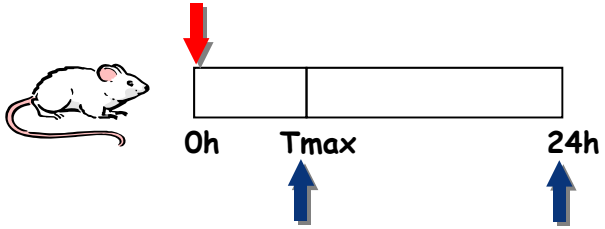
- Regulatory GLP studies outsourced
  - High investment to stay in compliance with GLP regulations versus the no. of studies conducted
- Screening studies conducted in-house
  - Short cycling-times
  - Testing multiple cpds in parallel in medium-throughput assays
  - Limited cpd shipment logistics
  - No method transfer (e.g. formulation, bioanalysis)

Study Design Aspects - In Vivo Comet Assay

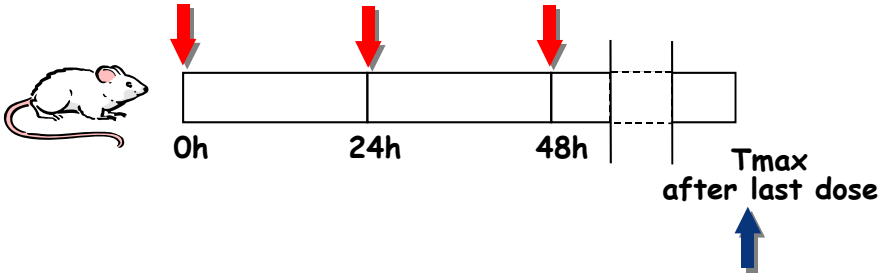
Detection of compounds inducing DNA damage in cells of various tissues by means of alkaline comet assay



Single dose / Multiple sampling



Multiple dose / Single sampling



Species: Rat / mouse / ....

Route: Oral / intravenous / ....

Samples: Liver / duodenum / ....

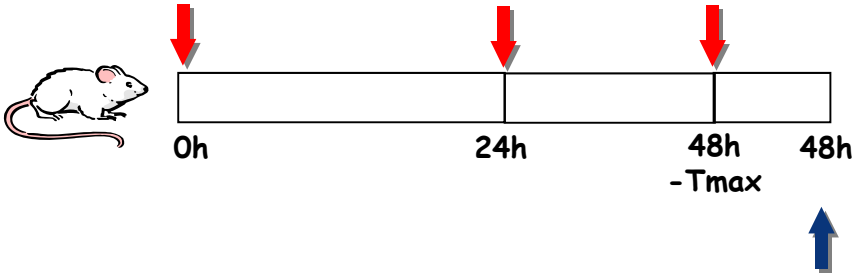
Dose groups:
- **Vehicle Control**
- **Low**
- **Medium**
- **High**
- **Positive Control**

MNT + Comet

3-day repeat dose study

Study Design Aspects - In Vivo Comet Assay

Multiple dose / Single sampling



MNT + Comet

3-day repeat dose study

Species: Rat / mouse / ….

Route: Oral / intravenous / ….

Samples: Liver / duodenum / ….

Dose groups: Vehicle Control
Low
Medium
High
Positive Control

- Formulation selection
- Top dose selection
- Tmax selection
- Tissue selection
- Single dose versus repeat dose

Screening to predict Regulatory

Formulation selection

- Formulation development is ongoing in the drug discovery phase
- Pharmacokinetic studies are available in support of formulation development

Selection of the discovery formulation close to regulatory formulation can be crucial

PK studies

Formulation	Dose (mg/kg)	Cmax (ng/mL)	AUC (ng.h/mL)
Discovery	200 SD	2670	31200
Regulatory	200 SD	7090	137000

3 to 4-fold higher exposure

Comet assay

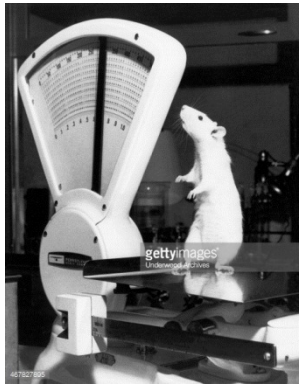
Formulation	Top dose (mg/kg)	Cmax (ng/mL)	Comet Response
Discovery	2000 3-day RD	5133	**Negative**
Regulatory	650 3-day RD	18700	**Weak Positive**

