Evaluating the Conceptual Framework for Genomic Damage: Benzene Case Study

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DISCLAIMER

This presentation is the work of the HESI GTTC Group. It does not represent any work product or opinions by the individuals involved or the employers of any individuals involved.

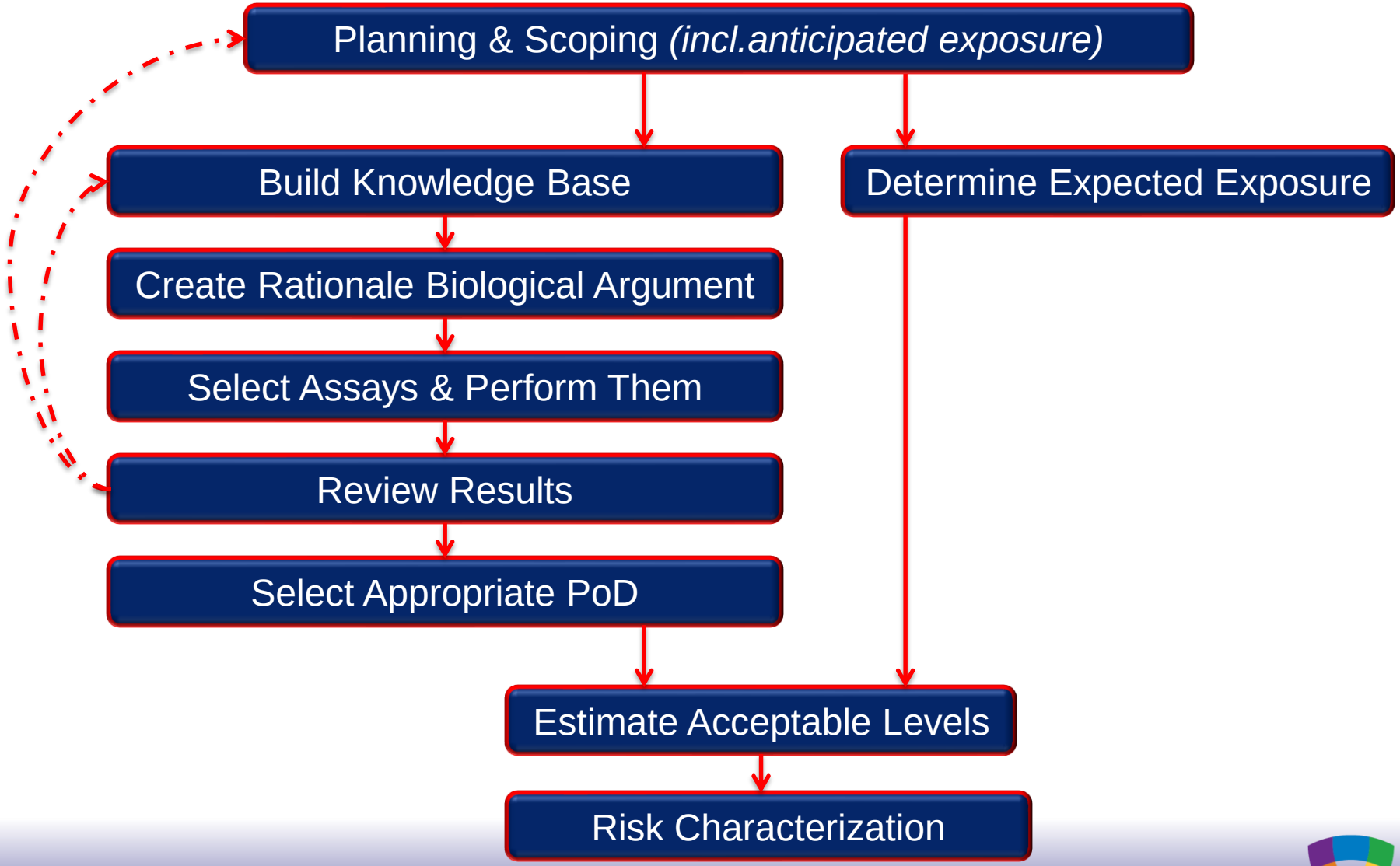
CURRENT US / EU APPROACH - RISK ASSESSMENT OF MUTAGENS

- EU and US = no existing Regulatory framework
- Focus is on Hazard identification
  - Cancer Risk assessment where data exist
  - EU – Classification and Labelling drives Risk Management
- There is a GAP!

A NEW APPROACH?

- Developed without taking into account current legislation
- Genetic toxicity assessed as an endpoint itself, BUT not evaluated in isolation
- Approach is ahead of existing regulatory frameworks
  - Transition needs to consider combining the current situation with the future

FRAMEWORK: TESTING STRATEGY FOR ASSESSMENT OF GENOMIC DAMAGE



Dearfield K.L., et al. (2016). Next generation testing strategy for assessment of genomic damage: A conceptual framework and considerations. *Environ Mol Mutagen.*

FROM CONCEPT TO CASES

Goal:

To evaluate usefulness/feasibility conceptual framework for various regulatory jurisdictions, incl. **industrial chemicals** and **pharmaceuticals**

Retrospective approach:

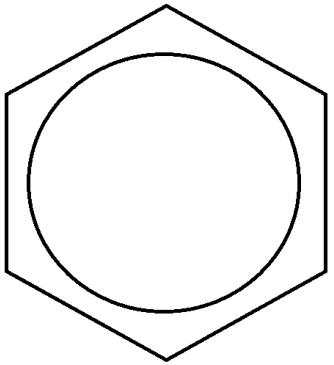
- Collect & review available data
- Review framework: would application of the framework and associated questions have led to data essential for risk assessment?

CASE STUDY: BENZENE

- Well known and studied industrial chemical
- Data rich (including toxicity, mechanisms, exposure potential)

  Toxicity Profile:

  – Human carcinogen (associated with acute myelogenous leukemia)
  – Hematotoxic
  – Genotoxic
  – Toxicokinetics well characterized
  – Data on human variability / susceptibility



EXPOSURE

Volatile substance: **inhalation** most important route of exposure

Different sources of exposure:

Occupational

- Petrochemical industry (benzene in crude oil, byproduct refining operations)
- Potential for exposure in low ppm range*
  - Carrieri et al, 2010: mean **0.014 ppm** (petrochemical plant, Italy)
  - Gaffney et al, 2010 (ExxonMobil refinery, Beaumont, USA):
    - Mean 'non-task' exposure levels, **<1ppm**,
    - Mean Task exposure levels **1.4 ppm** (air concentration, overall tasks,)**