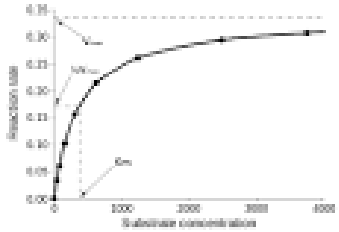
3 to 4-fold higher exposure

Top dose selection

OECD TG 489

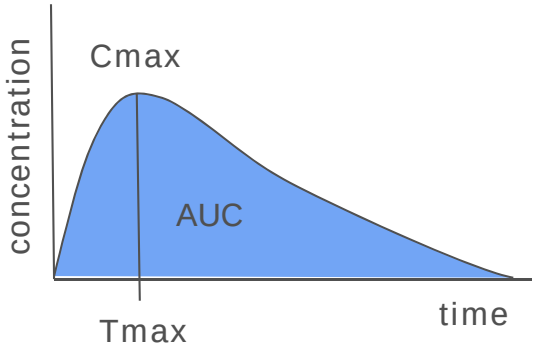
- Maximum tolerated dose
  - Toxicity studies (tolerability studies; SD and RD data)
- Maximum feasible dose
  - Formulation development data
- Maximum (limit) dose
  - Toxicity studies (non toxic); 2000 mg/kg
  - Exposure data (saturation)



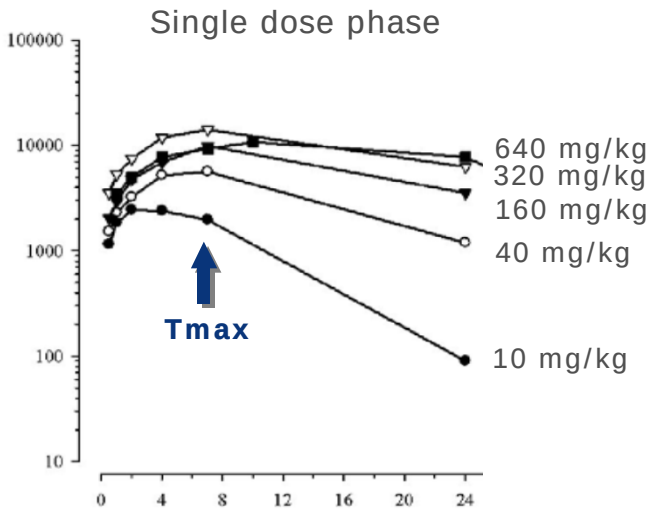


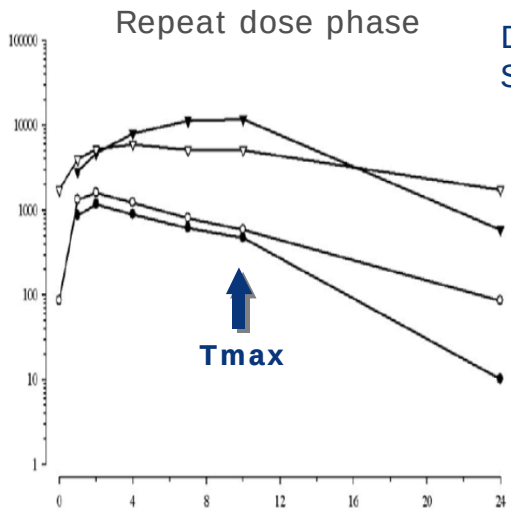
Tmax selection

- Concentration/time curves
  - Pharmacokinetic studies
  - Toxicity studies



Exposure data in relevant dose range





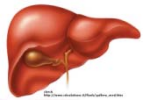
Dose range comet assay: 40-320 mg/kg/day
Selected Tmax: 7 hours

Tissue selection

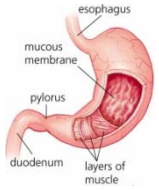
- PK/TK data
  - Absorption, clearance and metabolism data
  - Tissue distribution data are very limited in the drug discovery phase
  - PBPK modeling and simulation
- In vitro mammalian assay positive in absence or presence of metabolic activation system

Liver selected as standard tissue, occasionally additional tissue included in comet assay

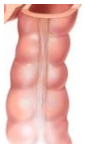
Liver



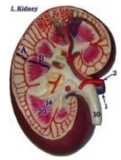
Stomach
Duodenum



Colon



Kidney



SD versus RD

- Repeat dose
  - Standard design (comet assay; combined MNT/comet assay)
- Single dose
  - Bone marrow toxicity (combined MNT/comet assay)
  - Tissue toxicity (comet assay)