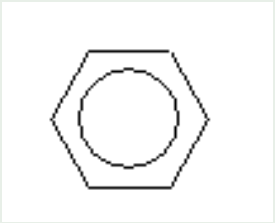
General population

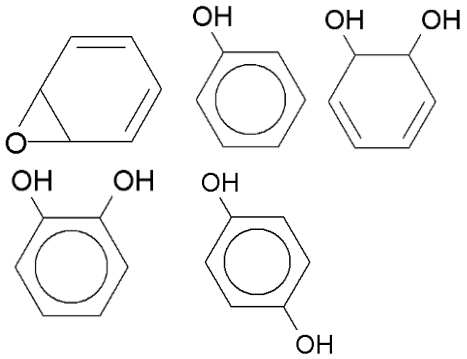
- Cigarette smoke, petrol filling station
- Exposure in ppb range - EU Air quality limit for benzene 5$\mu g/m^3$ (approx. 1.3ppb)

* EU and USA

** task based exposure levels **not** = to Exceeding OEL

IN SILICO

Benzene			
SMILES	c1ccccc1		
Structure			
Endpoints	DEREK	OASIS	
Software Version	Derek Nexus v.5.0.2	TIMES V.2.27.20	Relevance
Ames Mutagenicity			
Chromosome Damage (in vitro)			
Non-specific genotoxicity (in vitro)			



Benzene metabolites profiled in OECD Toolbox
Several alerts for DNA and protein binding, clastogenicity and carcinogenicity

< 70% similarity with successful AND <5% similarity with unsuccessful predictions;

Based on this What studies would we do?

- Ames?
- Consider Clastogenicity + importance of metabolism

IN VITRO GENOTOXICITY

Overview available data from commonly used in vitro tests

- Ames –ve – as predicted
- MNvit +ve
- CAvit +ve
- MLA +/- – consistent with ‘mutagenicity prediction?’

Key Point: Try to get the most out of the testing as possible

Are there opportunities to ‘update’ this assay set?

- *potentially giving insight into MoA or dose response at this early stage*

BENZENE TOXICITY

Before *in vivo* genotoxicity data are considered, what other information or data are available?

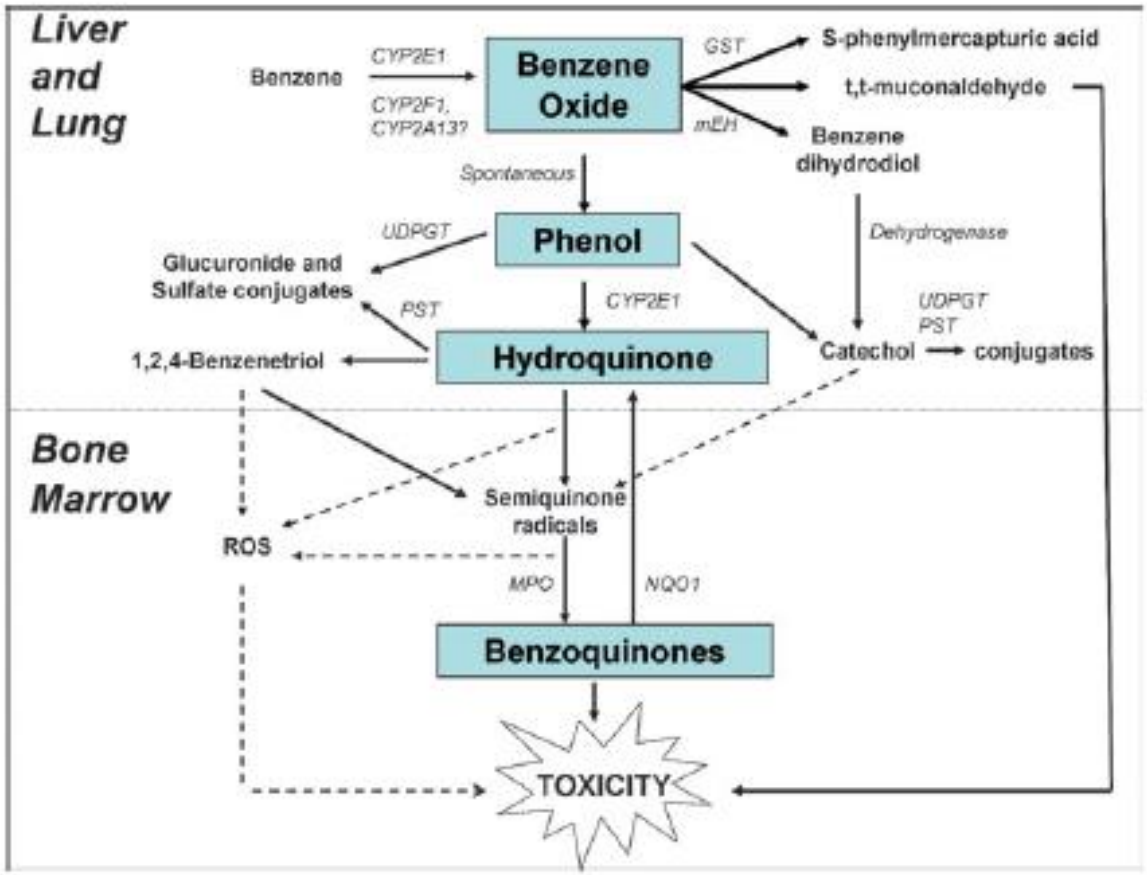
- AOPs
- *In vitro* data, e.g.
  - receptor activation (AhR; -ve in ToxCast)
  - oxidative stress
- *In vivo* data do we have or need?
  - 28-day repeated dose toxicity
  - 90-day repeated dose toxicity
  - developmental toxicity (screening)
  - toxicokinetics

These may inform how you plan in vivo follow up studies for genotoxicity

OTHER IMPORTANT TOXICITY FINDINGS

- Target organ toxicity (animals and humans):
  - Hematological system
    - Anemia, Leukopenia, thrombocytopenia, Pancytopenia
      - >10ppm, chronic inhalation, rat
  - Immune system – humoral and cellular immunological suppression

TOXICOKINETICS



From McHale, 2012

TOXICOKINETICS

- Rapidly absorbed through the lungs; approximately 50% of the benzene in air is absorbed
- Rapidly distributed throughout the body and tends to accumulate in fatty tissues
- Metabolism in the liver and lungs
  - production of several reactive metabolites
  - At low exposure levels, benzene is rapidly metabolized and excreted predominantly as conjugated urinary metabolites
  - At higher exposure levels, saturation of metabolic pathways → large portion of absorbed dose excreted as parent compound.
- PBPK model available (Watanabe 1994)

SUSCEPTIBLE GROUPS

Genetic variation

- Polymorphisms in the genes encoding for enzymes involved in the metabolism of benzene, e.g. CYP2E1, GSTM1 and GSTT1, can modify the toxicity of benzene (McHale et al 2012)
  - E.g. Garte et al. (2008) - Five metabolic loci studied in Bulgarian workers/controls to study effect on susceptibility to adverse effects: 5.5-fold difference between 'good' and 'bad' genotype (based on DNA Single strand breaks)

- *Possible involvement of detoxification pathways needs to be better incorporated into the framework.*
- *Other aspect is fold difference between genotypes. In current approach most likely accounted for by assessment factor. Next generation risk assessment will make use of more sophisticated assessment factors. Plus insight into uncertainty.*

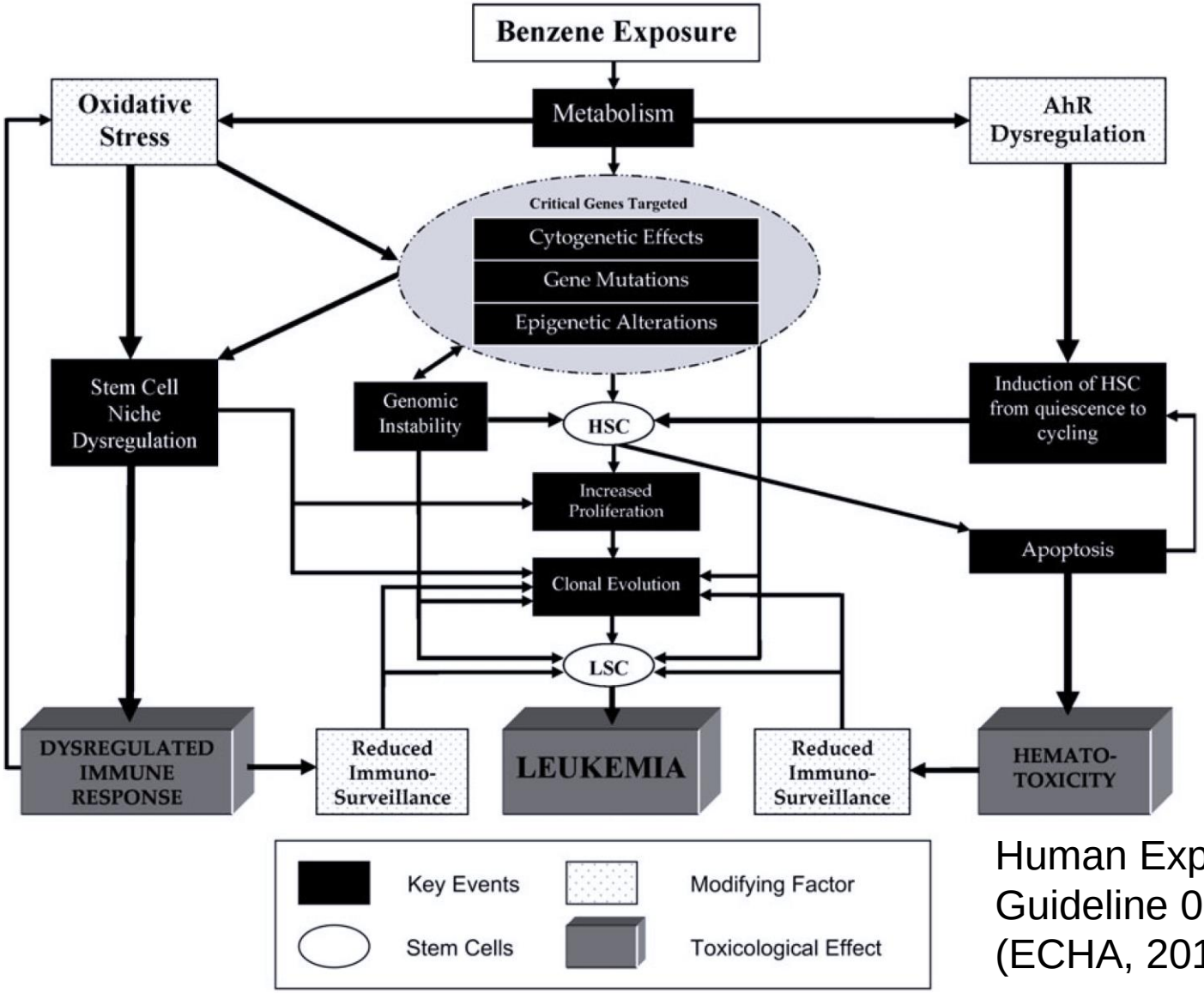
IN VIVO GENOTOXICITY

Overview of some available data from commonly used in vivo tests

- MN in vivo inbred/outbred mice - positive (inhalation, oral)
- CA in vivo - positive
- Oral TGR – positive (bone Marrow)
- Inhalation TGR – positive (lung and Spleen)
- Oral Comet – many studies, mix of negative / positive in Bone Marrow
- Inhalation Comet – positive in bone marrow

This is what we have, but what would we have done
Exposure route – Inhalation only?
Focus on Clastogenicity / Anugenicity versus mutation? – no TGR?

MODES OF ACTION



Human Exposure Guideline 0.1ppm (ECHA, 2017)

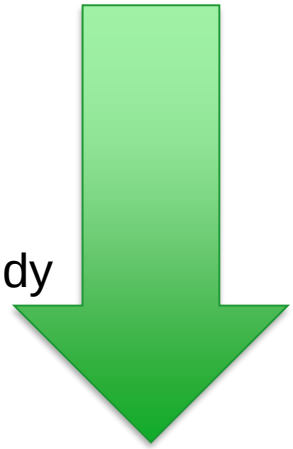
From McHale et al., Carcinogenesis, 2012

POD MODELLING / BMD APPROACH

Genomic damage endpoints:

- MN in vivo (inhalation) most sensitive (French et al. 2015)
- Diversity Outbred mice: BMC_{50} = 15 – 21.7 ppm
- B6C3F1 mice: BMC_{50} = 43.2 – 79.6 ppm

Derive human equivalent exposure level and Apply 'Assessment' or 'Uncertainty' factors (e.g. inter / intraspecies; study duration, etc.)



BUT: take into account other points of departure (non-genotoxicity; other genotoxicity PODs – keeping in mind mechanistic insight)

Exposure guideline value – relevant to purpose of assessment

KEY OBSERVATIONS ON FRAMEWORK

- Exposure – impact on assessment
  - More complex – Intended exposure versus indirect/unintended exposure
  - If driver for data needs – Scope of assessment is critical – General assessment versus specific worker?
- Importance of TK and other data
  - Study design / interpretation of genetox studies
  - Human susceptibility differences
- Study selection for genotoxicity POD
  - Use many, minimum criteria for study (group size/sex, dose ranges, etc.)
- What about PODs for other endpoints?
  - MoA for Benzene is complex
- Uncertainty factors – can decrease as ‘certainty increases’

KEY OBSERVATIONS VS CURRENT REGULATION

- Framework would diverge from current regulatory approach
- Different drivers
  - Framework = Risk assessment
  - Regulation = Classification and Labelling
- Can we move to avoiding the need for Cancer studies?