SD versus RD

Aflatoxin B1

- 3-day repeat dose comet assay in male rats
- Oral gavage: 0.25, 0.5, 1 mg/kg/day
- 1 mg/kg/day was MTD
- Liver sampling:
  3 h after final administration

Increase in %TI concomitant with tissue toxicity:
Inconclusive

Treatment	N	% Tail intensity Mean ± SD		Histopathology
Vehicle control 0 mg/kg/day	5	0.31 ± 0.15	T	
Aflatoxin B1 0.25 mg/kg/day	5	0.46 ± 0.32		Single cell necrosis (grade 1) Acute inflammation (grade 1)
Aflatoxin B1 0.5 mg/kg/day	5	0.95 ± 0.56	*	Single cell necrosis (grade 1-3) Acute inflammation (grade 2-3)
Aflatoxin B1 1 mg/kg/day	5	3.30 ± 1.42	**	Single cell necrosis (grade 2-4) Acute inflammation (grade 2-4) Hemorrhage (grade 4)
Positive control 200 mg/kg/day EMS	3	22.93 ± 2.47	°	

Significance versus vehicle control by one-sided t-test: ° $p < 0.0001$
Significance versus vehicle control by one-sided Dunnett's test: * $p < 0.05$, ** $p < 0.0001$
Significance by one-sided Jonckheere-Terpstra Trend test: T $p < 0.0001$

SD versus RD

Aflatoxin B1

- Single dose comet assay in male rats
- Oral gavage: 0.5, 1, 2, 4 mg/kg
- 4 mg/kg was MTD
- Liver sampling:
  3 and 24 h after administration

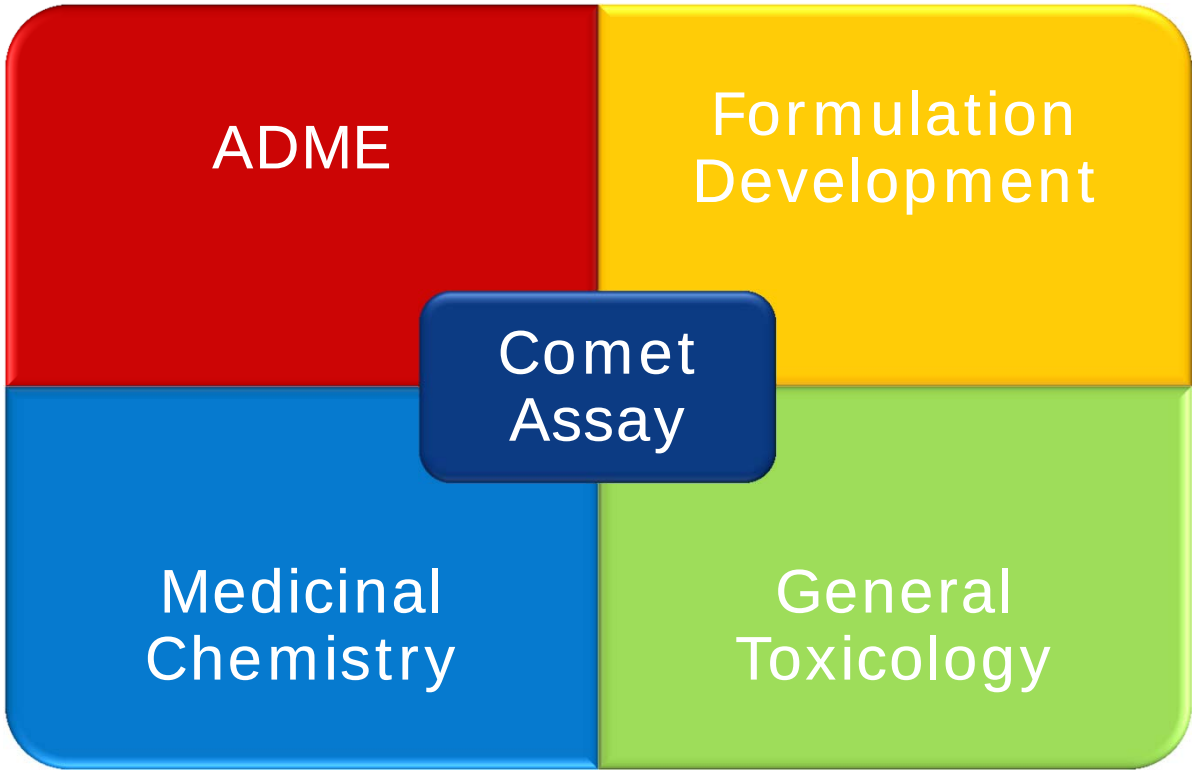
Increase in %TI
not concomitant
with tissue toxicity
at 0.5 and 1 mg/kg:
Positive