FINAL WORD

- It is possible to use Genetic damage for risk assessment
- The Framework demands:
  - Expertise
  - Making the best use of data and accepting that not every substance needs every study
  - Final outcome ultimately driven by purpose of assessment
    - Influenced by Exposure potential

ACKNOWLEDGEMENTS

HESI Genetic Toxicity Technical Committee

- Clean Sheet WG, esp. **Industrial Chemicals subteam**:
  - Nick Ball, Jan van Benthem, Bhaskar Gollapudi, Federica Madia, Stefan Pfuhler, Kristine Witt, Mirjam Luijten; Kerry Dearfield, Raffaella Corvi, Andrea Richarz

- Quantitative Analysis WG:
  - George Johnson, Paul White, Andreas Zeller

- Co-chairs & Management
  - Maik Schuler, Paul White, Stan Parish, Lauren Peel

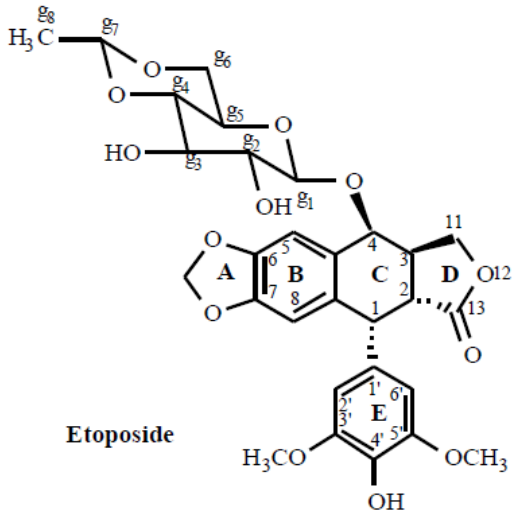
Next Generation Assessment of Genomic Damage – "The Clean Sheet"

Pharmaceutical case study

Workgroup:

Laura Custer, Azeddine Elhajouji, Mirjam Luijten, Timothy Mcgovern, John Nicolette, Mark Powley and Véronique Thybaud

PHARMACEUTICAL CASE STUDY ETOPOSIDE (VP-16-213)



CAS n°: 33419-42-0

Work still in progress:

- **Etoposide selected as data-rich pharmaceutical**
- **Evaluation of the possible use and usefulness of available information for proposed framework on testing strategy for assessment of genomic damage**

FRAMEWORK: TESTING STRATEGY FOR ASSESSMENT OF GENOMIC DAMAGE

Dearfield et al. (2016)

ANTI-CANCER DRUG
TOPOISOMERASE II INHIBITOR

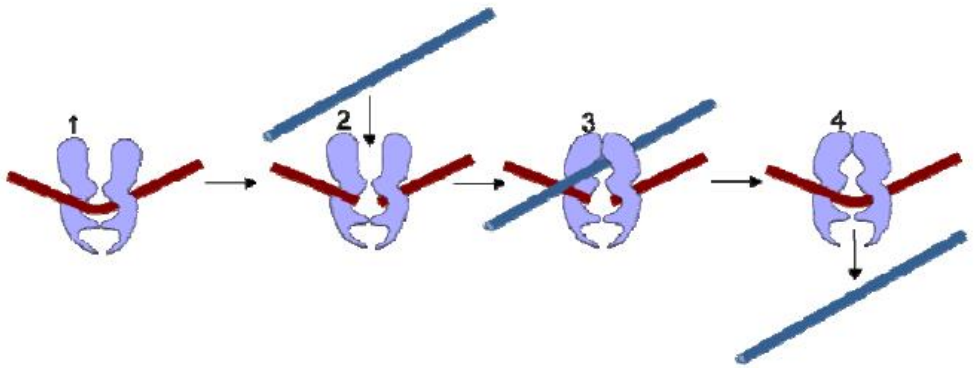
- **Semi-synthetic derivative of epipodophyllotoxin**
  - Discovered in 1960's and registered in 1980's
- **Widely prescribed for a variety of cancers**
  - Often combined with other cytotoxic agents
- **Inhibitor of topoisomerase II (Topo II poison)**
  - Mechanism discovered in mid 1980's
  - Clinical target is mainly the α isoform
    - Increased in rapidly proliferating cells (S and G2/M phases)
- **Secondary therapy related leukemia**
  - Acute myeloid leukemia in patients and in infant after in utero exposure.

POPULATIONS (POTENTIALLY) EXPOSED TO ETOPOSIDE

Population	Patients	Workers	General and environment
Exposure	Intended Concentrations in tissues large enough to allow inhibition of topoisomerase IIα	To be avoided / minimized Need to define: - precautionary measures - acceptable exposures	
Points to consider	In utero exposure in pregnant women patients: unintended exposure	Handling during synthesis, packaging, and at hospital	Destruction and control of wastes at industrial sites and hospital

- **Identification of the different (potentially) exposed populations is useful to define an appropriate strategy for risk characterization**
  - **for** Planning & Scoping (incl.anticipated exposure)

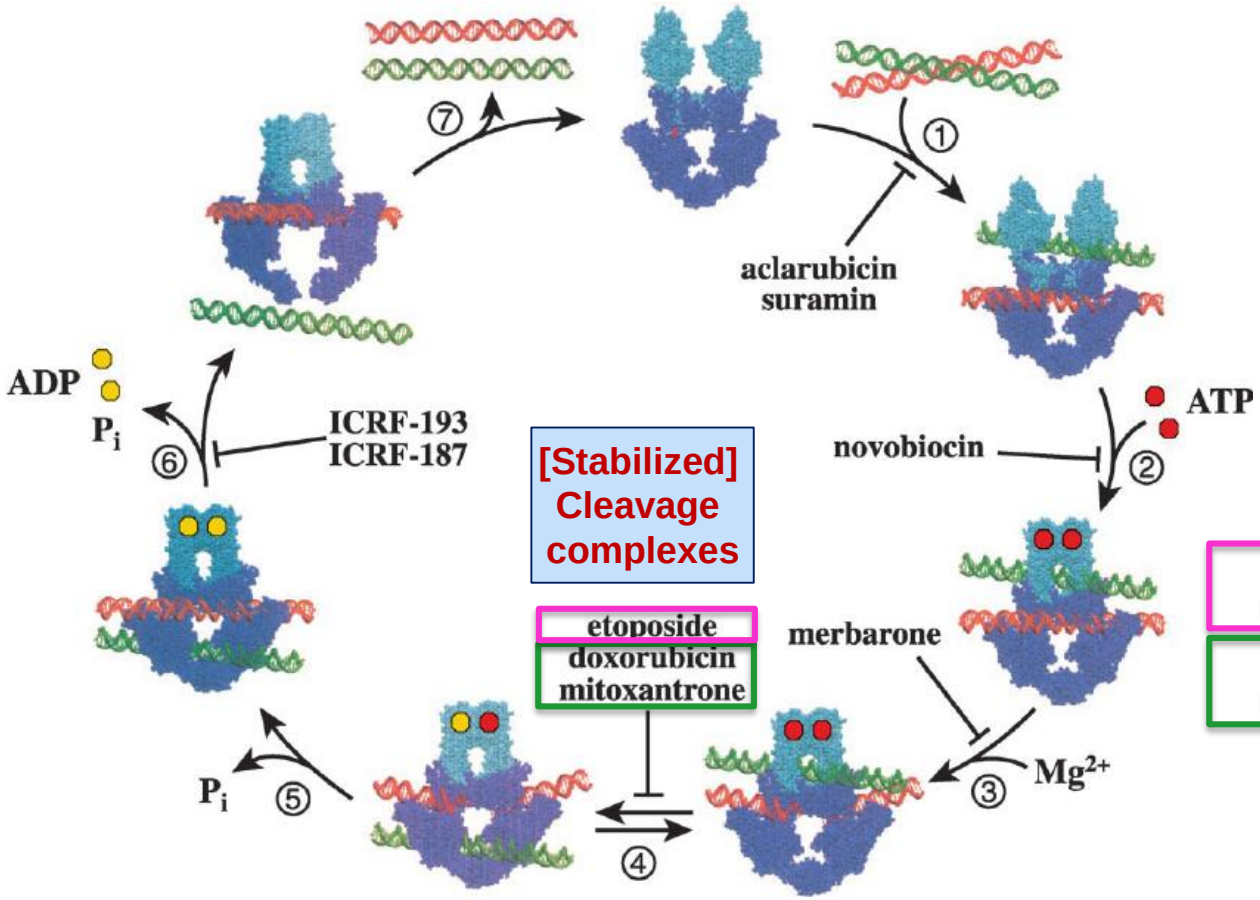
THE CATALYTIC CYCLE OF DNA TOPOISOMERASE II



Cowell and Austin 2012

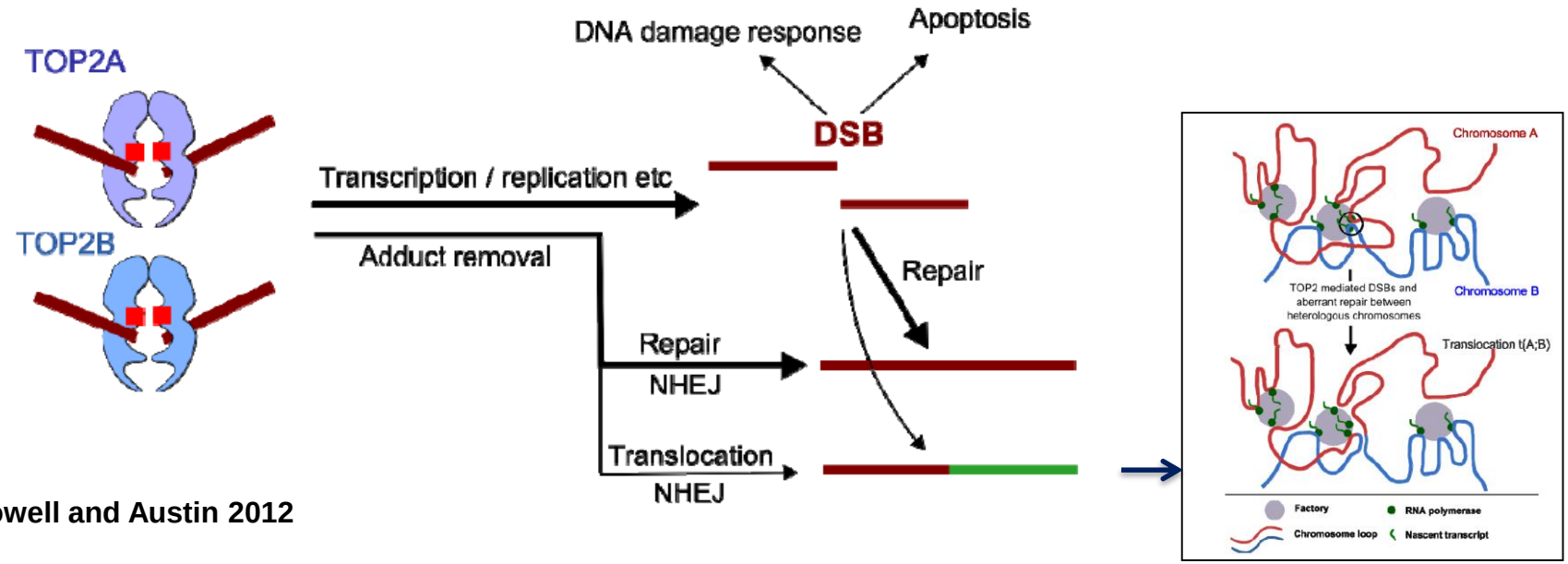
Figure 1. TOP2 mechanism. TOP2 cleaves both strands of a duplex DNA segment (brown, 1–2). A second DNA duplex (blue) passes through the transient enzyme-coupled break (2–3). The first duplex is then re-ligated and the products of the reaction are released from the enzyme (4).

TOPOISOMERASE II INHIBITORS



Larsen et al. 2003

CONSEQUENCES OF TOPOISOMERASE II INHIBITION BY ETOPOSIDE



Cowell and Austin 2012

Figure 3. TOP2 Poisons, downstream events. TOP2 poisons inhibit the religation step of the TOP2 reaction cycle, leading to accumulation of covalent TOP2-DNA cleavage complexes. These lesions are cytotoxic and lead to activation of the DNA damage response and potentially apoptosis. Alternatively these lesions are repaired, largely through the non-homologous end-joining pathway. Translocations observed in therapy-related leukemia are presumed to occur as a result of mis-repair, joining two heterologous ends.

MECHANISM OF ACTION (MOA) TOPOISOMERASE II INHIBITION

Molecular mechanisms:

- Low affinity for DNA
  - No intercalation or binding
- Covalent binding to topoisomerase II and/or DNA-topoisomerase II complexes
- Stabilization DNA cleavage complexes through inhibition of DNA religation

Cellular consequences:

- Blockade of the replication forks and transcription machinery
- DNA damage response
- Apoptosis
- DNA single and double-strand breaks
- DNA repair mainly NHEJ pathway
  - Possibly error prone NHEJ leading to translocation (e.g., mixed lineage leukemia (MLL) at locus 11q23)

- **Knowledge on MoA: useful to understand contribution of each key event to dose response and risk characterization**
  - **In** Build Knowledge Base **and** Create Rationale Biological Argument

AVAILABLE GENOTOXICITY DATA
AMES TEST

Test systems	Endpoints tests	Results	References
In microorganisms			
S. typhimurium TA100 (+/– S9)	Reverse mutation	–	Gupta *et al.*, 1987
S. typhimurium TA102 (+/– S9)	Reverse mutation	+	Gupta *et al.*, 1987
S. typhimurium TA1537 (– S9)	Reverse mutation	–	Ashby *et al.*, 1994
S. typhimurium TA1538 (+/– S9)	Reverse mutation	–	Ashby *et al.*, 1994
S. typhimurium TA98 (– S9)	Reverse mutation	–	Matney *et al.*, 1985
S. typhimurium TA98 (+/– S9)	Reverse mutation	+	Ashby *et al.*, 1994
S. typhimurium TA98 (+/– S9)	Reverse mutation	–	Gupta *et al.*, 1987
S. typhimurium (Other) (– S9)	Reverse mutation	+	Matney *et al.*, 1985
E. coli K 12	Forward/Reverse mutation	–	Gupta *et al.*, 1987
E. coli (other)	Reverse mutation	–	Gupta *et al.*, 1987
Neurospora crassa	Forward/Reverse mutation	–	Gupta, 1990