Treatment	N	% Tail intensity Mean ± SD		Histopathology
Vehicle control 0 mg/kg	5	0.37 ± 0.13	^{T}	
Aflatoxin B1 0.5 mg/kg	5	0.63 ± 0.23	**	
Aflatoxin B1 1 mg/kg	5	4.68 ± 2.31	***	
Aflatoxin B1 2 mg/kg	5	10.94 ± 9.53	***	Single cell necrosis (grade 1) Eosinophilic dark hepatocyte (grade 1)
Aflatoxin B1 4 mg/kg	5	17.64 ± 5.15	***	Single cell necrosis (grade 1) Eosinophilic dark hepatocyte (grade 1)
Positive control 200 mg/kg EMS	3	37.47 ± 8.53	°	

Significance versus vehicle control by one-sided t-test: ° $p < 0.0001$
Significance versus vehicle control by one-sided Dunnett's test: * $p < 0.05$, ** $p < 0.001$, *** $p < 0.0001$
Significance by one-sided Jonckheere-Terpstra Trend test: ^{T} $p < 0.0001$

Planning and communication

Many disciplines involved before study conduct



JaCVAM Validation Trial

- Validation trial with standardized protocol.
  - Pre-validation phase to optimize protocol
  - Assay reproducibility was confirmed among 13 laboratories (including Janssen R&D)
- 40 coded compounds were evaluated.
  - Janssen R&D tested 3 compounds
- The assay is concluded to be highly capable of identifying genotoxic compounds.
- The OECD TG is based on the standardized protocol of the trial.


Mutation Research 786–788 (2015) 2

Contents lists available at ScienceDirect

Mutation Research/Genetic Toxicology and Environmental Mutagenesis

journal homepage: www.elsevier.com/locate/gentox

Community address: www.elsevier.com/locate/mutres

Editorial

The JaCVAM-organized international validation study of the *in vivo* rodent alkaline comet assay

EFPIA Survey - Introduction

- Survey suggested by the EMA Safety Working Party and conducted by the EFPIA Preclinical Development Committee with the help of comet assay experts from different pharmaceutical companies.
- Investigation of the experience with the in vivo comet assay for regulatory purpose among European pharmaceutical companies with special attention to potential difficulties in interpretation of study data.
- The participating companies reported a total of 147 studies (conducted in-house or outsourced).

EFPIA Survey - Conclusions

- Final conclusion on the response in the in vivo comet assay was available for 136 pharmaceutical compounds.
- Occurrence of difficulties with interpretation of comet assay results is low.
  - Equivocal response (1 study); inconclusive response (3 studies).
  - With additional information (e.g. repeat assay, circumstantial information) a final conclusion could most probably have been drawn.
- The number of studies with a positive response is relatively low.
  - 14 studies (10%)
  - Positive comet assay results are not related to cytotoxic effects of the compound in the tissue examined.

EFPIA Survey - Conclusions

- The comet assay appears not over-sensitive.
  - Only 10% of the studies conducted as follow-up of a positive in vitro mammalian cell assay showed positive comet assay results.
  - No positive comet assay results with compounds having no genotoxic properties.
- The comet assay is considered an appropriate second in vivo genotoxicity assay as outlined in the ICH S2(R1) guidance.
  - 10 out of 12 compounds showed positive comet assay results are negative in the in vivo micronucleus assay.
- Regulatory acceptance of the ICH S2(R1) guidance to mitigate a positive in vitro mammalian cell assay with 2 negative in vivo assays (i.e. micronucleus and comet assay). All 46 cases accepted.

Conclusions

- The predictivity of a screening in vivo comet assay is highly dependent on the information available in the drug discovery phase.
- Participation in the JaCVAM validation trial greatly contributed to Janssen R&D experience with the in vivo comet assay.
- The EFPIA survey results demonstrate the robustness of the in vivo comet assay and regulatory acceptance of the current ICH S2 guidance.


Thank you for your attention
Questions?