Choudhury et al. 2004

AVAILABLE GENOTOXICITY DATA IN VITRO

In vitro			
Animal cells	DNA damage/strand break/X-linked	+	Jeggo *et al.*, 1989
			Kerrigan *et al.*, 1987
			Pommier *et al.*, 1988
			Gupta *et al.*, 1987
	Aneuploidy	+	Ashby *et al.*, 1994
Muntjac cells	Aneuploidy	+	Downes *et al.*, 1991
CHO cells	Gene mutation, SCE, CA	+	Singh & Gupta, 1983
	Cell death	+	Lock & Ross, 1990
			Berger *et al.*, 1991
Chinese hamster cells	SCE, CA, aneuploidy	+	Pommier *et al.*, 1988
Chinese hamster V79 cells	Deletion mutation & cell death	+	Berger *et al.*, 1991
	SCE, cell death, DNA strand break	+	Chatterjee *et al.*, 1990
Mouse lymphoma (not L1578Y)	Gene mutation	+	Gupta *et al.*, 1987
Mouse leukaemia L1210 cells	DNA single-, double-strand breaks	+	Wozniak & Ross, 1983
			Yang *et al.*, 1987
L5178Y cells, TK locus	Gene mutation	+	Ashby *et al.*, 1994
Mouse cells	CA	+	Ashby *et al.*, 1994
Cultured seminiferous tubules of rat	Meiotic MN	+	Sjoblom *et al.*, 1994
Human cells	DNA damage/strand breaks/X-links	+	Kerrigan *et al.*, 1987
			Sinha *et al.*, 1988
			Long *et al.*, 1986
Human lymphocytes	SCE	+	Tominaga *et al.*, 1986
	CA	+	Maraschin *et al.*, 1990
			Tominaga *et al.*, 1986
Human lung carcinoma cells	DNA breakage	+	Long *et al.*, 1986
Other human cells	CA	+	Caporossi *et al.*, 1993

Choudhury et al. 2004

AVAILABLE GENOTOXICITY DATA IN VIVO

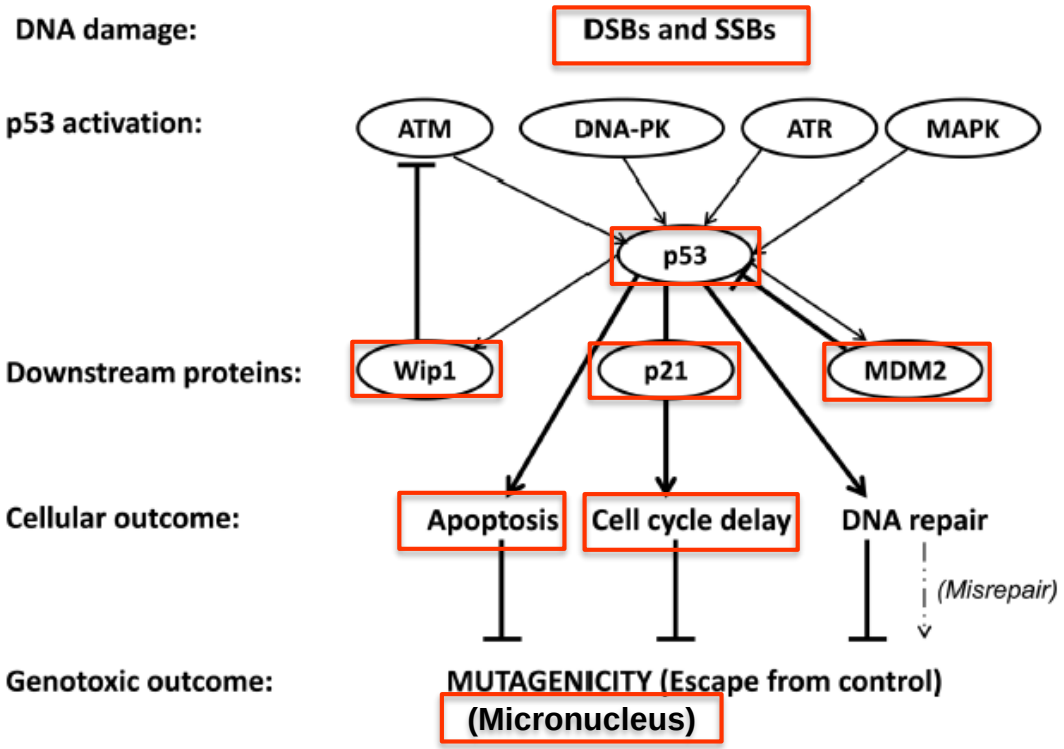
In vivo			Huang *et al.*, 1973
Mouse	MN	+	Nakanomyo *et al.*, 1986 Ashby *et al.*, 1994 Present study
	CA, SCE	+	Agarwal *et al.*, 1994 Sieber *et al.*, 1978
	CA	+	Present study
Mouse spermatid	MN	+	Kallio & Lahdetie, 1993
Rat spermatogenesis	Spermatid MN	+	Lahdetie *et al.*, 1994

Choudhury et al. 2004

Other and more recent data

Rat reticulocyte	Pig-a, PIGRET	-	Yamamoto et al. 2016 Kimoto at al. 2016
Mouse spleen	pKZ1 mouse mutagenesis model	+	Hooker et al. 2002

DNA DAMAGE RESPONSE IN VITRO DATA



All impacted

p-H2AX
p-p53 (s15)
p53
WIP1
MDM2
p21
Cell Cycle[e]
Apoptosis[f]
p-p53 (s46)
Micronucleus
Gene
Transcription

Clewell et al. 2014 and 2016

- HT1080 human fibrosarcoma cell line (p53 proficient)
- 24 - 28 hour treatment

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AVAILABLE GENOTOXICITY DATA SUMMARY

In silico:
- Negative prediction for Ames
- Positive prediction for chromosome damage in vitro and in vivo.

DNA damage:
- Single and double DNA strand breaks (γH2AX and Comet)
- DNA damage response

Mutagenicity data

In vitro:
- Conflicting results in Ames (no or small effects)
- HPRT negative

In vivo:
- Pig-a assays negative
- HPRT negative

Clastogenicity data

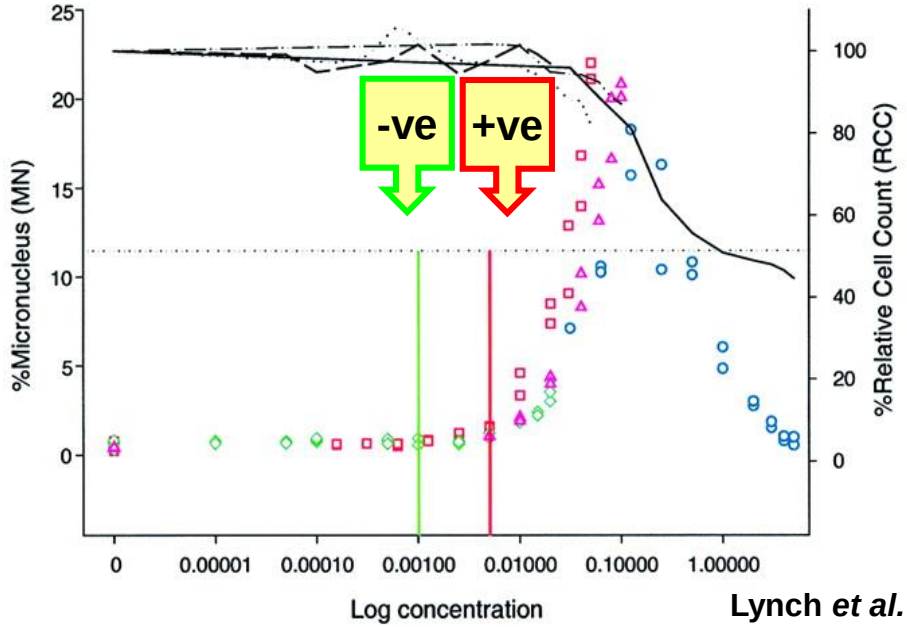
In vitro:
- Chromosome damage (MN, CA and SCE) and TK mutation tests positive in multiple cell types

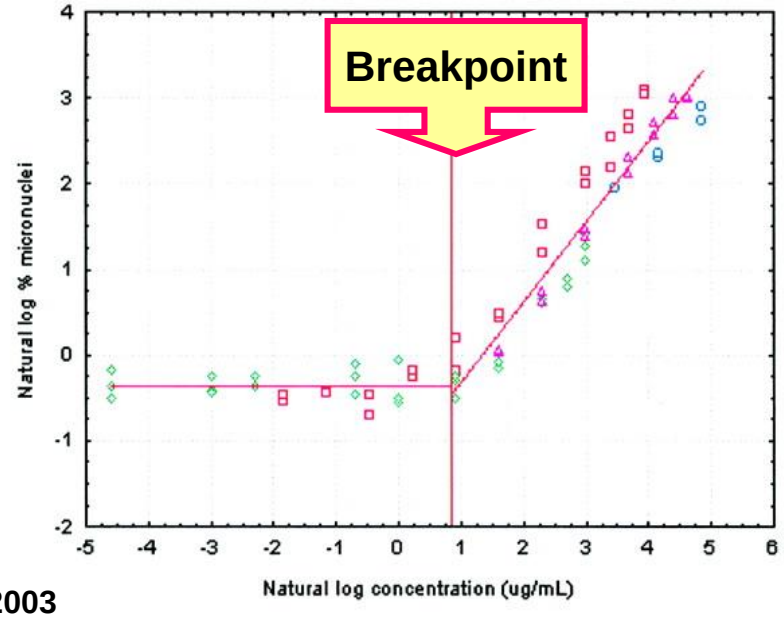
In vivo:
- Chromosome damage (MN and CA) test positive in bone marrow and spermatids

Recombination in vitro/ in vivo pKZ1 models:
- Increase at high doses / decrease at low doses

- **Available genotoxicity: What would be the most relevant data for risk characterization in the context of the proposed workflow?**
  - **In** Select Assays & Perform Them **and** Review Results

DOSE-RESPONSE IN VITRO DATA





Lynch *et al.* 2003

- **Micronucleus in mouse lymphoma L5178Y cells (p53 deficient)**
- **NOEL: 0.005 µg/mL (0.0085 µM)**
- **Breakpoint (Broken stick model): 0.00236 µg/mL (0.004 µM)**

Molecular weight: 588.557 g/mol

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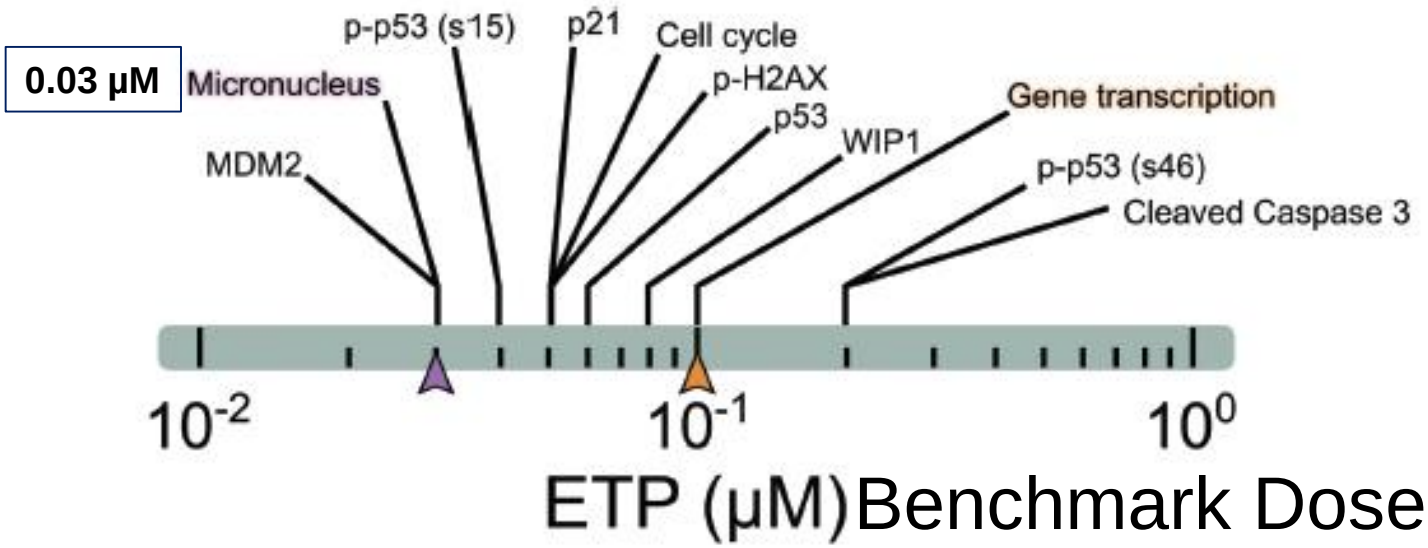
DOSE-RESPONSE IN VITRO DATA



Clewell et al. 2014 and 2016

- **HT1080 human fibrosarcoma cell line (p53 proficient)**
- **Activation of p53 and formation of micronuclei: point-of-departure concentrations of etoposide in the range of 0.01 to 0.1 µM.**

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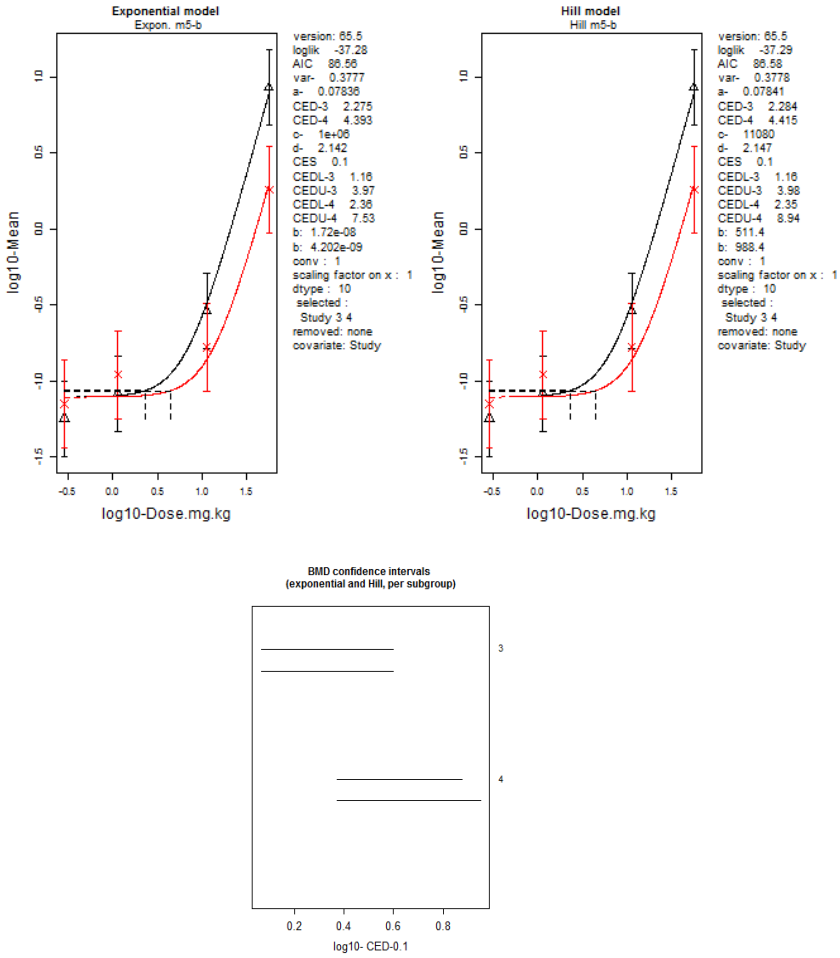
DOSE-RESPONSE IN VIVO DATA



- Bone marrow micronucleus in Fischer 344 rats, 14-day oral gavage, sampling 24h after last treatment (Garriot et al. 1995)
- Lower BMD confidence intervals in males
  - **$BMDL_{10}$ 1.16mg/kg** and **$BMDU_{10}$ 3.97mg/kg**
  - **$BMDL_{50}$ 2.89mg/kg** **$BMDU_{50}$ 7.42mg/kg**
- The only study identified to date adequate for the calculation of PoD, i.e., evaluating low enough doses to reach a no-effect dose (<5 mg/kg).

Analysis done by George Johnson

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ANALYSIS OF DOSES AND DOSE-RESPONSES

Plasma and/or tissue exposure in human
in µg/mL or µM

PoD in mammalian cell models
in µg/mL or µM

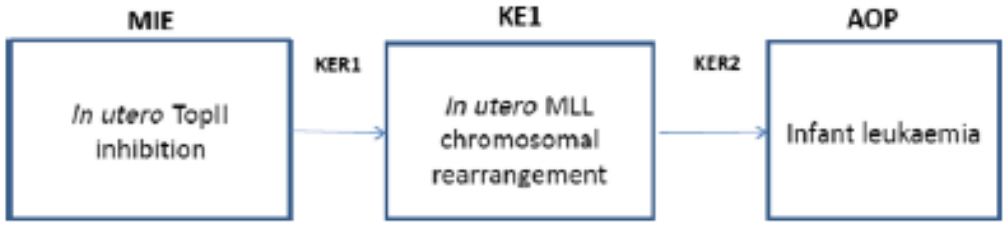
PoD in animal studies
in mg/kg
+
ideally corresponding plasma and/or tissue exposure
in µg/mL or µM

Work still in progress

- **What are the doses and dose-response data available for risk characterization in the context of the proposed workflow?**
  - **In** Select Appropriate PoD **and** Determine Expected Exposure
    Estimate Acceptable Levels

AOP FOR ACUTE MYELOID LEUKEMIA IN INFANT AFTER IN UTERO EXPOSURE

Adverse Outcome Pathway (AOP): *In utero* DNA topoisomerase II inhibition leading to infant leukaemia



The biological plausibility for this AOP is strong. The relationship between DNA double strand breaks, MLL chromosomal translocation and infant leukaemia is well established. The same pathway is reproducible in chemotherapy-induced acute leukaemia in patients following treatment with etoposide, a known Topo II poison.

EFSA 2017

AOP FOR ACUTE MYELOID LEUKEMIA IN INFANT AFTER IN UTERO EXPOSURE

Table B.2: Response–Response and temporality concordance for the tool compound etoposide

Concentration of etoposide	MIE *In utero* DNA topoisomerase II inhibition	KE1 *In utero* MLL chromosomal rearrangement	AO Infant leukaemia
0.01–0.1 μM, *in vitro (TopII enzymes and cells in culture)*	+++ (DNA damage response in various cells)	–	
0.1–1 μM, *in vitro cell cultures*	+++ (haematopoietic progenitor and stem cells)	+	
0.5–5 μM, *ex vivo,* mouse fetal liver HSC concentration[(a)]	+++ (inference from MLL cleavage)	+ (only MLL cleavage)	– (no leukaemia development)

Concentration of etoposide	MIE *In utero* DNA topoisomerase II inhibition	KE1 *In utero* MLL chromosomal rearrangement	AO Infant leukaemia
Max 5 μM, *ex vivo,* mouse fetal liver HSC concentration[(a)]	+++ (inference from MLL cleavage)	+ MLL fusions detected only in DNA repair deficient mice	– (no leukaemia development)
Max > 150 μM, plasma concs in etoposide-treated patients[(b)]	+++ (inference from MLL cleavage)	++ MLL-AF4 fusion gene and protein	+ treatment-related acute leukaemia

(a): A range of concentrations is linearly extrapolated on the basis of the concentration of 5 μM after the dose of 10 mg/kg.
(b): Plasma concentration of etoposide cannot be directly extrapolated to the concentration at the active site. Probably the actual active cellular concentrations of etoposide is much lower, perhaps 10% or less of the plasma concentration.

EFSA 2017

CONCLUSION

For etoposide risk characterization should consider

- **Different precautionary measures depending on the exposed population**
  - **Intended versus unintended exposure and acceptable level of risk**
- **Non DNA-reactive mechanism of action that might result in chromosome damage such as heritable translocation**
  - **At intermediate exposures depending on the fidelity of repair mechanisms, and when cells are not eliminated through apoptosis**
  - **Likely complex kinetics and equilibrium**
- **The most appropriate endpoint(s) to derive a PoD and to avoid/minimize genotoxicity risk**
  - **To be further evaluated.**

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THANK YOU

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